Association between Endocrine Pancreatic Secretory Granules and In-vitro-assembled Microtubules Is Dependent upon Microtubule-associated Proteins

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ABSTRACT By use of dark-field light microscopy, secretory granules isolated from the anglerfish endocrine pancreas were observed to attach to and release from microtubules assembled in vitro from brain homogenates. Secretory granules only bound to microtubules assembled in the presence of microtubule-associated proteins (MAPs) and not to microtubules assembled from purified tubulin. The addition of a MAP fraction to purified tubulin restored secretory granule binding. The secretory granules were released from MAP-containing microtubules by the addition of Mg-ATP but not by other nucleotides. The number of secretory granules bound to MAP-containing microtubules was increased in the presence of cyclic AMP. In addition to the associations of secretory granules with microtubules, MAP-containing microtubules also associated with each other. These laterally associated microtubules were dispersed by the addition of Mg-ATP. Electron micrographs confirmed that the associations between MAP-containing microtubules and secretory granules as well as the associations of mictotubules with one another were mediated by the high molecular weight MAPs known to project from the surface of in-vitro-assembled microtubules.

Numerous pharmacological and morphological studies have suggested that microtubules are involved in the intracellular transport of organelles and secretory granules (15, 31, 42, 43, 52). Studies of the endocrine pancreas have suggested that the secretion of insulin is a two-step process, dependent upon the presence of both microtubules and microfilaments (34). In the first step, insulin-containing beta granules lying near the cell periphery fuse with the plasma membrane to release insulin from the cell. The second step may involve the transport of beta granules from the cytoplasm to the periphery of the cell, near the plasma membrane. Based on pharmacological experiments, microfilaments appear necessary for the first step of secretion while microtubules appear to be important for the second step (31, 34).

More direct evidence for the involvement of microtubules in beta granule movements comes from morphological studies of beta granules in monolayer cell culture. In these cells, the membranes of the beta granules were frequently closely associated with or directly linked to microtubules (34, 40). Similar associations between microtubules and organelle membranes have been found in other cells (1, 20, 40, 42, 43, 52). While these studies suggest that various organelles are linked to microtubules in vivo, little is known about the nature of the interactions.

There has been some evidence and much speculation that microtubule-associated proteins, or MAPs, are important for the associations of secretory granules or other organelles with microtubules. Perhaps one of the most vexing problems in understanding the nature of organelle-microtubule interactions is the difficulty of studying what may be weak interactions between the organelles and microtubules. Similar difficulties have been encountered with in vitro studies of the interactions that occur between microtubules and/or MAPs and actin filaments (21, 47). Griffith and Pollard (21) developed a low-shear viscometric technique to study the associations between actin filaments and MAP-microtubules. Sattilaro et al. (47) found that the associations between MAPs and actin filaments were stable enough to permit centrifugation through sucrose gradients but attempts to reproducibly observe actin filaments attached to MAP-microtubules were not successful, possibly due to the very weak MAP-microtubule/actin attachments. Sherline et al. (49) studied the associations between microtu-

THE JOURNAL OF CELL BIOLOGY · VOLUME 93 APRIL 1982 164-174

bule proteins and secretory granule membranes isolated from mammalian pituitary using centrifugation techniques and reported that MAPs were capable of attaching to the secretory granule membranes. Unfortunately, no morphological evidence for the association of pituitary granules with microtubules has been published.

In this report we present direct morphological evidence showing that endocrine pancreatic secretory granules can reversibly associate with brain microtubules in vitro. Dark-field light microscopy, previously used to study microtubule assembly (30, 58) and the sliding of flagellar microtubules (55), was used to directly observe the interactions between microtubules and secretory granules in solution. The associations observed in the light microscope have been verified by examination by electron microscopy of fixed, embedded, and thin-sectioned microtubules and secretory granules. In addition to demonstrating that secretory granules can bind to microtubules in vitro, these data show that MAPs are necessary for the associations and that the associations are increased by cyclic AMP and decreased by ATP. Finally, evidence is presented showing that microtubules interact with one another, presumably via the MAP-filaments. Preliminary reports of these data have been previously presented (56, 57).

MATERIALS AND METHODS

Isolation of Secretory Granules

Anglerfish (*Lophius americanus*) were obtained during the summer months at the Marine Biological Laboratory, Woods Hole, MA. Secretory granules were isolated from the endocrine pancreas by differential centrifugation and sucrose step gradients according to the method of Noe et al. (39). All solutions were modified to include 1 mM $MgCl_2$ and 0.1 mM leupeptin (Sigma Chemical Co., St. Louis, MO, and generous gifts from Dr. Walter Troll, New York University Medical Center, New York). The final secretory granule fraction (F II B) was stored on ice in 2.0 M sucrose, 1 mM $MgCl_2$, and 0.1 mM leupeptin, and was used on the same day for experiments.

Preparation of Microtubule Proteins

Microtubule protein, which contained tubulin and microtubule-associated proteins (MAPs), was purified from chick brain homogenates by three cycles of temperature-dependent assembly and disassembly in PMEG (100 mM PIPES, pH 6.8, 1 mM $MgSO_4$, 1 mM EGTA, 1 mM GTP, and 2 mM dithiothreitol [DTT]) (13). The protein was stored on ice until needed and was used on the same day for experiments. Small aliquots of microtubule protein (0.2–0.5 ml) were warmed to 30°C to assemble microtubules just before each experiment.

Tubulin was purified from chick brain microtubules assembled in the presence of glycerol by phosphocellulose chromatography (65). The column was equilibrated in column buffer (CB) that included 50 mM PIPES, pH 6.8, 1 mM EGTA, 1 mM $MgSO_4$, 0.1 mM GTP, and 0.1 mM DTT. Microtubule protein (20–40 mg) was applied to a column that contained 10–20 ml of phosphocellulose (Whatman P11). Purified tubulin was eluted with column buffer and immediately made 1 mM in GTP and 1 mM in $MgSO_4$. The tubulin-containing fractions were pooled and made 10% in dimethyl sulfoxide (DMSO) (24), and microtubules were assembled by warming the preparations to 37°C. Microtubules were pelleted, resuspended at a concentration of 10–15 mg/ml in PMEG, and stored on ice until needed. Microtubules were assembled at 30°C for 15 min just before each experiment.

MAPs were eluted from the phosphocellulose column with 0.8 M KCl in column buffer as previously described (65). The MAPs were concentrated to 15–20 mg/ml using Aquacide and were desalted by dialysis against PMEG.

Dark-field Light Microscopy

Secretory granule–microtubule interactions were observed in solution by dark-field light microscopy. Microscope slides were washed in Micro (International Products Corp., Trenton, NJ), rinsed with distilled water, coated with Prosil (PCR Research Chemicals Inc., Gainesville, FL), and extensively rinsed again with distilled water. All solutions were filtered with 0.22-μm Millipore GS filters (Millipore Corp., Bedford, MA) to remove refractile particles. Assay components were mixed at 30°C in the following order: 20 μl of secretory granules (40–60 μg of protein), 20 μl of assembled microtubules (100–200 μl of protein), and 200 μl of assay buffer that contained 100 mM 2-(*N*-morpholino)-ethane sulfonic acid (MES), pH 6.5, 2 mM EGTA, 1 mM $MgSO_4$, 2 mM DTT, 2.0 M sucrose, 1 mM GTP and 0.1 mM leupeptin. Other components such as Mg-ATP, 3′,5′-cyclic AMP, ADP, and GTP were added when appropriate.

10 μl of each preparation were placed on a slide and were observed in a Zeiss WL microscope equipped with a Zeiss Universal mirror, Zeiss oil-immersion ultracondensor (N.A. 1.2/1.4), and a Zeiss 40 × N.A. 0.65 objective lens. Preparations were illuminated with a 1,000-W xenon arc lamp (Hanovia XBO 1000) in an Oriel lamp housing with a 48-mm f1.0 silica condensing lens and an Oriel 6242 power supply (Oriel Corp. of America, Stamford, CT). A heat filter was routinely used for observation and photography. Photographs were made with 1-s exposures on Kodak Tri-X film and were developed in Diafine (Baumann Chemical Corp., NJ).

Quantitation of Microtubule-Secretory Granule Associations

Quantitation of the number of microtubules associated with secretory granules was carried out by direct observation in the dark-field light microscope or by analysis of the photographs of the samples. In each case, only those microtubules or secretory granules that floated freely in solution were analyzed, to prevent selection of a population of organelles or microtubules that attached to the surface of the slide or cover slip. Since the organelles tended to attach to the cover slip after 5–10 min, as the slide dried out, samples were never observed for longer than 5–10 min. Fresh slides were made for each time point from a stock reaction mixture kept at 30°C.

Photographic negatives were analyzed by projecting their images onto a large sheet of white paper and tracing the microtubules and secretory granules. The number of microtubules with attached secretory granules was then scored from these tracings. In some experiments, tracings were made on acetate sheets placed over prints of the negatives and were analyzed in the same manner. All micrographs were scored before identification of the sample to prevent bias by the observer.

Electron Microscopy

Microtubules and secretory granules were fixed in solution by adding an equal volume of 0.1 M sodium phosphate buffer, pH 6.8, containing 1 mM $MgSO_4$, 1% tannic acid, and glutaraldehyde. The material was fixed at room temperature for 6–9 h. The samples were then pelleted by centrifugation, postfixed with 0.5% OsO_4 in 0.1 M phosphate buffer, stained *en bloc* with 1% uranyl acetate for 1–3 h, dehydrated in acetone, and embedded in Epon-Araldite. Thin sections were cut with a diamond knife and stained with 1% methanolic uranyl acetate (50%, vol/vol) and lead citrate (61). All samples were examined and photographed in a Zeiss EM 9S or a Philips EM 300 electron microscope.

Electrophoresis

Proteins were resolved on 7.5% acrylamide slab gels according to the method of Laemmli (32). Gels were stained for protein with Coomassie Blue by the procedure of Fairbanks et al. (16). Protein concentrations were determined by the method of Lowry et al. (33).

RESULTS

Secretory Granule and Microtubule Preparations

Secretory granules, isolated from the anglerfish endocrine pancreas, have cores of variable electron density that are surrounded by a single membrane (Fig. 1 *A* and *B*). The secretory granule fraction contained principally insulin-containing beta granules but also a small amount of glucagon- and somatostatin-containing granules (39). The anglerfish endocrine pancreas was used in this study because it provided a larger quantity of pure endocrine tissue than was available from other organisms.

Microtubules, purified from chick brain homogenates by three cycles of temperature-dependent assembly and disassembly, were composed of tubulins and microtubule-associated proteins (MAPs) (Fig. 2). Filamentous projections, known to be composed of high molecular weight MAPs (13, 28, 37), projected from the walls of these microtubules (Fig. 1 *C*). These

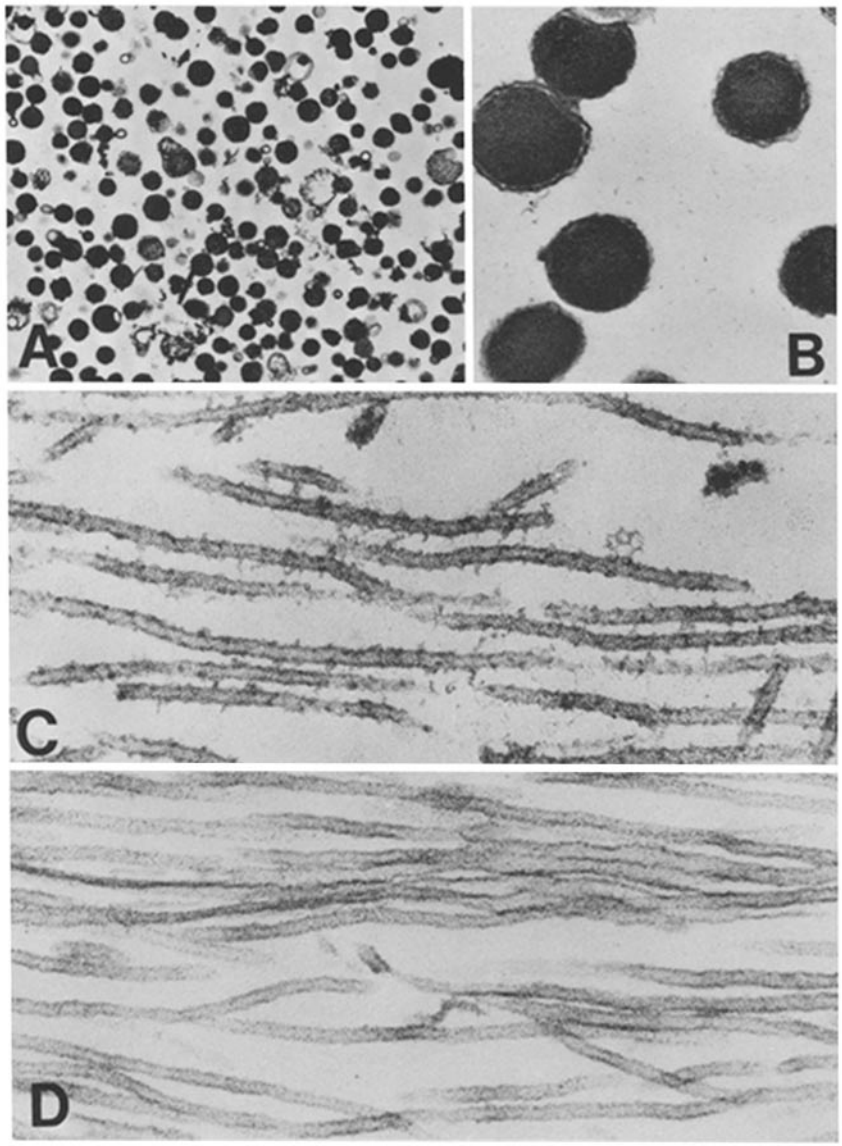

FIGURE 1 Morphological analysis of microtubule and secretory granule fractions. (*A* and *B*) Thin sections of a typical secretory granule preparation isolated from the anglerfish endocrine pancreas. The fractions contained principally secretory granules, most of which had intact membranes. (*C*) Chick brain microtubules assembled in vitro. Filamentous structures are attached to the surface of these MAP-containing microtubules. (*D*) Chick brain microtubules assembled from purified tubulin. These microtubules lack the MAP-filaments. (*A*) × 2,500, (*B*) × 60,000, (*C* and *D*) × 10,000.

microtubules will be referred to as MAP-microtubules. Tubulin that was purified by phosphocellulose chromatography of the microtubule protein lacked the high molecular weight MAPs (Fig. 2) and assembled into microtubules that had smooth filament-free walls (Fig. 1 *D*). These microtubules will be called MAP-free microtubules.

Assay Methods

Dark-field light microscopy was used to directly observe secretory granules and microtubules and to detect any associations that occurred between these organelles. Pancreatic secretory granules appeared as bright refractile spheres and were easily distinguished from the microtubules, which appeared as bright lines (Fig. 3). When secretory granules were bound to the microtubules, both organelles moved about together as a single unit in Brownian motion. When the organelles were not associated with one another, each moved about independently.

Two methods were used to quantify the associations that occurred between the secretory granules and microtubules. Since the associations were readily detectable by direct observation, each experiment was scored for the degree of microtubule-secretory granule associations observed using a scale of 0–5. A score of 0 represented the absence of observable associations between microtubules and secretory granules and a score of 5 represented the maximum number of associations that were observed, which was equivalent to ~50% of the microtubules having one or more secretory granules bound to them. Direct observation of the microtubules and secretory granules not only made the collection of data more efficient but also permitted the analysis of greater fields of microtubules by examining all planes of focus in the preparation.

The second method used to quantify the number of microtubules with bound secretory granules was to photograph randomly chosen fields of microtubules and secretory granules in each experiment and to count the number of associations from enlargements of the negatives. Although quantitation from micrographs might appear to be more accurate and is certainly less subjective than scoring from direct dark-field observations, the superimposition of secretory granules onto microtubules in the plane of the film occasionally made it difficult to unambiguously distinguish free from bound secretory granules. For example, while up to 8% of the MAP-free microtubules appeared to have one or more bound secretory granules, as assayed from light micrographs (Fig. 4; Table I), associations between secretory granules and MAP-free micro-

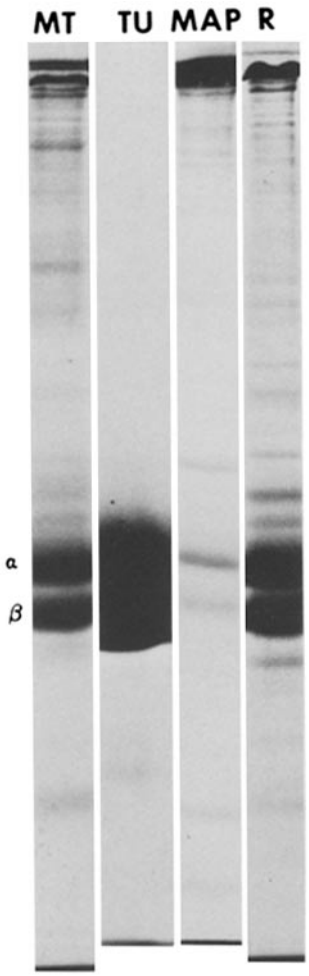

FIGURE 2 Electrophoretic analysis of microtubule fractions. Thrice-cycled microtubules (*MT*) were composed of alpha and beta tubulins and high molecular weight MAPs, whereas microtubules assembled from purified tubulin (*TU*) contained no MAPs. MAP fractions isolated from microtubule protein by phosphocellulose chromatography (*MAP*) coassembled with purified tubulin, as shown by the gel of pelleted microtubules formed from MAP and tubulin fractions (*R*).

tubules were never seen when the organelles were directly observed in solution (Table I). It is likely, therefore, that 1–8% of the apparent associations between microtubules and secretory granules scored from light micrographs represents superimposition of unassociated organelles in the film plane and does not represent the true number of associations that occurred in solution.

In Vitro Associations between Secretory Granules and Microtubules

Secretory granules were mixed with either MAP-microtubules or MAP-free microtubules, were incubated at 30°C for 5 min, and aliquots of the mixtures were placed on a clean microscope slide for observation in dark-field. Mixtures were kept at 30°C throughout the duration of the experiment and individual samples were observed for 5 min or less. In samples viewed for >5 min, secretory granules tended to begin to stick to the cover slip or slide as the preparation gradually dehydrated, making it difficult to assay organelles floating about freely in solution.

Secretory granules attached only to MAP-containing microtubules and were never found to associate with MAP-free microtubules (Fig. 3, Table I). The absence of any binding of secretory granules to MAP-free microtubules was reversed upon the addition of a MAP fraction to purified tubulin (Fig. 2). Microtubules assembled from MAPs and purified tubulin showed an even greater affinity for secretory granules than did the MAP-microtubules assembled directly from chick brain homogenates. Electron micrographs taken of secretory granules and microtubules revealed that the secretory granules were linked to the microtubules by the filamentous MAPs (Fig. 5). When secretory granules were mixed with microtubules assembled from purified tubulin and the MAP fractions, filamentous structures were found to be attached to the secretory granule membranes. Since secretory granule membranes that had not been exposed to MAPs had comparatively smooth membranes, we presume that free MAPs attached to the granule membranes. Preliminary studies showed that MAP-microtubules attached only to the secretory granule membranes and not to mitochondrial or microsomal membranes, which were present in low levels as contaminants in the secretory granule preparations. As one control for nonspecific attachment of particles to microtubules, we mixed polystyrene beads (0.1 μm diameter) with MAP-microtubules. We found no evidence that the beads attached to the microtubules.

Once made, the associations between microtubules and secretory granules appeared to be stable over a period of up to 2 h. The secretory granules rarely detached from the microtubules and resumed independent Brownian motions unless ATP was added to the mixtures. Movement of the granules along the length of the microtubules was never observed under any of the conditions tested.

Effects of Mg-ATP, Mg-ADP, and 3',5'-Cyclic AMP on the Associations of Secretory Granules with MAP-Microtubules

Once it was established that MAPs were required for the association of secretory granules with microtubules in vitro, it was important to determine the optimal conditions for the associations as well as the conditions that induced the release of the linked organelles. Since ATP inhibited the association of pituitary secretory granules with microtubules and MAPs in vitro (49) and the interactions between MAPs and actin filaments (21, 47), the effect of ATP on the attachments of pancreatic secretory granules to microtubules was examined.

As is shown in Table I, few, if any, secretory granules were attached to the microtubules during the first 15 min after microtubules and secretory granules were mixed together in the presence of ATP. After 30–90 min, however, an increased number of secretory granules were linked to microtubules. The frequency with which granules were bound to microtubules after 30–90 min was similar to that observed in the presence of ADP (Table I). This result suggested that ATP must have been hydrolyzed during the 30- to 90-min period, possibly by ATPases or protein kinases in the microtubule and/or secretory granule preparation. Further evidence for this proposal is the observation that the addition of 0.1 mM ATP and an ATP regenerating system composed of 0.1 mg/ml pyruvate kinase and 5 mM phosphoenol pyruvate completely inhibited the association between secretory granules and microtubules for up to 2 h.

The effect of 3',5'-cyclic AMP on the associations was examined because (*a*) Sloboda et al. (51) reported that cyclic AMP stimulated the phosphorylation of MAP-2, (*b*) our results indicated that one of the high molecular weight MAPs is responsible for linking secretory granules to microtubules, and (*c*) cyclic AMP has been found to be important for the secretion of insulin by beta cells (6, 8, 60). The addition of 0.1 mM cyclic AMP and 0.1–0.2 mM ATP to the assay buffer resulted in both an increase in the number of microtubules to which secretory

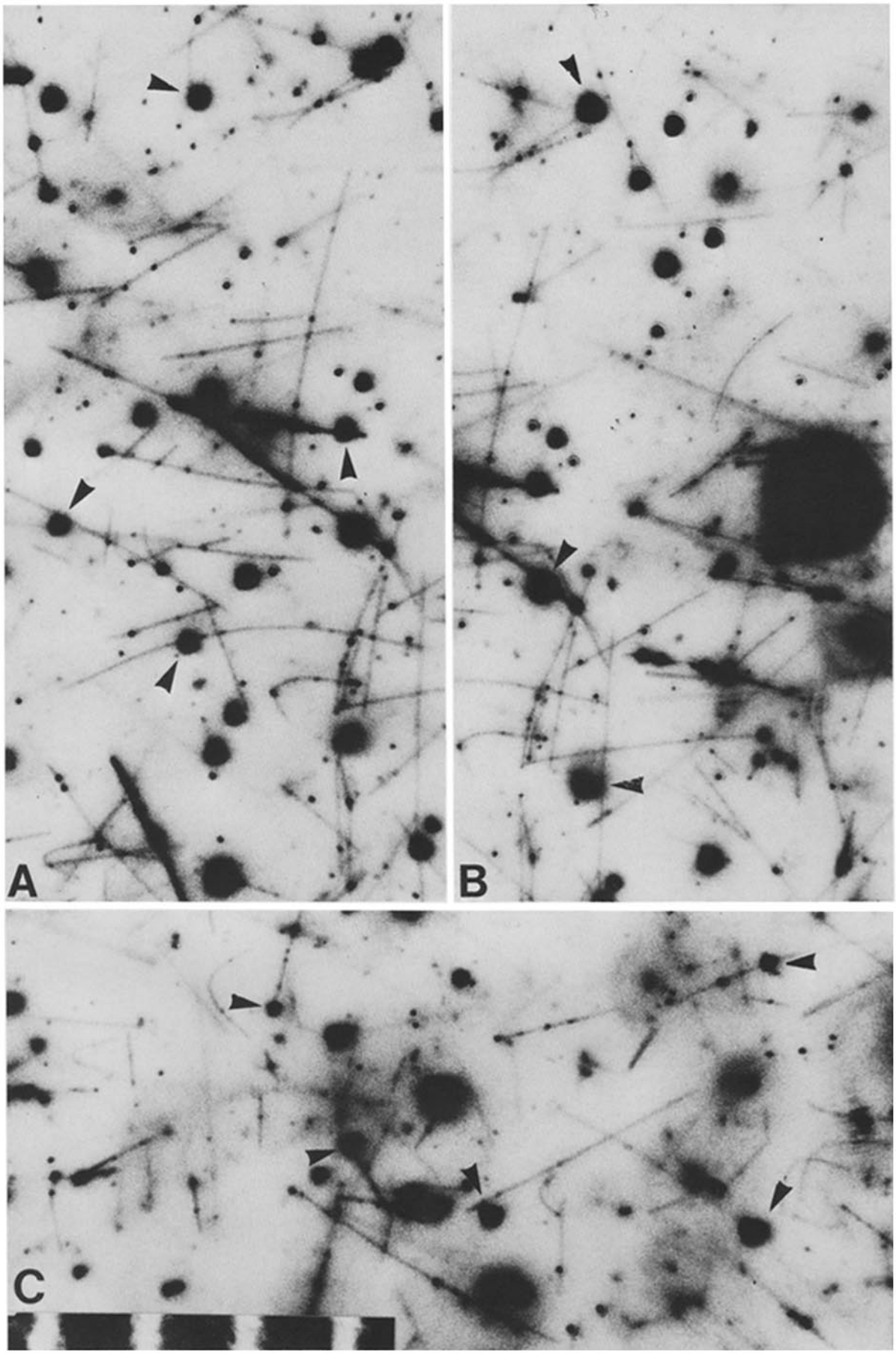

FIGURE 3 Dark-field light micrographs of MAP-microtubules (thin lines) and secretory granules (large dots) in assay buffer. The micrographs have been printed in negative contrast to more clearly visualize microtubules and secretory granules. Scale divisions at lower left = 0.01 mm.

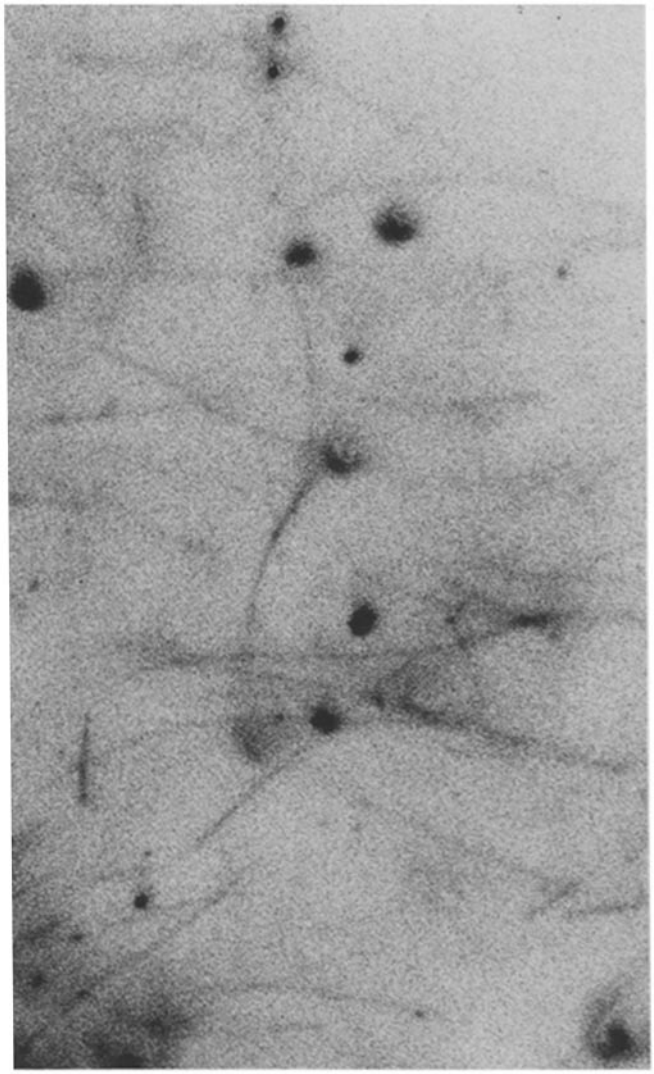

FIGURE 4 Dark-field light micrograph of MAP-free microtubules, assembled from purified tubulin, and secretory granules. Since there were no secretory granules that bound to microtubules, as directly observed in solution, the apparent association between secretory granules and these MAP-free microtubules is due to superimposition of the two organelles in the plane of the negative. The MAP-free microtubules are distinguishable from MAP-containing microtubules in that they are considerably less refractile and therefore appear somewhat thin and wispy. × 1,700.

granules were bound (Table I) and an increase in the number of secretory granules that were attached to any single microtubule. Cyclic AMP also induced the formation of large clusters of secretory granules and microtubules (Fig. 6). No effect of cyclic AMP on associations was observed if ATP was replaced by ADP, and cyclic GMP had no observable effect on associations.

Secretory granules that attached to microtubules in the presence of any of the solutions discussed above (see Table I) rapidly released from the microtubules upon the addition of 5 mM Mg-ATP alone or the addition of 0.1 mM ATP and the ATP regenerating system. After 5 mM Mg-ATP released the granules, they gradually reattached to the microtubules. Within 60–90 min, the number of secretory granule–microtubule interactions was nearly that observed before the addition of Mg-ATP. The addition of fresh 5 mM Mg-ATP again removed some of the reassociated secretory granules but generally not to the same extent as the initial release. In the presence of the ATP regenerating system, the secretory granules did not reassociate with microtubules during a 2-h period. The release of secretory granules appeared to be specific for ATP, since 5 mM concentrations of ADP, AMP, GTP, GDP, ITP, EDTA, or 25 mM KCl or NaCl had little or no observable effect on the microtubule-granule associations.

Lateral Associations of MAP-Microtubules

In addition to the interactions observed between secretory granules and microtubules, MAP-microtubules often associated with one another to form bundles of microtubules. These associations were observed in both the presence and absence of secretory granules and are, therefore, properties of the MAP-microtubules themselves. Larger clusters of microtubules were induced to form by the addition of 0.1 mM cyclic AMP and 0.1 mM ATP (Figs. 6 and 7). The addition of 2–5 mM Mg-ATP resulted in the rapid dispersal of the microtubule clusters and the apparent sliding apart of laterally associated microtubules (Fig. 6). This dispersal was probably not due to the net disassembly of microtubules because the addition of 5 mM Mg-ATP to an identically prepared sample of microtubules resulted in the loss of only 12% of the polymer, as assayed by turbidometric measurements at 350 nm. After dispersal by ATP, the microtubules gradually reaggregated over a 1- to 2-h period at 30°C.

Preparations of bundled microtubules were fixed in solution and examined in thin section using the same techniques as were used for the associations between secretory granules and microtubules. As is shown in Fig. 7, the microtubules appeared to be linked to one another by filamentous MAP projections. Lateral associations of MAP-free microtubules were never observed by dark-field light microscopy or by electron microscopy in our experiments.

DISCUSSION

If microtubules are directly involved in the translocation of secretory granules or other membrane-bounded organelles, the microtubules and the organelle membranes must be linked under certain physiological conditions. A number of morphological studies have indicated that such linkages occur *in situ* (1, 20, 34, 40, 42, 43, 52, 59) and that the connections appear to be mediated by filamentous structures similar to the high molecular weight MAPs associated with the walls of microtubules assembled in vitro from brain homogenates (13, 28, 37). Sherline et al. (49) reported that MAP-microtubules co-sedimented with pituitary granules in vitro and that MAPs were attached to the secretory granule membranes, but they did not present morphological evidence to prove that microtubules were directly attached to secretory granule membranes by the filamentous MAPs. We initially attempted to use sedimentation assays to study the interactions between MAP-microtubules and endocrine pancreatic secretory granules, but, upon morphological examination of fractions separated on sucrose gradients, it was difficult to determine whether the co-sedimentation of microtubules and secretory granules was due to specific interactions or to nonspecific trapping of granules in a microtubule meshwork.

The results reported here provide direct evidence for the association between secretory granules and MAP-microtubules. Since both microtubules and secretory granules floated freely in solution, the attachment of one organelle to another was easily and unambiguously detected by direct observation under

TABLE I

Quantitation of the Binding of Secretory Granules to Microtubules Assembled In Vitro

Assay components	Time of observation after mixing components: 5–15 min	30–90 min
MAP-microtubules	1	1–2
MAP-microtubules + 0.1 mM Mg-ATP	0–1 10 ± 5%, n = 434 17 ± 3%, n = 150	1–2 44 ± 5%, n = 351 43 ± 6%, n = 524
MAP-microtubules + 0.1 mM Mg-ADP	1–2	2–3
MAP-microtubules + 0.1 mM cAMP + 0.1 mM Mg-ATP	4–5 51 ± 23%, n = 190 51 ± 20%, n = 47 57 ± 7%, n = 295 Then add 5 mM Mg-ATP* or 0.1 mM ATP and ATP regenerating system	0–1 12 ± 6%, n = 138
MAP-microtubules + 0.1 mM cAMP + 0.1 mM Mg-ADP	1–2 Then add 5 mM Mg-ATP or 0.1 mM ATP and ATP regenerating system	0–1
MAP-free microtubules	0	0
MAP-free microtubules + 0.1 mM Mg-ATP	0 8 ± 4%, n = 344‡ 1 ± 1%, n = 83	0
MAP-free microtubules + 0.1 mM Mg-ADP	0	0
MAP-free microtubules + 0.1 mM cAMP + 0.1 mM Mg-ATP	0–1§	0
MAP-free microtubules + 0.1 mM cAMP + 0.1 mM Mg-ADP	0	0

Microtubules were mixed with secretory granules and appropriate reagents in assay buffer as described in Materials and Methods and these mixtures were examined using dark-field light microscopy. The extent of microtubule-secretory granule associations observed was quantified on a relative scale of 1–5. A score of 5 represented the maximum degree of associations observed (in which ~50% of the microtubules had one or more secretory granules bound to them) and a score of 0 represented a condition in which no secretory granules were observed to associate with microtubules. The number of microtubules with one or more secretory granules attached to them was also quantified from light micrographs. The data from these observations are expressed as a percentage of the microtubules with attached secretory granules as opposed to the total number of microtubules observed in a single micrograph. The number of microtubules scored are indicated. Each percentage listed represents a separate experiment.

* Mg-ATP was addd 30–90 min after the initial mixing and observations were made 5–15 min after the addition of Mg-ATP.

‡ No secretory granules were ever observed to be associated with MAP-free microtubules under these conditions. The associations recorded from light micrographs likely represents superimposition of granules and microtubules in the plane of the film and are regarded as "background" in all experiments.

§ In one experiment, secretory granules were observed to be associated with MAP-free microtubules. When analyzed by SDS PAGE, however, a small quantity of MAPs were associated with these microtubules.

the dark-field light microscope. In addition, solutions containing different nucleotides or salts were perfused under the cover slip and their effects on the microtubule-secretory granule interactions were directly observed. Although the results reported here were recorded on photographic film, which required long exposure times and the use of dense sucrose solutions to slow the Brownian motion, preliminary experiments using video microscopy have shown that cytoplasmic microtubules can easily be observed in dark field and recorded without using the viscous solutions (Dentler, unpublished results; 2, 26).

MAPs were required for the attachment of secretory granules to microtubules in vitro. As analyzed by both dark-field light microscopy and electron microscopy, secretory granules had no tendency to associate with MAP-free microtubules under any of the conditions tested. Examination of the mixtures of MAP-microtubules and secretory granules revealed that the granule membranes were linked to the microtubules by filamentous MAP-proteins. Secretory granules bound to microtubules that were purified by several cycles of assembly and disassembly as well as to microtubules that were assembled from mixtures of MAP-free tubulin and MAP-fractions. Since MAPs were necessary for the association between secretory granules and microtubules in vitro and since MAP-filaments linked the organelles to one another in a manner similar in appearance to the linkage of organelles to microtubules *in situ*, it is tempting to propose that MAPs are responsible for the attachment of organelles to microtubules in vivo. Further evidence in support of this proposal is provided by the observation that cyclic AMP stimulated the binding of secretory granules in vitro. Since MAP-2 is one of the major components of the filaments attached to the walls of microtubules assembled from brain homogenates (13, 28, 37) and since cyclic AMP stimulates the phosphorylation of MAP-2 (51), the regulation of secretory granule–microtubule interactions in vivo may be related to the phosphorylation of MAP-2. This proposal must

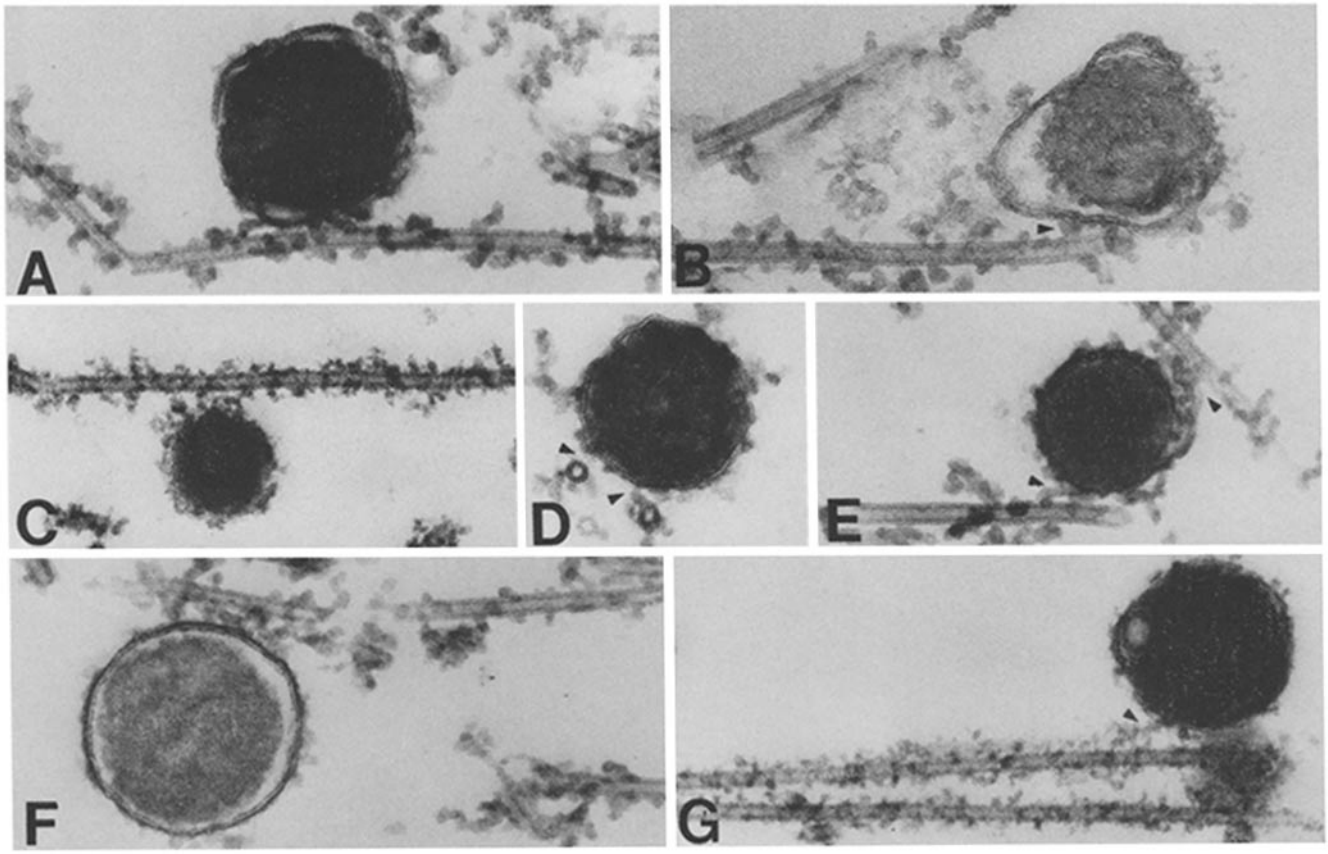

FIGURE 5 Thin sections of MAP-containing microtubules and secretory granules that were fixed in solution and subsequently pelleted and processed for electron microscopy. Secretory granules were attached to microtubules by the MAP-filaments (arrowheads). (*A*, *B*, and *D–F*) × 100,000, (*C*) × 72,000, (*G*) × 80,000.

be viewed cautiously, however, because there are a number of proteins in addition to MAP-2 that are associated with microtubules assembled in vitro (Fig. 2 and references 3, 10, 46, 50), some of which are phosphorylated in the presence of cyclic AMP (51). Since both microtubules and secretory granules were incubated in the presence of cyclic AMP, one should not overlook the possibility that proteins attached to the secretory granule membranes were also effected by cyclic AMP and that membrane-associated proteins are important regulators of the MAP-dependent associations with microtubules.

If the high molecular weight MAPs, particularly MAP-2, are responsible for the attachment of secretory granules to microtubules, it is curious that there were only one to four secretory granules attached to any single microtubule. The major portion of the microtubule surface was free of secretory granules even though examination of the microtubules by electron microscopy revealed that most of the surface was covered with MAP-filaments. Since the periodicity of MAP-2 can be up to one filament every 32 nm (28), it remains to be determined whether only a few of the MAP-2 molecules are in the appropriate conformation to bind secretory granules or whether some other microtubule-associated protein, present in smaller quantities than MAP-2, is responsible for the observed interactions. Alternatively, if cyclic AMP-stimulated phosphorylation is necessary for MAP-secretory granule associations, only a few of the MAP-2 molecules may have protein kinases associated with them and, therefore, only a small population of the MAP-2 molecules would be phosphorylated. Finally, since our experiments showed that rarely more than 50% of the microtubules in a single preparation had secretory granules bound to them, there may have been two or more classes of microtubules (with different MAPs) in the preparations.

Several reports have indicated that both microtubule preparations (4, 19, 23, 63, 64) and pancreatic secretory granules (18) have ATPase activities. ATPases may regulate the associations between MAPs or microtubules and other organelles since it has been shown experimentally that ATP inhibits the association between MAP-microtubules and actin filaments (21), pituitary secretory granules (49), and coated vesicles (48), and between purified MAPs and actin filaments (21, 48). The results reported here show that ATP inhibited the associations between endocrine pancreatic secretory granules and MAP-microtubules. During the first 5–15 min in the presence of 0.1 mM Mg-ATP, very few microtubules bound secretory granules but the granules readily associated with microtubules in the same time period when incubated in 0.1 mM Mg-ADP. After 30–90 min, secretory granules and microtubules incubated in ATP gradually associated with one another and reached the same extent of binding as did the preparations incubated in ADP. These results suggest at least two possibilities. ATP may be inhibitory to vesicle-microtubule interactions, and the attachment of secretory granules to microtubules after 90 min in the presence of ATP may be due to the hydrolysis of ATP. Alternatively, the attachment of secretory granules to microtubules during the 90-min period may be due to a low level of MAP-phosphorylation in the absence of cyclic AMP (51). No attachments were observed in the continuous presence of ATP (maintained by an ATP regenerating system) and ATP released secretory granules from microtubules whether or not the preparations had been previously treated with cyclic AMP. The

release of granules from microtubules was more striking in the cyclic AMP–treated preparations because a greater number of secretory granules were attached to microtubules in these preparations in comparison with preparations not treated with cyclic AMP.

In addition to the interactions between secretory granules and microtubules, MAP-microtubules also tended to group into large refractile clusters in the presence of cyclic AMP, regardless of the presence or absence of secretory granules. Electron micrographs confirmed that the filamentous MAPs were responsible for the lateral associations between microtubules. These results suggest that MAPs may be similar to ciliary and eukaryotic flagellar dyneins that bind tightly to one microtubule (the A-microtubule of a doublet) and more weakly to an adjacent microtubule (the B-microtubule of a doublet). Although Haimo et al. (22) reported that dynein-decorated

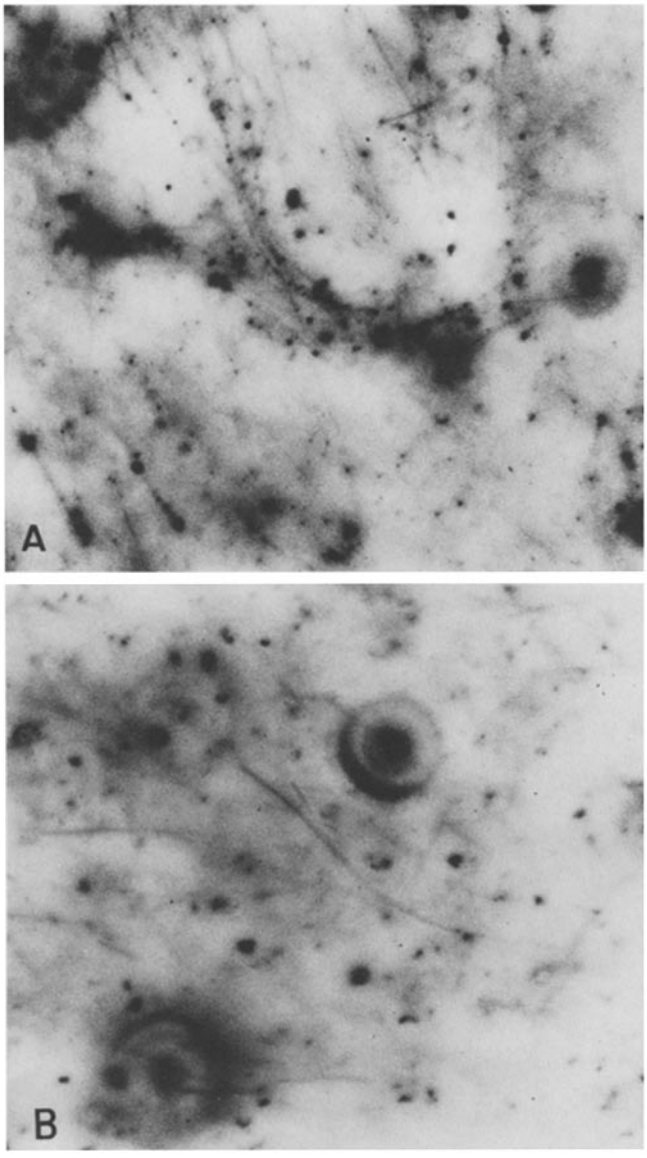

FIGURE 6 Dark-field light micrographs of MAP-containing microtubules incubated with 0.1 mM cyclic AMP. (*A*) Secretory granules and microtubules are frequently clustered together and secretory granules are bound to microtubules regardless of whether they are clustered. (*B*) The same field as in *A* but after the addition of 5 mM ATP. The clustered microtubules were rapidly dispersed and the secretory granules were released from the microtubules. × 6,300.

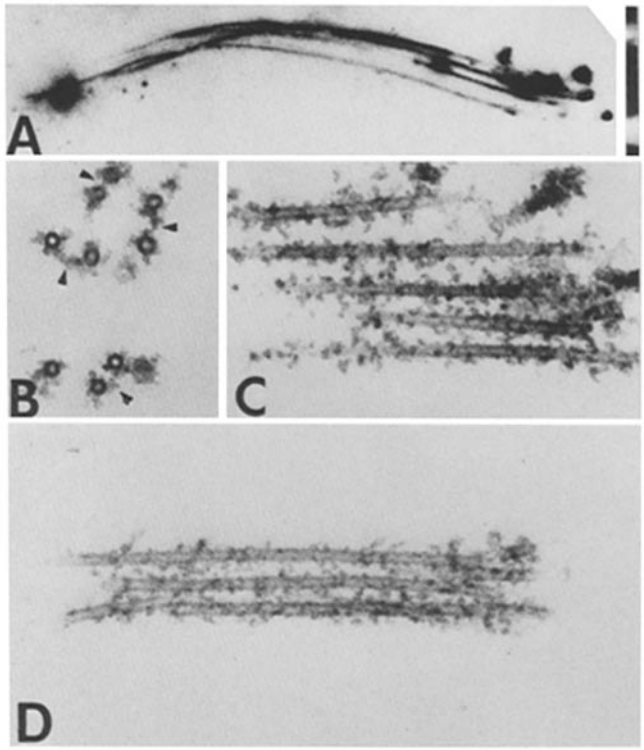

FIGURE 7 Aggregation of MAP-containing microtubules. The associations of MAP-containing microtubules with one another can be seen both in dark-field light micrographs (*A*) and in thin sections of clusters shown in *A* fixed in solution. The bridging of microtubules appears to occur via the MAP-filaments. Similar clustering was never observed in preparations of MAP-free microtubules. (*A*) Marker = 0.1 mm. (*B*) × 114,000; (*C*) × 95,000; (*D*) × 76,000.

microtubules could aggregate via the dynein arms, this is the first report that MAPs can link microtubules together in vitro. The aggregates of microtubules were rapidly dispersed upon addition of Mg-ATP, similar to the dissociation of secretory granules and microtubules by Mg-ATP. Further studies of the MAP-mediated microtubule-microtubule interactions are clearly warranted. Bundles of microtubules linked by filamentous structures have also been observed in extracts of endocrine pancreatic tissue homogenized in microtubule-stabilizing buffers (12).

If the associations between MAP-microtubules and secretory granules observed in these experiments mimic interactions that occur in vivo, it is expected that the specificity of the interactions must be regulated either by specific MAPs or by specific lipids associated with the secretory granule membranes. Even though brain MAPs mediated the linking of anglerfish secretory granules to microtubules in vitro, it is known that not all cells contain the same MAPs (9, 26, 62). Matus et al. (36) recently reported that antibodies against MAP-2 stain neuronal dendrites, but neither axonal microtubules in the same cell nor adjacent glial cells were stained. Specific MAPs may, therefore, be associated with specific microtubules or with specific functions within a cell. It will be important to examine pancreatic cells to determine whether molecules similar to brain MAPs are responsible for linking secretory granules to microtubules in vivo. While specific MAPs have not been identified in pancreatic cells, a preliminary study has shown that microtubules and secretory granules are tightly linked in fractions obtained from the anglerfish endocrine pancreas after homogenization in buffers that contained either hexylene glycol or

taxol to stabilize cytoplasmic microtubules (12, 56). The analysis of these microtubule–secretory granules is currently under way.

As with the MAPs, little is known about the composition of the membranes of endocrine pancreatic secretory granules. Since tubulin has been found to be associated with ciliary membranes (11, 53), brain membranes (see reference 11; 17, 38, 53, 66), liver membranes (44), and phospholipid vesicles (7, 29), it is possible that the MAPs could bind to membrane-associated tubulin as well as to the tubulin of the microtubule wall. The specificity for microtubule-membrane associations may be regulated by the lipid composition of the membrane, which could determine the ability of tubulin to partition in the membrane. Alternatively, actin, known to be associated with secretory granule membranes in vitro (5, 25, 41), may link the secretory granules to MAPs, since other studies have shown that MAPs or MAP-microtubules can associate with actin in vitro (21, 47). Future studies of the complexes of microtubules and secretory granules isolated from tissues, as well as studies of the interactions that occur in vitro between purified MAPs, actin, microtubules, and membrane vesicles, should clarify both the nature of the proteins and lipids involved in the interactions and the mechanisms by which the associations are made or broken.

Since microtubules appear to be important for the sustained release of insulin in vivo (31, 34, 40) and since our results demonstrate that endocrine pancreatic secretory granules can directly attach to microtubules in vitro, it is likely that the association between these organelles is important for the movements of beta granules before insulin release as was first proposed by Lacy, Malaisse, and colleagues (31, 34). Our results are not, however, inconsistent with the passive diffusion model of insulin secretion proposed by Matthews (35), based on calculations of cytoplasmic viscosity and mechanical restraint. If the beta granules are not actively transported to the cell surface but arrive instead by Brownian motion, it is possible that the beta granules are bound to and stored along microtubules and are, therefore, prevented from moving to the cell surface until needed. The release of the granules from the microtubules and their subsequent movement to the cell surface may be dependent upon phosphatases, protein kinases, and ATPases that are associated with the microtubule-membrane complexes.

We would like to thank Dr. Brian Noe for his help in the isolation of anglerfish secretory granules, Dr. Ray Stephens for his encouragement and laboratory space (supported by National Institutes of Health (NIH) grant GM 20644), and Dr. L. I. Rebhun for permitting one of us (K. A. Suprenant) to spend the time at the Marine Biological Laboratory (MBL) to complete this project.

This research was supported by grants to the Physiology Course, MBL (AM 00265) and to W. L. Dentler (NIH grant AM 21672 and University of Kansas grants RRO 7037 and 3045).

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Received for publication 4 May 1981, and in revised form 16 October 1981.

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