MICROTUBULE-MEMBRANE INTERACTIONS IN CILIA

I. Isolation and Characterization of Ciliary Membranes from *Tetrahymena pyriformis*

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ABSTRACT

Tetrahymena ciliary membranes were prepared by four different techniques, and their protein composition was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), electron microscopy, and two-dimensional thin-layer peptide mapping. Extraction of the isolated cilia by nonionic detergent solubilized the ciliary membranes but left the axonemal microtubules and dynein arms intact, as determined by quantitative electron microscopy. The proteins solubilized by detergent included a major 55,000-dalton protein, 1–3 high molecular weight proteins that comigrated, on SDS-PAGE, with the axonemal dynein, as well as several other proteins of 45,000–50,000 daltons. Each of the major proteins contained a small amount of carbohydrate, as determined by PAS-staining; no PAS-positive material was detected in the detergent-extracted axonemes. The major 55,000-dalton protein has proteins quite similar to those of tubulin, based on SDS-PAGE using three different buffer systems as well as two-dimensional maps of tryptic peptides from the isolated 55,000-dalton protein. To determine whether this tubulin-like protein was associated with the membrane or whether it was an axonemal or matrix protein released by detergent treatment, three different methods to isolate ciliary membrane vesicles were developed. The protein composition of each of these different vesicle preparations was the same as that of the detergent-solubilized material. These results suggest that a major ciliary membrane protein has properties similar to those of tubulin.

KEY WORDS membranes · cilia and flagella · microtubules · tubulin · dynein

Although the structure, function, and biochemistry of the 9 + 2 axoneme of cilia and flagella have become relatively well understood, little is known about either the structure or the biochemistry of the membranes that surround the axonemes. Recent studies, however, have demonstrated that the membranes of cilia may be involved in modulating the planar waveform by which cilia beat (R. E. Stephens, unpublished observations). It is, therefore, important to study both the composition of ciliary membranes and the mechanism by which the ciliary membranes interact with the ciliary microtubules at this time.

Recent studies have also demonstrated that the distal tips of both the central-pair microtubules

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and outer-doublet microtubules of flagella (16) and cilia (15) are linked to the surrounding membrane by specialized structures (central microtubule caps and distal filaments). Since these structures are associated only with the distal tips of axonemal microtubules, it is likely that they are involved in the elongation of the microtubules during ciliary and flagellar growth (15, 16). Other studies have demonstrated that lateral associations, or bridges, physically link the ciliary and flagellar membrane to the outer-doublet microtubules along the long axis of the axoneme (3, 17, 18, 34, 36). More recent data suggest that these bridges are composed of a dynein-like, high molecular weight protein and, furthermore, that this protein may be important in the interactions of the axoneme during ciliary movement (17, 18).

Stephens (42) recently reported that molluscan gill ciliary membranes which were solubilized by nonionic detergent were composed principally of a glycosylated tubulin and, moreover, that vectorial labeling techniques demonstrated that the tubulin was localized primarily within the lipid bilayer of the membrane (44). The results reported here both confirm and extend Stephen's observations: using three different techniques, intact *Tetrahymena pyriformis* ciliary membranes were isolated and were compared with proteins released from cilia by detergent extraction (42). The results show that ciliary membranes are composed principally of a weakly glycosylated 55,000-dalton protein as well as several 45,000- to 50,000-dalton proteins and a class of high molecular weight proteins that comigrate, on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), with certain of the axonemal dyneins. Thin-layer peptide mapping was used to identify the 55,000-dalton membrane protein as being very similar to tubulin.

A preliminary report of some of this research was presented to the American Society for Cell Biology (19).

MATERIALS AND METHODS

Isolation of Tetrahymena Cilia

T. pyriformis, strain BIV, were grown to late log phase (3–6 × 10^5 cells/ml) in 2% proteose peptone (Difco Laboratories, Detroit, Mich.) in 500-ml Erlenmeyer flasks on an orbital shaker at room temperature. Cilia were isolated by the procedure of Thompson et al. (45). Approximately 10^9 cells were harvested, washed with fresh medium, and resuspended in 40 ml of fresh medium with 0.3 mM phenylmethylsulfonylflouride (PMSF). The cells were then stirred in an Erlenmeyer flask, and 40 mg of Dibucaine-HCl, dissolved in fresh medium, was rapidly added at 4°C. Deciliation was monitored with a phase microscope and was generally complete within 3–4 min. Immediately after deciliation, fresh medium was added to a final volume of 200 ml. Deciliated cells were sedimented by centrifugation for 4 min, 920 *g*, 4°C, using a JA-20 rotor in a Beckman J-21C centrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The supernate, containing cilia, was decanted and centrifuged at 25,000 *g* for 25 min at 4°C in a JA-21 rotor. The cilia formed a tight, white pellet beneath a large pellet of mucus. The mucus was dislodged by squirting a jet of wash buffer (30 mM Tris, pH 7.6, 5 mM $MgSO_4$, 250 mM sucrose, 0.5 mM EGTA, 0.5 mM EDTA, 0.5 mM DTT) with a Pasteur pipette at the mucus; the dislodged mucus was then decanted, leaving the white pellet of cilia at the bottom of the tube. Cilia were resuspended in wash buffer and washed twice by resuspension and centrifugation before subsequent treatments.

Isolation of Ciliary Membranes

DETERGENT-SOLUBILIZED FRACTION: Ciliary membranes were dissolved using the procedure of Stephens (42). Isolated and washed cilia were resuspended in 0.5–2.0 ml of wash buffer with 0.2–1% Nonidet P-40 (Particle Data Laboratories, Elmhurst, Ill.) and were incubated for 10–15 min, 4°C. The axonemes were sedimented by centrifugation at 11,400 *g*, 4°C, using a JA-20 rotor. The supernate, called detergent-solubilized fraction, was carefully removed and immediately reduced for electrophoresis. Routinely, the cilia were extracted twice by this procedure; a third extraction solubilized very little protein. The final pellets, containing demembranated axonemes, were either fixed for electron microscopy or reduced for electrophoresis.

DETERGENT-RELEASED MEMBRANES: Intact ciliary membranes were released from axonemes by resuspending washed cilia in 2–3 ml of wash buffer made 0.02% in Nonidet P-40 and incubating for 5–10 min at 4°C; frequent agitation with a Pasteur pipette was necessary to release the membranes. Membranes were isolated by layering the extracted cilia over a sucrose step gradient composed of 3 ml of 30% sucrose-Wash buffer layered over 50% sucrose-Wash buffer in 15-ml centrifuge tubes. The gradients were centrifuged for 60 min, 17,170 av. *g*, 4°C, in a JS-13 swinging bucket rotor. Detergent-released membranes were collected from the interface between the 30 and 50% sucrose layers with a Pasteur pipette. The membranes were diluted with Wash buffer and sedimented at 15,300 *g*, 30 min, 4°C, in a JA-20 rotor. The very small amount of membrane obtained in this manner was divided into two aliquots, one of which was reduced for electrophoresis and the other was fixed for electron microscopy. Partially demembranated axonemes, which sedimented through the 50% sucrose to form a pellet, were either fixed for electron microscopy or reduced for electrophoresis.

VORTEX-RELEASED MEMBRANES: Intact ciliary membranes were also released from cilia by mechanical agitation similar to the method used by Snell (38). Washed cilia were resuspended in 3–5 ml of wash buffer with 0.3 mM PMSF and 10 mM sodium azide and were shaken on a vortex mixer (Lab-Line Instruments Super Mixer, Lab-Line Instruments, Inc., Melrose Perk, Ill.) at setting number three for 12 h at 4°C. Membranes released from the cilia were isolated by the sucrose gradient procedure described above. Partially demembranated axonemes, which formed a pellet beneath the 50% sucrose, were reduced for electrophoresis, fixed for electron microscopy, or were extracted with potassium iodide (see below).

KI-EXTRACTED MEMBRANES: Intact ciliary membranes

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were prepared by dissolving the microtubular components of cilia with 0.6 M KI (25). Intact, washed cilia were resuspended in 20–40 ml of either unbuffered 0.6 M KI or wash buffer + 0.6 M KI and were incubated at 4°C for 30 min. The best preparations of ciliary membranes (see Results) were obtained by extracting partially demembranated axonemes, obtained after either brief detergent extraction or vortex treatment, with either buffered or unbuffered 0.6 M KI. In each case, extracted membranes were sedimented by centrifugation at 15,300 *g*, 30 min, 4°C, in a JA-20 rotor. Membranes were routinely extracted and sedimented two to four times before reduction for electrophoresis or fixation for electron microscopy. In some experiments, membranes were washed by resuspension in 30 mM Tris HCl, pH 7.6, to remove salts which prevented the resolution of the two tubulin subunits.

Saponin treatment of KI-extracted membranes was carried out using the procedure of Castle and Palade (13). Membranes were suspended in either 0.3 M Na_2SO_4 or Na_2SO_4 + 15–30 μg/ml saponin and incubated at 4°C for 30 min with frequent mechanical agitation. In some experiments, 0.6 M KI was added to the saponin to attempt to release the axonemal components from membrane vesicles. Membranes were sedimented as described above, and the extraction procedure was repeated two to three times. Sedimented membranes were reduced for electrophoresis and fixed for electron microscopy.

Salt Extraction of Ciliary Dynein

Dynein was solubilized from detergent-extracted axonemes (after detergent-solubilization of membranes) by the procedure of Kincaid et al. (29). Salt-extracted axonemes were sedimented by centrifugation at 11,400 *g*, 15 min, 4°C, and the pellets were fixed for electron microscopy.

Electrophoresis

SDS-PAGE was routinely carried out using 5% acrylamide gels in the Tris acetate system described by Fairbanks et al. (21). Resolution of the two tubulin subunits was carried out by either using 5% Tris-glycine gels (11, 41) or by the method of Laemmli (31), in which a 9% separating and a 3% stacking gel was used. Gels were poured and run either in 0.5 × 9.5-cm tubes (in a Canalco apparatus, Ames Co., Div. Miles Lab Inc., Elkhart, Ind.) or in 0.75 × 120 × 176-mm slabs (in a Hoefer SE500 apparatus, Hoefer Scientific Instruments, San Francisco, Calif.). Gels were routinely stained for protein with Coomassie blue (21). For quantitation of protein, the protein was fixed and SDS was removed from the gels using the procedure described by Fairbanks et al. (21), except that the stain was omitted. The gels were then equilibrated in 7% acetic acid, were subsequently stained with fast green (27), and were scanned at 650 nm with a Gilford model 240 recording spectrophotometer (Gilford Instrument Co., Oberlin, Ohio) equipped with a linear transport. Carbohydrate was detected by the PAS procedure (21), and PAS-stained gels were scanned at 540 nm with the Gilford spectrophotometer. Gel scans were integrated using either a compensating polar planimeter or a Hewlett-Packard Model 9810A calculator equipped with digitizer.

Electron Microscopy

Whole cilia, demembranated axonemes, and isolated membranes were sedimented as described above. The small pellets were fixed for 1–2 h with 1% glutaraldehyde (Electron Microscope Sciences, Fort Washington, Penn.) in 0.2 M sucrose, 0.1 M sodium phosphate, pH 7.0, at 4°C, and postfixed with 1% osmium tetroxide in phosphate-sucrose buffer. The pellets were then washed thoroughly with distilled water and were stained for 30 min, room temperature, with 1% aqueous uranyl acetate. Samples were dehydrated in an acetone series and embedded in Spurr resin (39). Thin sections were cut with a diamond knife, stained with methanolic uranyl acetate and lead citrate, and examined and photographed in a Philips EM 300 electron microscope.

Peptide Mapping

Two-dimensional peptide mapping was carried out using thin-layer silica gel plates as described by Stephens (43). Protein was prepared by detergent-extraction (see above) of isolated cilia. The solubilized protein (supernate) and the axonemal protein (pellet) were immediately reduced in guanidine-HCl (Schwartz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.) and subsequently alkylated with iodoacetimide (Sigma Chemical Co., St. Louis, Mo.). After exhaustive dialysis against distilled water, the precipitated protein was stored at −80°C. The protein was then resuspended, dansylated, and run on 7% Tris-glycine preparatory gels (41). The dansylated protein was visualized using a mineral light, and the 55,000-dalton protein bands were cut out. Subsequent extraction, digestion with trypsin, chromatography, and electrophoresis were carried out exactly as described by Stephens (43).

RESULTS

Detergent-solubilized Fraction

When isolated *Tetrahymena* cilia were extracted with 1% Nonidet P-40, most of the ciliary membrane dissolved, although small patches of membrane occasionally remained attached to the outer-doublet microtubules (15–18). Most of the membrane was solubilized with one detergent wash, and the remainder was solubilized with a second detergent wash; additional detergent washes solubilized very little protein. Morphological analysis of the axonemal pellet after two or more detergent washes showed that virtually all of the microtubules and their major associated structures remained intact (Table I). The detergent-solubilized proteins must, therefore, have come from the dissolved ciliary membrane or the ciliary matrix.

Electrophoretic analysis of the detergent-solubilized fraction is shown in Figs. 1 and 3. When analyzed using Tris-acetate SDS-PAGE (Fig. 1), the major solubilized proteins were a 55,000-dalton protein, which co-migrated with axonemal tubulin and which comprises 60% of the solubilized protein (Table II), and several high molecular weight proteins which co-migrated with axonemal dynein (Fig. 1). The major protein band was further analyzed using both Tris-glycine and Tris-Cl SDS-PAGE (Figs. 1–3), in which the microtubule protein tubulin is split into two approximately

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TABLE I

Occurrence of Axonemal Structures after Detergent and Salt Extraction of Cilia

Experiment	No. of outer-doublet microtubules with:			Central microtubules	
	2 Arms	1 Arm	0 Arms	Present	Absent
Detergent-extracted					
Exp. 1	97% (254)	3% (7)	0	95% (55)	5% (3)
Exp. 2	99% (188)	1% (1)	0	95% (21)	5% (1)
Exp. 3	98% (243)	1% (2)	1% (3)	95% (82)	5% (4)
Salt-extracted					
Exp. 1	7% (21)	4% (12)	89% (255)	76% (25)	24% (8)
Exp. 2	1% (3)	2% (6)	97% (19)	50% (19)	50% (19)

Cross-sections of ciliary axonemes were quantitated for the presence of 0, 1, or 2 arms attached to the A-microtubule of each outer-doublet microtubule and for the presence or absence of the central microtubules after extraction of the axonemes with 1% Nonidet P-40 or 1% Nonidet P-40 plus salt (29). Axonemes with only one central microtubule were scored as having no central microtubules. The numbers of axonemes or outer-doublet microtubules scored are in parentheses.

equimolar bands: α and β tubulin. The results show that with both Tris-glycine SDS-PAGE (Fig. 1) and Tris-Cl SDS-PAGE (Fig. 3), the 55,000-dalton band was split into two approximately equimolar bands. This suggests that the 55,000-dalton membrane protein may be tubulin. It is important to realize that the separation between the *Tetrahymena* axonemal tubulins and the membrane proteins is very small when compared with the separation of tubulins from either molluscan ciliary tubulins (42) or tubulins isolated from bovine brain by in vitro assembly procedures (unpublished data); this is characteristic of *Tetrahymena* tubulins in a variety of different SDS-PAGE systems that have been tested.

In contrast to the results obtained with Tris-acetate (Fig. 1) or Tris-glycine (Figs. 1 and 2) SDS-PAGE, Tris-Cl SDS-PAGE (Fig. 3) resolved the 55,000-dalton protein band into two classes of polypeptides: one of which co-migrated with axonemal tubulin at 55,000 daltons and another class of 1–3 proteins which migrated between 45,000 and 50,000 daltons. The number of proteins in the 45,000- to 50,000-dalton region and their amount relative to the 55,000-dalton proteins varied somewhat from preparation to preparation but the 55,000-dalton proteins generally comprised 61–78% of the proteins in the 45,000- to 55,000-dalton region of the gel and 40–60% of the total membrane protein, as determined by quantitative analysis of fast-green-stained gels (Table II). While the 45,000- to 50,000-dalton proteins comprised ~15% of the detergent-solubilized membrane fraction (and up to 30% of the intact membrane vesicles), they comprised <4% of the total ciliary proteins and were not detectable in gels of demembranated axonemes; it is likely, therefore, that these proteins are unique to the ciliary membrane.

Since Stephens (42) previously reported the presence of a glycosylated tubulin in scallop gill ciliary membranes, the 55,000-dalton protein in *Tetrahymena* ciliary membranes was analyzed for carbohydrate by the PAS procedure. Using Tris-acetate SDS-PAGE, in which there was a single 55,000-dalton protein band, most of the PAS-positive material was in the 55,000-dalton protein band, the remainder being in the high molecular weight region, over the dynein-like proteins (19). No PAS-positive material was found in the axoneme fractions. In the Tris-Cl SDS-PAGE, in which the 55,000-dalton band is resolved into two 55,000-dalton proteins and the 45,000- to 50,000-dalton proteins, the PAS-positive material was in both of the major bands as well as over the high molecular weight region containing the proteins which comigrated with dynein (compare Figs. 3 and 4). Virtually identical results were found with each of the two detergent washes (Fig. 4). The small amount of PAS-positive material present in the demembranated axoneme fraction (Fig. 4) was likely nonspecific staining, as the gel was loaded with ~20 times as much protein as was loaded on the membrane gels. In contrast to the membrane samples, in which the PAS-positive bands were visible to the naked eye (Fig. 4), the diffuse PAS-positive material in the axoneme fractions was only apparent after spectrophotometric analysis.

In addition to the major 55,000- and 45,000- to 50,000-dalton proteins, ~20 higher and lower molecular weight proteins were present in membranes

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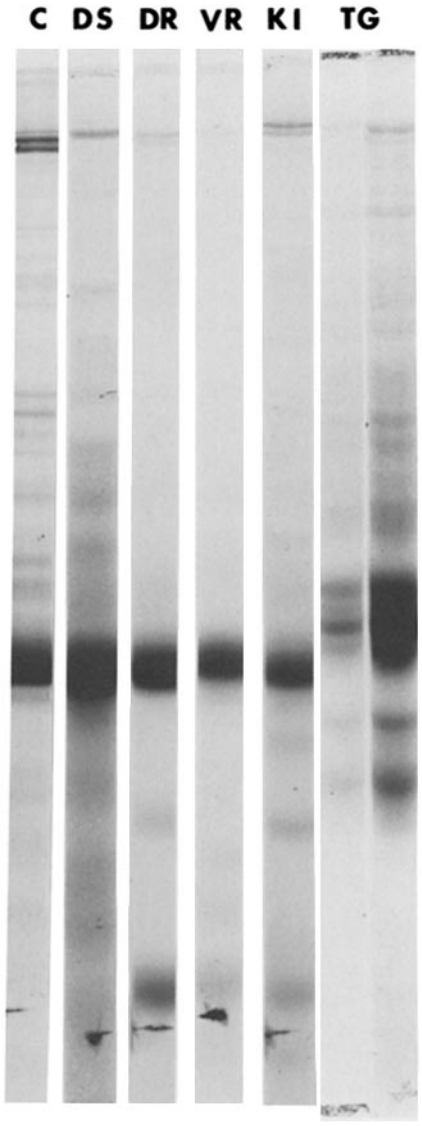

FIGURE 1 SDS-Tris-acetate-PAGE analysis of *Tetrahymena* ciliary membrane fractions stained with Coomassie blue. *C*, whole cilia; *DS*, detergent-solubilized fraction; *DR*, detergent-released membrane; *VR*, vortex-released membrane; *KI*, membrane vesicles obtained after 0.6 M KI extraction of partially demembranated axonemes; *TG*, two loadings of detergent-solubilized membrane in which the tubulin subunits were resolved after reduction and alkylation by 5% Tris-glycine SDS-PAGE. See Materials and Methods for preparation techniques for each fraction.

but not axonemes (Fig. 3). Of particular interest were the high molecular weight proteins. Detailed electrophoretic analysis (Fig. 7) showed that the

TABLE II

Quantitation of the Major Tetrahymena Ciliary Membrane Proteins

Preparation	SDS-PAGE	Dynein	55,000-dalton proteins	45,000- to 50,000-dalton proteins
		%	%	%
Cilia	TAc	15	60	
	TCl	25	49	3
Detergent-solubilized	TAc	2	60	
I	TCl	6	40	16
II	TCl	4	54	14
Detergent-released	TAc	1	60	
	TCl	1	54	26
KI	TAc	12	60	
	TCl	7	53	24

Gels of membrane proteins were stained with fast green and were scanned in a spectrophotometer. Data from Tris-acetate SDS-PAGE (TAc), in which one major 55,000-dalton protein band was observed (see Fig. 1), and Tris-chloride SDS-PAGE (TCl), in which two 55,000-dalton and one to three 45,000- to 50,000-dalton protein bands were observed (see Fig. 3) are presented.

detergent-solubilized fraction contained a group of 3–4 proteins which co-migrated with the lower molecular weight axonemal dyneins (26). Since other results demonstrated that these detergent-released proteins had a sedimentation coefficient and ATPase activity similar to those of axonemal dynein (17, 18), it was important to determine whether this dynein was released from the outer-doublet microtubules. Since dynein comprises the arms which are attached to the outer-doublet microtubules (25), quantitative electron microscopy was carried out to assay for the presence of arms in detergent-extracted axonemes. At the same time, the breakdown of the central-pair microtubules was quantified to insure that the 55,000-dalton protein(s) solubilized by detergent was not due to the breakdown of axonemal microtubules. The central-pair microtubules were scored because they are the most labile of all of the axonemal microtubules. The results (Table I) show that, in three separate experiments, 97–99% of the outer-doublet microtubules had both arms attached to the A-microtubules and that 95% of the axonemes had both central microtubules intact. By contrast, in axonemes that had been extracted with salt to solubilize dynein arms, only 3–11% of the arms remained attached to the outer-doublet microtubules. In four other experiments, 97 ± 2% (2,349 scored) of the outer-doublet microtubules had

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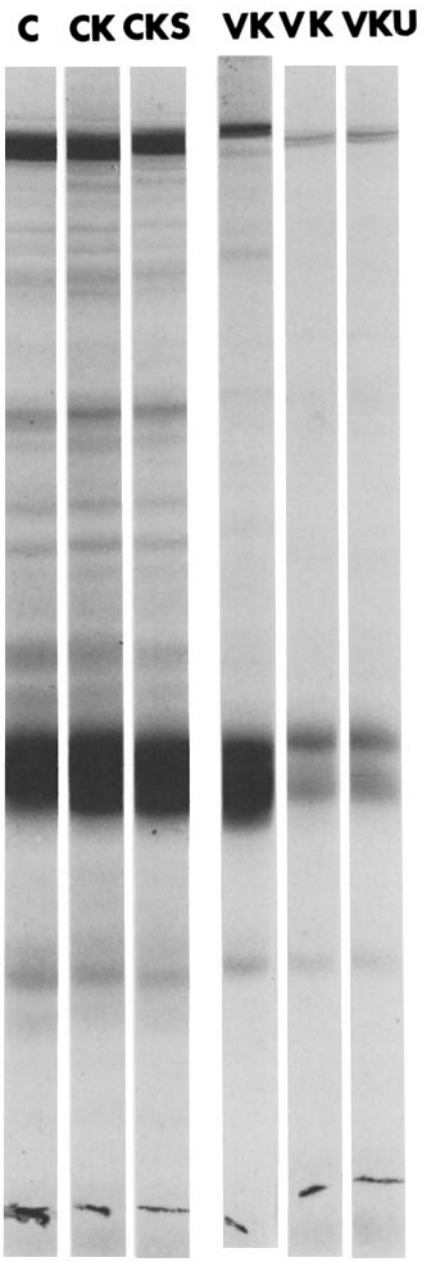

FIGURE 2 SDS-Tris-glycine-PAGE analysis of membrane vesicles prepared by 0.6 M KI extraction of whole cilia (*C*, *CK*, and *CKS*) and partially demembranated cilia (*VK* and *VKU*). The compositions of whole cilia (*C*) and cilia extracted with KI (*CK*) or KI and saponin/Na_2SO_4 (*CKS*) were identical while many of the protein bands migrating between tubulin and dynein were removed when partially demembranated axonemes were extracted with either buffered (*VK*) or unbuffered (*VKU*) KI. The two tubulin subunits were resolved in the KI-membrane fractions when lower amounts of protein were applied to the gels (*VK* and *VKU*). See Fig. 6 for electron microscope analysis of the membrane fractions.

both arms intact and 99 ± 2% (401 scored) of the axonemes contained both central microtubules, even after up to four detergent washes. These results show that the major 55,000-dalton protein(s) and the high molecular-weight dynein-like proteins solubilized by detergent are probably not due to the release of axonemal components and are, therefore, components of the membrane or, possibly, the matrix.

Detergent-released and Vortex-released Membranes

Although ciliary membranes were tightly linked to outer-doublet microtubules (17, 18), a small fraction of intact membranes could be released from cilia by mechanical agitation in the presence of low concentrations of nonionic detergent (detergent-released membranes) or by vigorous agitation of cilia using a vortex mixer (vortex-released membranes) and subsequently isolated from the partially demembranated axonemes on sucrose gradients. Both methods yielded preparations of empty membrane vesicles which were free of either microtubules, axonemes, or intact cilia, as assayed by thin-section (Fig. 5) and negative-stain electron microscopy. High-magnification electron microscopy (Fig. 5 *B* and *D*) showed that each of the membrane preparations contained typical bilayer unit membranes. The two preparations of membrane were, however, morphologically different: while the detergent-released membrane vesicles were of approximately uniform diameter (Fig. 5 *C* and *D*), the vortex-released membranes were quite heterogeneous and appeared as small flattened disks, hollow vesicles, and, occasionally, small tubular vesicles (Fig. 5 *A* and *B*). Although the vortex-released membranes exhibited a variety of different forms, thin sections generally revealed these vesicles to be composed of typical membrane bilayers.

Electrophoretic analysis showed that the same major proteins found in detergent-solubilized fraction were also present in each of the intact membrane vesicle preparations. In Tris-acetate SDS-PAGE (Fig. 1), the major membrane protein migrated at 55,000 daltons. Tris-Cl SDS-PAGE (Fig. 3) showed both the 55,000- and 45,000- to 50,000-

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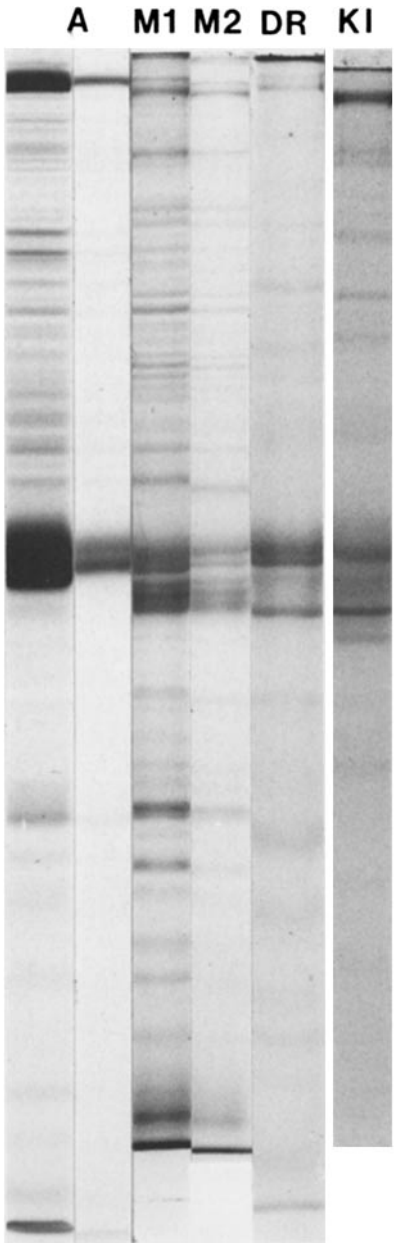

FIGURE 3 SDS-Tris-Cl-PAGE analysis of ciliary membrane fractions. The major membrane proteins that were associated with all ciliary membrane fractions were proteins which co-migrate with tubulin, 1–3 proteins between 45,000 and 50,000 daltons, and proteins which comigrate with axonemal dynein. *A*, detergent-extracted ciliary axonemes; *ML* and *MS*, fractions solubilized by one and two detergent extractions of cilia, respectively; *DR*, membrane vesicles released by mechanical agitation in low concentrations of detergent; *KI*, membrane vesicles prepared from partially demembranated cilia by KI extraction.

dalton proteins. Although the relative proportions of the 55,000- and 45,000- to 50,000-dalton proteins varied somewhat from preparation to preparation, both of the major protein bands were always present in the isolated membrane preparations. Although the high molecular weight proteins which comigrated with dynein were also present in the intact membranes, they were generally present in lower amounts than in either the detergent-solubilized fraction or the KI-membranes (see below).

Although quantitation of the breakdown of axonemal components was not carried out after brief detergent-extraction or after vortexing, it is unlikely that a great amount of breakdown occurred because (*a*) more severe detergent-extraction did not disrupt the axonemes (Table I), (*b*) most of the axonemes appeared intact after vortex-treatment (Fig. 6*A* and *B*), and (*c*) the membranes were washed extensively to remove any solubilized axonemal components before electron microscopy and SDS-PAGE (see also reference 8).

Potassium Iodide-extracted Membranes (KI-Membranes)

When isolated intact cilia were extracted with either unbuffered 0.6 M KI or 0.6 M KI in Wash buffer, most of the microtubules were rapidly dissolved, leaving a population of membrane vesicles (Fig. 6*E* and *F*). Electron microscopy of thin-sectioned membranes revealed, however, that a majority of the membrane vesicles were filled with amorphous material and, occasionally, intact axonemes and parts of outer-doublet microtubules. The amorphous-appearing material was not released from the vesicles either by extractions in large volumes of 0.6 M KI or after osmotically shocking the vesicles by resuspension in 10 mM Tris-Cl, pH 7.6. The extracted membranes were also washed with 15–30 μg/ml saponin in 0.3 M Na_2SO_4, a method that has been used to extract contaminants from secretory granule membranes (13). None of these procedures, however, released the amorphous material from the vesicles. Electrophoretic analysis of these membrane preparations showed no significant differences in the composition of whole cilia, KI-extracted cilia, or cilia that had been treated with KI and saponin (Figs. 2 and 3). Apparently, the ciliary membranes sealed tightly around the dissolved axonemes and prevented the release of their contents.

To circumvent the trapping of dissolved axonemal components, partially demembranated

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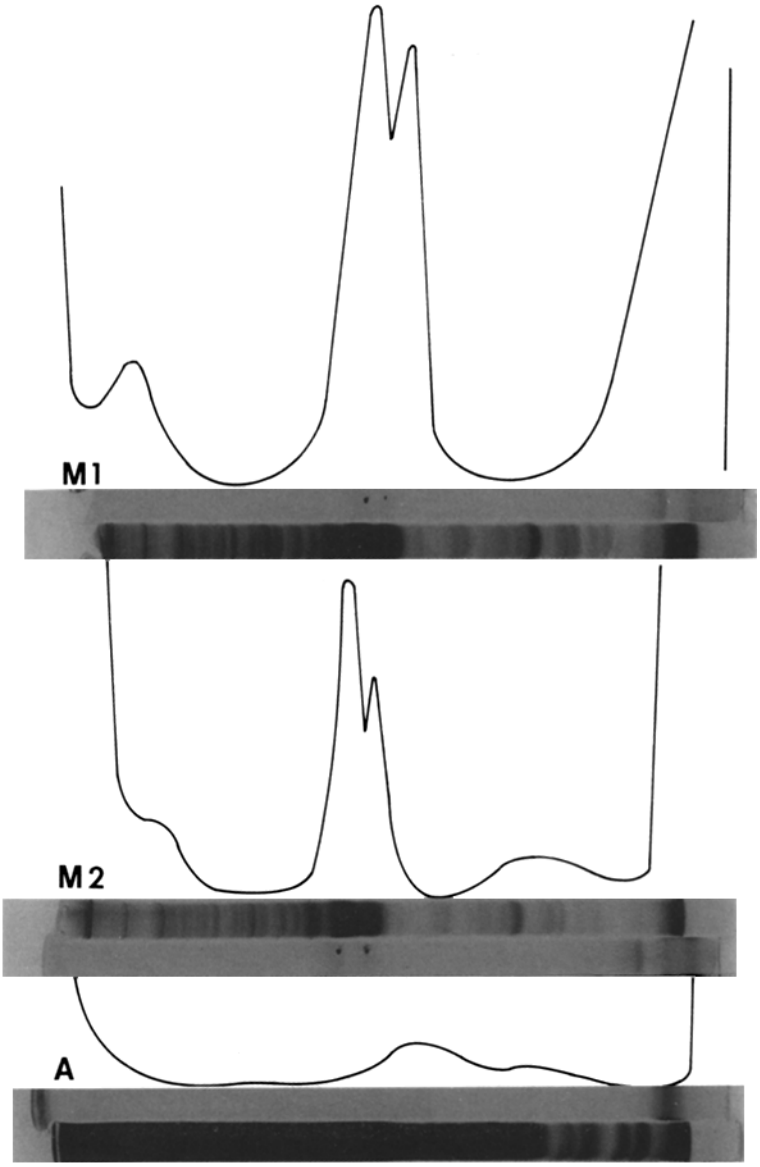

FIGURE 4 Analysis of carbohydrate in detergent-solubilized fractions (*M1* and *M2*) and axonemes after detergent-extraction (*A*). Densitometric scans (560 nm) and the gels stained by the PAS procedure are shown with their Coomassie blue-stained counterparts. Dots of India ink were applied to the PAS-positive bands after scanning. PAS-stained gels of the detergent-solubilized protein were loaded with four times the amount of protein loaded on the Coomassie blue-stained gels. Axonemal protein was loaded at eight times the concentration applied to the Coomassie blue-stained gel. The principal PAS-positive bands in the solubilized fractions are those with mol wts of 55,000 and 45,000–50,000; a small amount of PAS-positive material is also in the high molecular weight region of the gel, although it is not possible to assign a specific protein to this PAS-stained region. The small amount of PAS-positive material in the axoneme fraction is probably nonspecific staining due to the excessive amount of protein loaded on this gel. The top of the gels is on the left. Bar, 0.05 OD unit.

cilia, after 12 h of vortexing (Fig. 6*A* and *B*) or after the preparation of detergent-released membranes, were extracted with 0.6 M KI (vortex-KI-membranes and KI-membranes, respectively). In contrast to the KI-extracted whole cilia, these preparations were composed of morphologically

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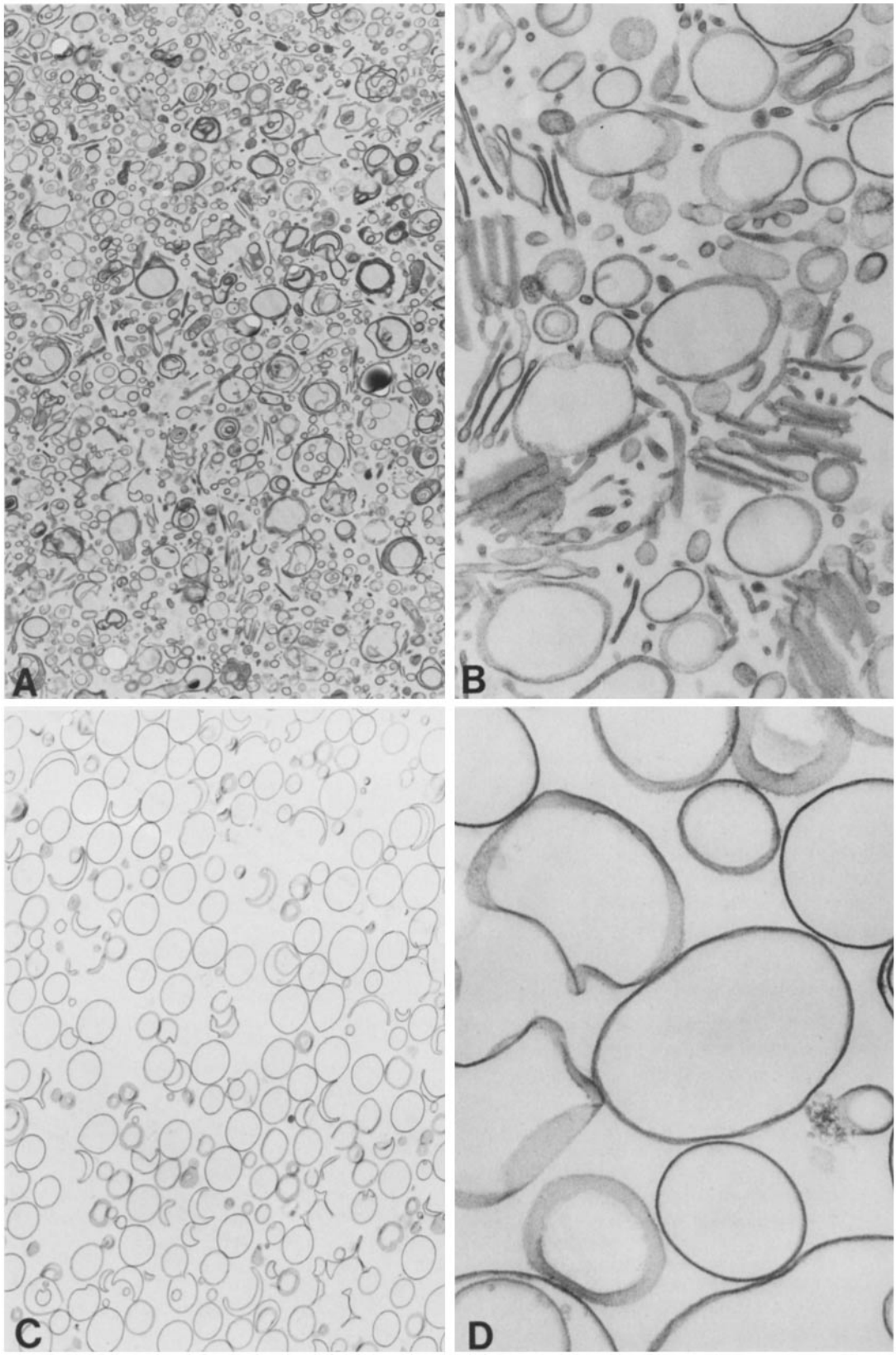

FIGURE 5 Thin sections of vortex-released (*A* and *B*) and detergent-released (*C* and *D*) ciliary membranes. Whereas detergent-released membranes formed apparently empty vesicles of approximately uniform diameter, the vortex-released membranes appeared more flexible and formed a variety of different shaped vesicles. Membranes in each fraction were composed of the typical "unit membrane" structure. *A* and *C*, × 8,500; *B* and *D*, × 52,500.

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empty membrane vesicles (compare Fig. 6 *C* and *D* with *E* and *F*). The small amount of amorphous material that did remain with these membranes was generally associated with the membrane surface.

Electrophoretic analysis of the KI-membranes (Figs. 1–3) showed that the major membrane proteins were virtually identical to those found in each of the other membrane preparations. Tris-acetate SDS-PAGE showed one major 55,000-dalton band and high molecular weight dynein-like proteins. The 55,000-dalton band was split into two bands on Tris-glycine SDS-PAGE and into two 55,000 bands and the 45,000- to 50,000-dalton bands on Tris-Cl SDS-PAGE.

Peptide Mapping

The major proteins in each of the *Tetrahymena* ciliary membrane preparations appeared quite similar to the membrane-associated tubulin found in scallop gill ciliary membranes (42). Both proteins co-migrated with tubulins on different SDS-PAGE systems and both contained a small amount of PAS-positive material. The 55,000-dalton membrane proteins were released from cilia by detergent-extraction (detergent-solubilized fraction) and were isolated by SDS-PAGE as described by Stephens (41). Detergent-solubilized fractions were used because their major proteins were identical to those found in intact membranes and they yielded a greater amount of protein than did membrane vesicle preparations. Since *Tetrahymena* α and β tubulins could not be reliably resolved on SDS-PAGE, the combined 55,000-dalton protein band from axonemes and from membrane fractions were compared with one another. The 45,000- to 50,000-dalton proteins did not significantly contaminate the 55,000-dalton protein because they could be distinguished from the 55,000-dalton band by running the preparatory gel for a sufficient time to resolve the 55,000 and 45,000–50,000 bands by inspection with a mineral light (41). Moreover, quantitative densitometry (Table II) showed that 70–80% of the protein in the 55,000- to 45,000-dalton region of the gel is composed of the 55,000-dalton protein.

The results (Figs. 8 and 9, Table III) show that, at both pH 3.5 and pH 6.5, the 55,000-dalton protein is remarkably similar to the tubulin isolated from the ciliary microtubules. By comparing the number of peptides in each individual preparation (membrane 55,000-dalton protein = M, axonemal tubulin = A) with the maps of the mixed peptides (equal amounts of A and M = AM), the percentage of axonemal tubulin peptides that have counterparts in the membrane 55,000-dalton protein can be determined to be between 74 and 77%. This high degree of coincidence strongly suggests that the ciliary-membrane 55,000-dalton protein is a tubulin-like molecule.

DISCUSSION

The results reported here show that *Tetrahymena* ciliary membranes are composed of a 55,000-dalton protein, one to three proteins of 45,000–50,000 daltons, and high molecular weight proteins that co-migrate with dynein, the protein responsible for force production during ciliary beating (Table II; references 25, 26, 29). Moreover, the major 55,000-dalton protein has properties very similar, but not identical, to those of tubulin, based on its co-migration with *Tetrahymena* axonemal tubulin on three different SDS-PAGE systems and by the coincidence of ~75% of its tryptic peptides when compared with two-dimensional peptide maps of axonemal tubulin. These results suggest that a tubulin-like protein is present in ciliary membranes of *Tetrahymena*.

Is the 55,000-dalton tubulin-like protein really associated with the membrane or is it simply a contaminant of membrane fractions due to the breakdown of ciliary microtubules or of soluble proteins in the ciliary matrix (i.e., all proteins that are not associated with the membrane or the axonemal microtubules)? Certainly, the principal method used to isolate the protein, detergent-extraction of the whole cilium, should solubilize both membrane and matrix proteins. The axonemal microtubules, however, were not significantly disrupted by detergent-extraction, on the basis of quantitation of the amount of breakdown from electron micrographs (Table I). Moreover, if microtubule breakdown were to account for the tubulin-like protein in the detergent washes, it would be expected that second, third, or even fourth washes would yield more soluble tubulin-like protein. In the experiments reported here, a second detergent wash releases ~20% of the protein released by the first wash; further extractions solubilize virtually no protein. In experiments designed to analyze bridges that link the ciliary membrane to the microtubules (17), up to four detergent washes over a long period of time were routinely used (with and without the cross-linking reagent)

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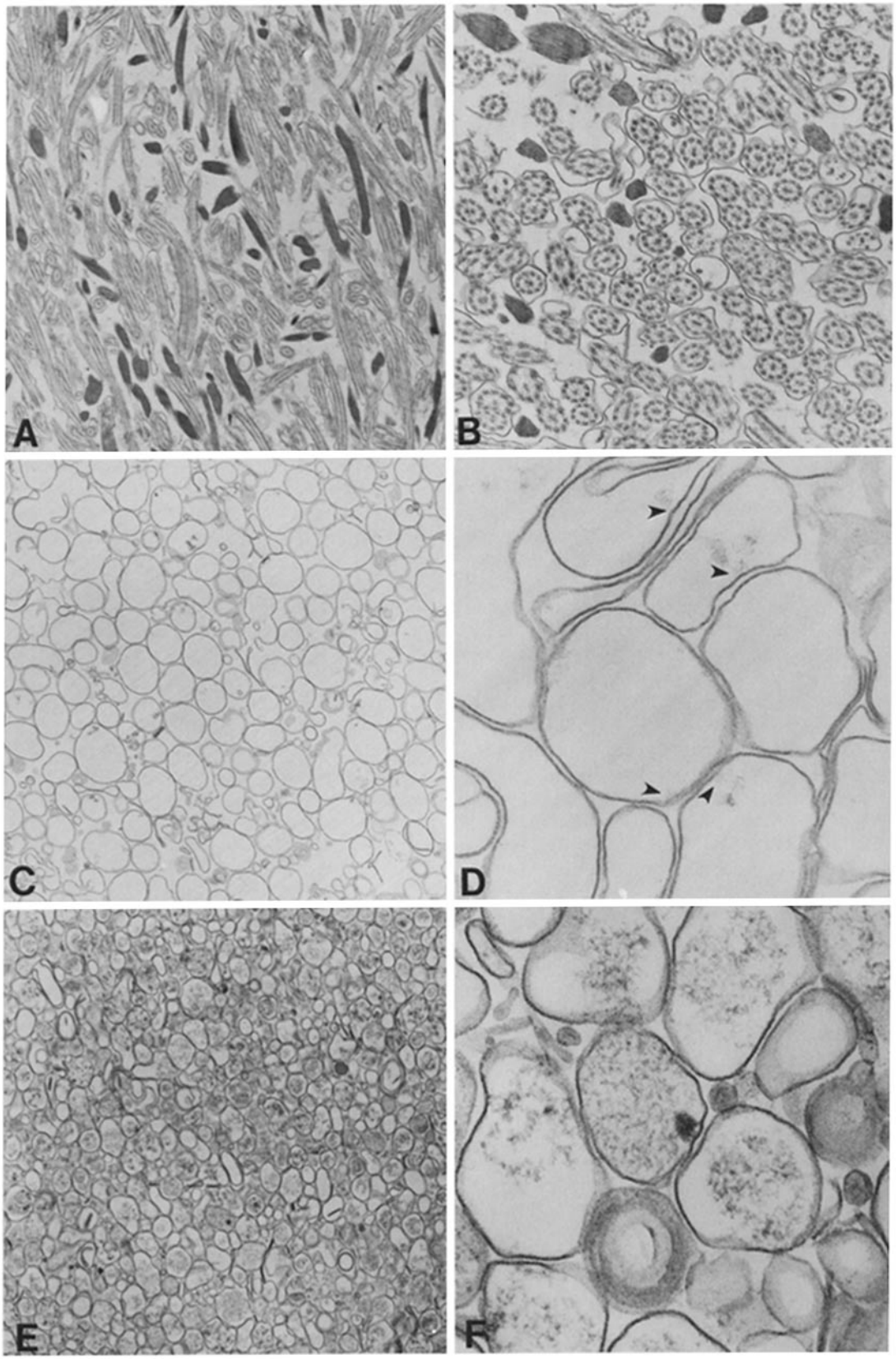

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and the ciliary microtubules remained intact, as assayed by thin-section electron microscopy (see Fig. 4 *B*, reference 17). The presence of the 55,000-dalton protein in detergent washes cannot, therefore, be accounted for by the breakdown of ciliary microtubules.

To determine whether the 55,000-dalton protein was a matrix protein that was released by detergent extraction of the cilia, ciliary membrane vesicles were prepared by three different methods. When analyzed by SDS-PAGE, each of these membrane preparations had a protein composition that was essentially the same as the proteins released by detergent-extraction, although differences were observed in the proteins that migrated between 55,000 and ~250,000 daltons. In addition to the major 55,000-dalton protein, each of the membrane vesicle fractions, as well as the proteins released by detergent-extraction, were enriched for 1–3 proteins of 45,000–50,000 daltons. These proteins comprised 15–25% of the membrane preparations but only 3% of the intact cilia and were not detectable in the detergent-washed axonemes. It seems unlikely that both the 45,000- to 50,000-dalton proteins and the 55,000-dalton protein are present in each of the membrane preparations due to nonspecific trapping of soluble matrix components because the relative proportions of these proteins remained the same in each of the membrane vesicle preparations as well as the detergent-solubilized fractions. If simple trapping of solubilized proteins were to account for the presence of these proteins in membrane vesicles, then membranes isolated after solubilization of the microtubules (KI-membranes) would be expected to be exceptionally rich in the 55,000-dalton protein; this is not, however, observed. The KI-membrane preparations contain essentially the same major proteins in the same proportions as do each of the other membrane preparations.

It is possible that the 55,000-dalton tubulin-like protein is a matrix protein that is released by detergent-extraction and is selectively absorbed by the membrane vesicles, since Caron and Berlin (12) recently reported that in vitro assembled brain tubulin is selectively absorbed by artificial phospholipid vesicles. If this were the case, however, it is not clear why the membrane vesicles would only

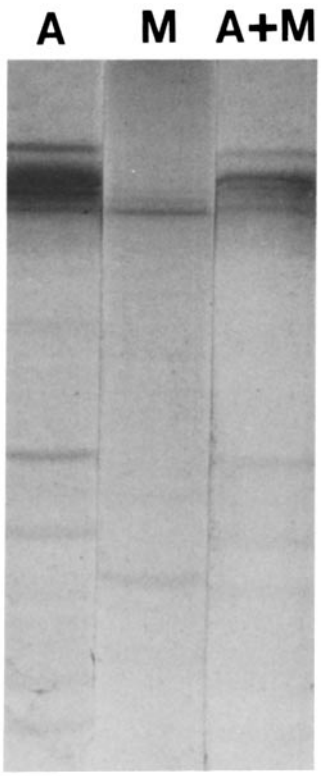

FIGURE 7 SDS-Tris-Cl-PAGE of the high molecular weight proteins in axonemes (*A*), detergent-solubilized fraction (*M*), and mixed axoneme and detergent-solubilized fraction (*A* + *M*). The detergent-solubilized, high molecular weight proteins co-migrated with the three or four faster migrating axonemal dyneins.

FIGURE 6 Thin sections of ciliary axonemes after vortex treatment and before extraction with potassium iodide (*A* and *B*), membranes obtained by KI-extraction of the vortex-treated axonemes (vortex-KI-membranes) (*C* and *D*), and membranes after potassium iodide extraction of whole cilia (*E* and *F*). Extraction of vortex-treated cilia, which contained partially disrupted ciliary membranes (*A* and *B*), yielded a population of apparently empty vesicles which were morphologically similar to the detergent-released membranes. The small amount of amorphous material which was associated with these vortex-KI-membranes was generally observed to be attached to the inner surface of the membrane vesicle (arrows in *D*). Cilia extracted with potassium iodide produced membrane vesicles which contained large amounts of amorphous material and, occasionally, intact ciliary microtubules (*E* and *F*). *A*, *C*, and *E*, × 8,500; *B*, *D*, and *F*, × 52,500.

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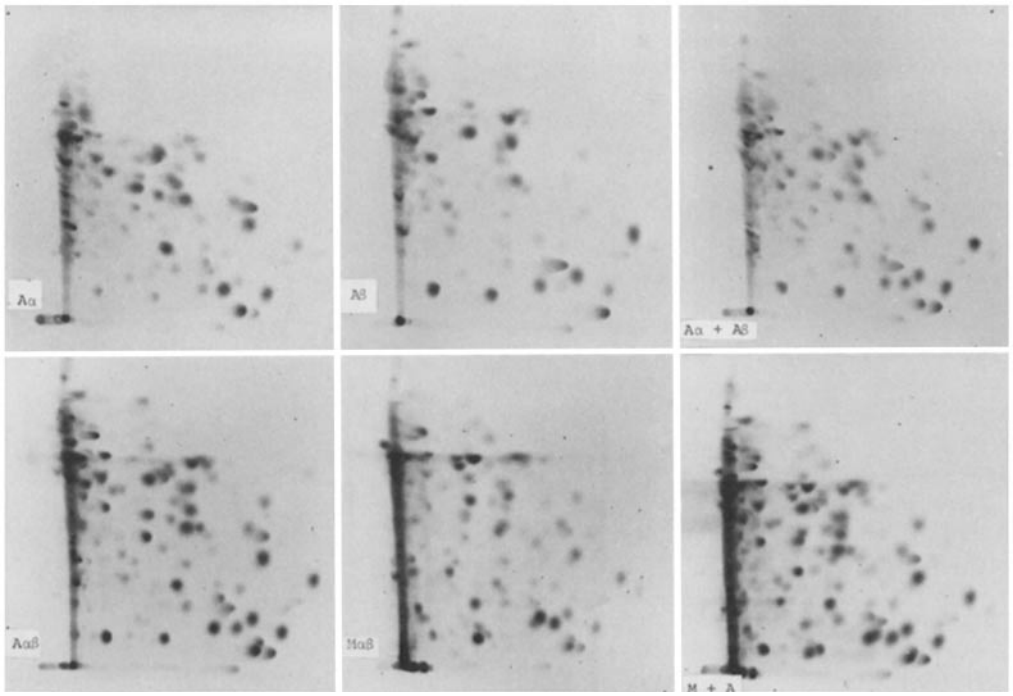

FIGURE 8 Comparative tryptic peptide maps (pH 6.5) of axonemal tubulin and the detergent-solubilized 55,000-dalton protein. Since the detergent-solubilized protein could not be reliably separated into two subunit bands, they were eluted from preparatory gels as a single large band and were compared with axonemal tubulins prepared in the same manner. Peptide maps of the separated ($A\alpha$ and $A\beta$) and mixed axonemal tubulins ($A\alpha + A\beta$) appear virtually identical to those of axonemal tubulins that were eluted and mapped together ($A\alpha\beta$). Maps of the detergent-solubilized 55,000-dalton ($M\alpha\beta$) appear very similar to those of the axonemal tubulins ($A\alpha\beta$) when individual maps are compared with co-migration of equal amounts of each protein ($M + A$). Chromatography was in the ascending direction and electrophoresis was with the anode on the left.

absorb the protein during the fractionation and isolation procedure; if the membrane can absorb the 55,000-dalton protein it would likely do so in the intact cilium and, therefore, the 55,000-dalton protein would become a membrane-associated protein. Moreover, other studies have suggested that microtubule protein may not be absorbed by biological membranes even though they may partition into phospholipid vesicles (12). Bhattacharyya and Wolff (8) mixed radioactively labeled tubulin with tubulin-containing brain membranes and reported that the labeled tubulin could be washed away by using procedures less vigorous than those used in the present report. Sherline et al. (37) reported that pituitary secretory granule membranes would adsorb microtubules that contained the high molecular weight microtubule-associated proteins (MAPs) but that pure tubulin did not absorb to the secretory granules. Their assay was, however, the amount of colchicine-binding protein that could be released from the secretory granules after cold treatment, a method that may depolymerize microtubules but not release proteins from the secretory granule membrane. Furthermore, the membrane-associated tubulin, if any, might not be competent to bind colchicine, although Stephens (42) reported that ciliary membrane-associated tubulin did bind colchicine. Finally, the likelihood that the 55,000-dalton protein is nonspecifically absorbed to the membrane fractions is reduced by the results from cross-linking studies presented in the accompanying manuscript (17). In these studies, the cross-linking reagent 4,4′-dithiobisphenylazide can only bind either to intrinsic membrane proteins or to proteins that lie immediately adjacent to the membrane surface; the observation that the 55,000-dalton protein was cross-linked in these studies supports its localiza-

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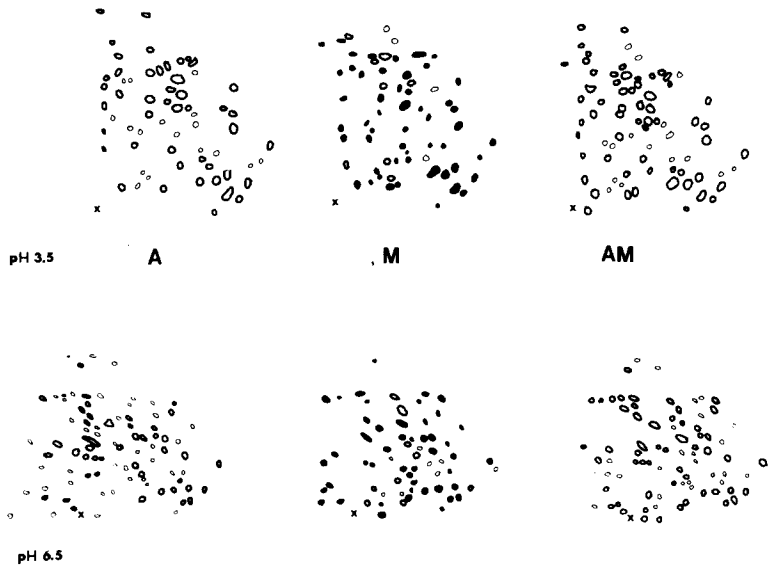

FIGURE 9 Tracings of two-dimensional tryptic peptide maps of *Tetrahymena* axonemal tubulin (*A*) and the detergent-solubilized 55,000-dalton protein (*M*) and equal amounts of axoneme and detergent-solubilized protein mixed together (*AM*). Peptide maps at both pH 6.5 (see also Fig. 8) and pH 3.5 are shown. Filled-in spots on the map of detergent-solubilized protein (*M*) are peptides that are common with those in the axonemal microtubules. See Table III for quantitative analysis. Chromatography was in the ascending direction and electrophoresis was with the anode on the left. The origins are marked with ×.

TABLE III

Coincidence of Tryptic Peptides of Axonemal Tubulin and Membrane 55,000-Dalton Protein

	pH 3.5	pH 6.5
	%	%
Peptides common to axoneme and membrane	77	74
Unique membrane peptides	23	26

tion as either in or very closely associated with the ciliary membrane.

Is the membrane-associated 55,000-dalton protein tubulin? It does behave very much like tubulin on different SDS-PAGE systems and it shares a remarkable number of tryptic peptide fragments with tubulin obtained by solubilization of ciliary microtubules. The tryptic peptide maps are not, however, identical. Similar results were reported in membrane fractions prepared by detergent extraction of scallop gill cilia (42). Other differences also exist between the membrane-associated, 55,000-dalton protein and tubulin prepared from microtubules. As judged by PAS-staining of the polyacrylamide gels, the 55,000-dalton protein as well as the 45,000- to 50,000-dalton proteins contain a small amount of carbohydrate. The amount of carbohydrate, however, appears to be very small since the gels had to be very heavily loaded with protein to detect the PAS-positive material. This amount of PAS-positive material is not likely to be nonspecific since no PAS-positive material was observed in axonemal proteins even when gels were loaded with several times the amount of protein that was loaded on the gels of detergent-solubilized material. The possibility remains that there is another protein, heavily glycosylated but present in very small quantities, that co-migrates with the 55,000-dalton protein; heavily overloaded gels would, therefore, be necessary to detect this protein. If this were the case, however, this other protein would have to migrate as a single band on Tris-acetate SDS-PAGE (19, 42) and as two major bands of 55,000 and 45,000–50,000 daltons on Tris-Cl SDS-PAGE (Fig. 4).

In both *Tetrahymena* and *Aequipecten* (42) there appears to be very little carbohydrate associated with the 55,000-dalton protein. Although it is generally expected that proteins with bound carbohydrate tend to migrate at a higher apparent molecular weight than is the case, the small amount of carbohydrate associated with the 55,000-dalton

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protein might not be enough to significantly alter the protein's mobility. Moreover, Stephens (42) suggested that the carbohydrate may not be covalently bound to the 55,000-dalton protein because both acetone treatment and carboxymethylation of the protein drastically reduced the amount of bound carbohydrate. The 55,000-dalton protein may, therefore, have considerably more bound carbohydrate in vivo than is detected by the PAS-staining of the polyacrylamide gels.

Within the past several years tubulin-like proteins have been reported in association with a variety of cell membranes. Electrophoretic analysis has suggested that tubulin is present in synaptosomal membranes (9, 23, 30), postsynaptic densities (5, 14, 46), cervical-ganglia plasma membranes (20), in lilly microspore nuclear membranes (28), as well as in ciliary and flagellar membranes (this report and references 1, 33, 42, 44). The binding of colchicine to membranes has also suggested the presence of tubulin-like protein in synaptosomal membranes (22, 32), pigeon erythrocyte membranes (48), brain and thyroid membranes (7), liver nuclear and microsomal membranes (40), and in Golgi and plasma membranes (4) (see Stephens [42] for a more complete discussion of these results). The identification of tubulin-like proteins in membranes is quite controversial in part because of the difficulty of insuring that none of the membrane-associated tubulin is a result of contamination by free cytoplasmic tubulin or the depolymerization of microtubules during the isolation procedure. The membrane-associated tubulin-like proteins are not, however, identical to the tubulin that forms microtubules, on the basis of tryptic peptide maps of both synaptosomal proteins (9, 22, 30, 46) and ciliary proteins (this report and reference 42), although the membrane tubulin-like proteins and the microtubule tubulins are very similar. Moreover, while tubulin in either axonemal or cytoplasmic microtubules has no associated carbohydrate, the tubulin-like proteins in synaptic plasma membranes (24) as well as in ciliary membranes (this report and reference 42) do contain a small amount of carbohydrate, on the basis of PAS-staining. The presence of sugar residues suggests that portions of the molecule are exposed at the surface of the membrane; additional evidence for the surface localization comes from vectorial labeling studies using iodination (20, 44), partial digestion (20, 44), and fluorescent labeling with membrane probes (44). Finally, photoactivatable cross-linking reagents that penetrate the membrane bilayer have been shown to dimerize the membrane-associated 55,000-dalton protein into a 110,000-dalton species (17, 18).

The results reported here and elsewhere (17, 18) support the identification of a ciliary membrane-associated dynein-like protein. Depending on the method of isolation, high molecular weight proteins which co-migrate with the lower molecular weight axonemal dynein bands comprise between 1 and 12% of the total membrane protein. Other data show that the dynein-like protein has a native molecular weight similar to that of axonemal dynein (17, 18) and that it has an ATPase activity which is very similar to that of axonemal dynein (17, 18). PAS-staining suggests that this dynein-like protein may be glycosylated, although higher resolution methods must be used to unambiguously distinguish it from other high molecular weight membrane proteins.

While the roles of both membrane-associated tubulin-like proteins and dynein are not well understood, chemical cross-linking studies suggest that these proteins may interact with one another as well as with the outer-doublet microtubules to form a bridge which links the ciliary membrane to the outer-doublet microtubules (17, 18). Since the dynein-like protein may be PAS-positive, it may be exposed to the external surface of the ciliary membrane and also penetrate the membrane to form the membrane-microtubule bridge. The bridge must not be a permanent connection, however, because stabilization of the bridge with cross-linking reagents also arrests ciliary beating (17, 18). The presence of a dynein-like protein which may extend from the external cell surface and interact with ciliary microtubules is particularly interesting at this time since Bloodgood (10) has recently reported the movement of particles along the surface of flagellar membranes. We have recently attached these particles to the surfaces of *Tetrahymena* cilia and demembranated axonemes and have found that they bind via bridge structures, as visualized in the electron microscope. The binding of these particles is likely a result of interactions with proteins since both intact cilia and demembranated axonemes fail to bind particles if they are treated with low concentrations of proteases (unpublished results).

The protein composition of *Tetrahymena* ciliary membranes is remarkably similar to the protein composition of both detergent-solubilized (42) and intact (Dentler and Stephens, unpublished results) scallop-gill ciliary membranes. The results re-

ported here, however, differ from those reported by others. Baugh et al. (6) and Satir (35) reported the absence of a 55,000-dalton protein in *Tetrahymena* ciliary membranes. Adoutte et al. (2) reported the absence of a 55,000-dalton protein in *Paramecium* ciliary membranes whereas Otter (33) described a PAS-positive ciliary membrane tubulin in the same organism. Moreover, Adair and Goodenough (1) report a PAS-positive tubulin in *Chlamydomonas* flagellar membranes whereas earlier reports by Snell (38) and Witman et al. (47) show little, if any, membrane tubulin. In light of these reports, however, it is important to note that, in our hands, the amount of membrane-associated tubulin as well as proteins with molecular weights of ~60,000 and 30,000 daltons varies somewhat from preparation to preparation. Moreover, the amount of the 55,000-dalton protein will often decrease with increasing time of storage of the reduced and frozen sample. It is possible, therefore, that some of the variabilities that are found in different laboratories may be due to differences in the preparation methods employed, partial proteolysis of membrane proteins, and, possibly, variations in the method of SDS-PAGE employed.

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