Reconstitution of Ciliary Membranes Containing Tubulin

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ABSTRACT Membranes from the gill cilia of the mollusc *Aequipecten irradians* may be solubilized readily with Nonidet P-40. When the detergent is removed from the solution by adsorption to polystyrene beads, the proteins of the extract remain soluble. However, when the solution is frozen and thawed, nearly all of the proteins reassociate to form membrane vesicles, recruiting lipids from the medium. The membranes equilibrate as a narrow band (d = 1.167 g/cm³) upon sucrose density gradient centrifugation. The lipid composition of reconstituted membranes (1:2 cholesterol:phospholipids) closely resembles that of the original extract, as does the protein content (45%). Ciliary calmodulin is the major extract protein that does not associate with the reconstituted membrane, even in the presence of 1 mM calcium ions, suggesting that it is a soluble matrix component. The major protein of reconstituted vesicles is membrane tubulin, shown previously to differ hydrophobically from axonemal tubulin. The tubulin is tightly associated with the membrane since extraction with 1 mM iodide or thiocyanate leaves a vesicle fraction whose protein composition and buoyant density are unchanged. Subjecting the detergent-free membrane extract to a freeze-thaw cycle in the presence of elasmobranch brain tubulin or forming membranes by warming the extract in the presence of polymerization-competent tubulin yields a membrane fraction with little incorporated brain tubulin. This suggests that ciliary membrane tubulin specifically associates with lipids, whereas brain tubulin preferentially forms microtubules.

Membrane-associated tubulin was first described in synapto
dosome preparations, identified as such on the basis of colchicine
binding (9), electrophoretic migration, and peptide mapping
(10). The protein was also reported to be bound to thyroid
membranes, wherein it had a greater thermal stability and
higher optimal colchicine binding temperature than soluble
tubulin (2). In contrast to the cytoplasmic form, membrane-
bound brain alpha-tubulin was found to be free of C-terminal
tyrosine but it could be tyrosylated when freed from the
membrane (23). Differential solubility studies implied that
alpha-subunit of brain membrane tubulin was an integral
protein, whereas the beta-subunit was peripheral (13, 31).
Furthermore, proteins co-migrating with tubulin chains were
shown by iodination to be exposed on the surface of neurites
growing in culture (7). Similar results were obtained recently
with leukemic human lymphoid cells (25). Consistent with the
idea of tubulin as an integral membrane protein was the earlier
observation that tubulin associated with synaptosomes was a
glycoprotein while soluble, axonal tubulin was not (12).

The major protein in detergent-solubilized molluscan gill
ciliary membrane preparations is a PAS-positive tubulin, in
contrast to that of molluscan sperm flagella, which contains a
like amount of a single glycoprotein with a fourfold higher
subunit molecular weight (26). In addition to carbohydrate
content, ciliary membrane tubulin may be distinguished from
9 + 2 tubulins through hydrophobic differences in amino acid
composition, tryptic peptide maps, and detergent charge-shift
electrophoresis (28), negating the argument that membrane
tubulin is derived from the axoneme by Triton solubilization
(1). Using sucrose gradient purification, a similar PAS-positive
tubulin can be isolated from *Tetrahymena* ciliary membrane
vesicles, prepared by a variety of methods (5). Both it and
molluscan membrane tubulin can be cross-linked within the
lipid bilayer, using a lipophilic, photoactivated, cleavable, bi-
functional reagent (6). Moreover, a preliminary account shows
ciliary membrane alpha-tubulin to be accessible to surface
labeling by both iodination and vectorial fluorescent probes,
whereas beta-tubulin is more accessible to trypsinization (27).

In spite of differences suggesting a specific, glycosylated,
hydrophobically unique membrane tubulin, polymerization-
competent, soluble brain tubulin may be incorporated into
phospholipid bilayers. When colchicine is added to suppress
the normal formation of in vitro microtubules, high speed
supernatants of brain, when warmed, will yield membranes
containing tubulin and MAPs (11), evidently recruiting phos-
pholipids from the homogenate. Tubulin and MAPs, purified
through two cycles of polymerization and depolymerization
will incorporate into liposomes of dimyristoyl phosphatidyl
choline (4). Phosphocellulose-purified tubulin will associate with dipalmitoyl phosphatidyl choline liposomes, eliciting dye release from the liposomes when the protein binds at the phase transition temperature (18). The tubulin thus associated is more alpha-helical, has reduced tryptophan fluorescence, and is less susceptible to tryptic cleavage (19). If solubilized, membrane tubulin from brain will copolymerize with cytoplasmic tubulin (3), whereas soluble brain tubulin will not adsorb to tubulin-containing membranes.

As a result of these observations, it appears clear that tubulin can exist in both soluble and membrane-bound forms, although differing in conformation. What is not clear is whether a subpopulation of tubulin is selectively associated into reconstituted membranes. Because brain tubulin is heterogeneous with respect both to cellular source and to glycosylation and tyrosylation, it would be desirable to reinvestigate the behavior of tubulin derived exclusively from membranes. This report deals with the direct reconstitution of ciliary membranes, uncomplicated by the presence of cytoplasmic tubulin or added lipids.

**MATERIALS AND METHODS**

**Protein Preparations:** Cilia were prepared from the gills of the bay scallop, *Aequipecten irradians*, as published previously (20), but modified to substitute a 120-min deciliation step at 20°C with gentle stirring, followed by filtration through two layers of Miracloth (Calbiochem-Behring Corp., La Jolla, CA). This modification resulted in higher yields of very pure cilia, uncontaminated by either mucus or exfoliated gill cells. Phenyl methyl sulfonylfuoride (PMSF) at 0.05% was included during both isocionic and hypotonic wash steps and during membrane solubilization. The membrane-matrix was solubilized by extracting the purified cilia with 10 volumes of 0.25% Nonidet P-40 in 30 mM Tris-HCl (pH 8.0), 3 mM MgSO₄, 0.1 mM EDTA, and 0.1% 2-mercaptoethanol, with additions or substitutions in accord with the experimental protocol. The extract was centrifuged at either 25,000 or 100,000 g for 15 min or 1 h, respectively. The clarified supernatant was treated with gentle agitation, for 2 h at 4°C with 1/3 volume of SM-2 Bio Beads (Bio-Rad Laboratories, Richmond, CA) to adsorb the detergent (14). UV absorption measurements indicated that this treatment removed all detectable NP-40 within 90 min.

Skate brain tubulin was prepared by two cycles of polymerization-depolymerization at 25°C and 0°C, respectively, by a previously published method (21). To improve the yield, 1 mM ATP and 1 mM diethiothreitol were included in all steps.

**PAGE and Electrofocusing:** Samples were run stoichiometrically (i.e. pellets were resuspended to the original sample volume) on 1.5-mm thick slab gels, using the SDS-containing, discontinuous ionic system of Laemmli (20). The separating gel was either uniform, of 7.5T/2.5C composition, or employed a linear gradient, 5T-15T/2.5C, to resolve both high and low molecular weight constituents. Duplicate samples, in adjacent wells, sometimes contained either 2.5 mM CaCl₂ or 2.5 mM EGTA in order to detect calmodulin by a mobility shift across lanes (30).

Isoelectric focusing was performed on 1.5-mm thick slabs, using the O'Farrell (24) formulation, substituting a pH 4-6 ampholyte for the prescribed pH 5-7 one (28). Samples containing 0.1 M MES buffer were dialyzed against 2.5 mM Tris-HCl (pH 8.0) before use in either electrophoresis or electrofocusing.

Gel slabs were stained with either Coomassie Blue or Fast Green, using the general methods of Fairbanks et al. (8). Isoelectric focusing slabs were first fixed by 20 volumes of 10% trichloroacetic acid (1 h) and then soaked 2 h in 0.25% NP-40 in 0.1 M ammonium bicarbonate as the extraction buffer, then treated with 2.1 chloroform-methanol (1 ml per mg of residue). Alternatively, a detergent-free extract was subjected to direct extraction (Folch wash [15]). The combined extracts were evaporated under nitrogen. Total lipid and total protein were determined by weighing. Lipids were further fractionated into neutral, glyco-, and phospholipids by silica gel chromatography and the resulting eluates were evaporated under nitrogen and weighed. Cholesterol was determined colorimetrically while phospholipids were resolved by one- or two-dimensional thin-layer chromatography on silica gel G, the relative amounts being estimated by comparison with known quantities of standards run in parallel (15).

**RESULTS**

**Reconstitution by Detergent Removal/Freeze-Thaw**

It is not unreasonable to expect that removal of detergent from a solubilized ciliary membrane preparation could result in lipid micelle formation and subsequent protein incorpora-

![Figure 1](https://via.placeholder.com/150)
tion into membrane vesicles. When NP-40 is removed from scallop ciliary membrane extracts, no measurable amount of any protein is selectively adsorbed to the polystyrene surface of the SM-2 Bio Beads. Recovery is virtually quantitative (Fig. 1a vs. b). Although there is a slight increase in light scattering after Bio Bead treatment, little of this appears to be a consequence of either protein denaturation or vesicle formation, because less than 1% of the total protein is sedimentable at 100,000 g after detergent removal (Fig. 1c vs. d). A barely perceptible pellet is obtained.

However, when the detergent-free high speed supernatant is frozen in liquid nitrogen and then thawed, an opalescent suspension results. At least 85% of the protein in the extract sediments at 25,000 g (Fig. 1e vs. f). The major protein remaining in the freeze-thaw supernatant is calmodulin, readily detectable through a migration shift between adjacent lanes in the presence and absence of calcium. Calmodulin remains in the supernatant, even when the calcium ion concentration is 1 mM. Electrophoresis in the absence of mercaptoethanol indicates no significant intramolecular disulfide bonding (data not shown).

Differential-interference or phase-contrast microscopy reveals that the opalescent suspension obtained from the detergent-free membrane extract after the freeze-thaw step consists of a variety of membranous structures. The specific morphology is a function of the rapidity of the freeze-thaw cycle. Slow freezing (as in a conventional freezer at -20°C) results in large, bulbous or tubular structures with a background of smaller vesicular particles, whereas rapid freezing (cryotube in liquid nitrogen) produces mainly small, fairly uniform vesicles (Fig. 2a vs. b). High speed sedimentation and subsequent resuspension erases any difference between the two modes of preparation since the larger vesicles evidently are dispersed into smaller ones.

At an ultrastructural level, the initial pellet obtained after the freeze-thaw cycle consists of a heterogeneous population of membrane vesicles, often occurring in apparent strings or as multilamellar spheres (Fig. 3a-b). Regardless of the relative position within the pellet, only vesicles or fragments thereof are seen, and there are no obvious differences in vesicle morphology with position. Protofilaments or filament bundles have never been observed. The morphology of the vesicles is the same whether ATP, GTP, or calcium ions are included at 1 mM concentrations in the original extract.

**Extraction with Chaotropic Agents**

The mere formation of vesicles and the coincident sedimentation of certain proteins with them does not prove that the
proteins are membrane-associated. When a vesicle preparation is layered on a 20–60% (wt/wt) continuous linear sucrose gradient and centrifuged to equilibrium, a single, sharp band is obtained at 36.5–37.5% sucrose, corresponding to a density range of 1.164–1.169 g/cm³ (Fig. 4a). When the band is removed quantitatively and run in parallel with a stoichiometric aliquot of the original vesicles on SDS PAGE, recovery is nearly complete and there is no selective loss of any of the constituent proteins as a consequence of equilibrium gradient centrifugation (Fig. 4b–c). Insoluble or denatured protein alone, if not lipid-associated, should sediment to the bottom of the gradient. None can be detected.

When reconstituted membrane vesicles are sedimented, resuspended in 50–100 volumes of 1 M solutions of KCl, KI, or KSCN, extracted for 30 min on ice, resedimented, and then applied to a sucrose gradient, no significant change in the average buoyant density takes place as a result of the extraction, although the density distribution broadens somewhat (Fig. 5a). An electrophoretic comparison of pellets and supernatants from such a procedure reveals that KCl extracts 9% of the total protein, KI extracts 17%, and KSCN extracts 23%. If 1 mM EDTA is included in the extraction to eliminate divalent cations, the amounts solubilized are 15%, 24%, and 32%, respectively, for the three salts. The 10 mM Tris-EDTA buffer alone extracts no more protein than KCl. There is no obvious selective extraction of the major protein components (Fig. 5b–e). A very high molecular weight protein is not extracted under any condition whereas a very low molecular weight protein is extracted under all conditions (asterisks).

These results are more consistent with membrane redispersal than with protein extraction. Preparations treated with KCl (minimal extraction, serving as a mechanical control) and KSCN (maximal extraction) were sedimented and examined ultrastructurally. Although both preparations consist of distinct vesicles, the KSCN-EDTA treated preparation appears less intact (Fig. 6a vs. b–c). Thus, in spite of extensive extraction by KSCN treatment, retention of ultrastructure, buoyant density, and general protein profile would imply that the observed proteins are tightly associated with the membranes.

Membrane Lipid Composition

If lipid-protein interactions are fairly specific, one might expect the composition of reconstituted vesicles to mimic that of the original membrane. Chloroform-methanol extraction of whole cilia, lyophilized total ciliary membrane extract and reconstituted membrane vesicles yields lipid fractions that are almost indistinguishable, with the exception of a marked relative depletion of glycolipids in the reconstituted membranes (Table I). The preparations contain neutral and phospholipids in a 1:2 ratio, constituent lipids of the latter class being present in the same proportions (Fig. 7). They also contain nearly equivalent amounts of total protein, particularly if one assumes that some of the proteins that do not reassociate with the membrane fraction (e.g. calmodulin) were originally free in the ciliary matrix.

Using the protein and lipid composition determined here, plus known densities for protein and lipids, one may calculate the expected buoyant density of the reconstituted membrane to be 1.168. An average value of 1.167 was found by equilibrium centrifugation.

Membrane Formation in the Presence of Exogenous Tubulin

Because brain tubulin can be incorporated into liposomes,
the presence of tubulin in reconstituted ciliary membranes does not prove that it belongs there. When scallop ciliary membranes are solubilized with 0.25% NP-40 in 0.1 M MES (pH 6.4), 1 mM EGTA, and 1 mM Mg-GTP (i.e., microtubule polymerization buffer) and the detergent is removed with SM-2 BioBeads, membranes may be reconstituted by the freeze-thaw procedure but with a comparative yield of only 30–40% and a somewhat different morphology (Fig. 8 b). The decreased membrane yield is a consequence of pH and ionic strength differences under the two sets of reconstitution conditions. As mentioned above, the presence of nucleotide is inconsequential to the reconstitution. Membranes reconstituted in microtubule polymerization buffer have a substantially lower bouyant density (1.146 vs. 1.167; Fig. 8 a) and protein content (38% vs. 45%). If the reconstitution is performed in the presence of an eightfold molar excess of skate brain tubulin, there is no significant increase in total membrane-associated protein nor is there any change in buoyant density.

Because the scallop and skate tubulin subunits differ significantly in isoelectric point, membranes prepared by "co-freezing" scallop ciliary membrane extract and skate brain tubulin may be analyzed by isoelectric focusing (Fig. 9 a). No brain tubulin can be detected when equal amounts of the two

| Table 1 Lipid and Protein Composition of the Ciliary Membrane-Matrix, the Membrane-Matrix Extract, and Reconstituted Membrane Vesicles |
|----------------------------------------------|
| Neutrals | Glycolipids | Phospholipids | Protein |
| % | % | % |
| CILIA |
| #1 | 14.3 | 6.6 | 38.1 | 41.0* |
| #2 | 15.5 | 7.2 | 34.1 | 43.2* |
| Average | 14.9 | 6.9 | 36.1 | 42.1* |
| EXTRACT |
| Folch wash | 14.6 | 8.3 | 27.4 | 49.7 |
| Lyophilize | 13.0 | 6.9 | 32.8 | 47.4 |
| Average | 13.8 | 7.6 | 30.1 | 48.6 |
| VESICLES |
| #1 | 17.8 | 1.2 | 35.8 | 45.2 |
| #2 | 19.3 | 0.6 | 36.1 | 44.0 |
| Average | 18.6 | 0.9 | 36.0 | 44.6 |

NOTE: Neutral lipids, in all cases, analyzed as >90% cholesterol. Phospholipids (Fig. 7) consisted of PE:PC:PS:other in a ratio of 6:1:2:0.5, where "other" is mainly sphingomyelin. Protein content for the ciliary membrane-matrix (*) was calculated, given that it represents 18.4% of the total ciliary protein (30).
proteins are present during freezing (lane 1 vs. 2) and even with an eightfold excess of brain over membrane, there is no more brain tubulin present in the reconstituted membranes than is sedimentable when brain tubulin is frozen alone (Lane 4 vs. 6 and 7), asterisks).

If, instead of freezing a solution of ciliary membrane extract in microtubule polymerization buffer, the extract is warmed to 25°C for 30 min, about 25% of the protein is sedimentable (Fig. 9b, lane 1 vs. 2), appearing as membrane vesicles (Fig. 10a). Twice-cycled skate brain is >90% polymerizable under comparable conditions (Fig. 9b, lane 3 vs. 4). When the two are mixed and “copolymerized” by warming, both membranes and microtubules are formed (Fig. 10b). When the microtubules are cold-depolymerized, the (insoluble) membrane and (soluble) microtubule fractions may be analyzed by isoelectric focusing to determine which species of tubulin are present (Fig. 9, lanes 5–6 vs. 7–8). Brain tubulin is not detectable in the membrane fraction although there appears to be significant membrane tubulin in the microtubule fraction. Thus scallop membrane tubulin is associated almost exclusively with the reconstituted membrane fraction, whereas the skate brain tubulin primarily forms microtubules. Fig. 9 is intended to make the qualitative point that exogenous skate brain tubulin is not appreciably incorporated into reconstituted ciliary membranes; a more quantitative study of microtubule formation by copolymerization of scallop membrane and skate brain tubulin through several cycles is now in progress.

DISCUSSION

Simply freezing and thawing a scallop gill ciliary membrane extract, solubilized by and freed of non-ionic detergent, results in the formation of membrane vesicles having a protein and lipid composition closely resembling that of the original membrane. The reconstitution is independent of added nucleotide or divalent cation, but is somewhat dependent upon ionic strength. Detergent removal from solubilized membrane proteins, followed by freeze-thawing in the presence of sonicated lipids, is an accepted procedure (17). The efficacy of the freeze-thaw method presented here is probably a direct consequence of concentrating the soluble ciliary membrane proteins and micellar lipids during ice formation. Membranes also can be produced by concentrating the detergent-free extract directly but the process is inefficient and generally produces membrane sheets rather than vesicles, accompanied by some protein denaturation. The fact that a myriad of proteins are incorporated

FIGURE 8 Membrane formation after a freeze-thaw cycle of the extract in microtubule polymerization buffer. (a) A single, sharp band reaches equilibrium on a 20-60% (wt/wt) sucrose gradient at a density of 1.146 g/cm³, corresponding to a 15% lower protein content. (b) The resulting membrane vesicles and leaflets are somewhat smaller than those obtained at lower ionic strength. Bar, 100 nm. X 80,000.

FIGURE 9 Isoelectrophoretic analysis of “cofreeze-thaw” and copolymerization of scallop ciliary membrane extract and skate brain tubulin. All samples were 0.1 M in MES (pH 6.4) and 1 mM in MgCl₂, EGTA, GTP, ATP, and DTT; 4 M glycerol was present in (b). Final protein concentrations were 0.35 mg/ml for scallop membrane and either 0.40 or 2.0 mg/ml for skate brain. (a) Cofreeze-thaw: Lane 1: membrane pellet obtained by cofreezing equal concentrations of membrane and brain samples. The relative positions of scallop and skate brain tubulin subunits are designated by (=) and (>>), respectively. Lane 2: membrane pellet obtained by freezing membrane sample alone. Lane 3: supernatant from (2); loading = 1/4. Lane 4: pellet obtained by freezing brain tubulin alone. Lane 5: supernatant from (4); loading = 1/4. Lanes 6 and 7: pellet obtained by cofreezing membrane and brain tubulin in a 1:8 ratio. Asterisks (*) designate the relative position of skate brain tubulin subunits. Lane 8: 10 mM Tris (pH 8.0) wash from the pellet in (6 and 7); loading = 1/4. Lane 9: initial supernatant from (6 and 7); loading = 1/4. (b) Copolymerization: lane 1: pellet obtained by warming the membrane extract alone. Lane 2: supernatant from (1); loading = 1/4. Lane 3: pellet obtained by warming brain tubulin alone. Lane 4: supernatant from (3); loading = 1/4. Lanes 5 and 6: membrane pellet obtained by warming the membrane extract and brain tubulin in a 1:8 ratio, then cold-depolymerizing the microtubules. Lanes 7 and 8: depolymerized microtubules; loading = 1/4. Lane 9: supernatant from the copolymerization; loading = 1/4.
Because mammalian brain tubulin can be incorporated into liposomes (4, 18), the association of tubulin with membranes might be regarded as experimentally induced. The use of skate brain tubulin to test this idea is not unequivocal since the ciliary tubulin came from a molluscan species, although this is several steps closer than using mammalian brain tubulin. On isolectric focusing, the elasmobranch protein runs as two distinct major bands, easily distinguished from the two equimolar pairs of scallop ciliary tubulin chains (29). Brain and membrane tubulin from the same or related species would not allow this distinction. Thus the possibilities remain that the relative ability of skate and scallop tubulin to absorb to ciliary membranes could simply reflect the specificity of the lipids, or that conditions favoring microtubule vs. membrane formation could differ for the two tubulins. That the two types of tubulin are not dramatically different, however, is demonstrated by the fact that they will copolymerize to some extent (Fig. 9 b).

Tubulin is the dominant protein of reconstituted ciliary membranes, behaving, with respect to solubility, as an integral membrane protein. Since exogenous brain tubulin is not appreciably incorporated into vesicles during reconstitution by freeze-thawing or during simultaneous membrane formation and microtubule polymerization, it appears as if these two protein species are able to "sort out" in accord with their organellar origin, at least under the experimental conditions used and with the reservations expressed above. These results provide further support for the reality of a membrane-specific tubulin, but do not provide any further insight into the biological function of this enigmatic protein.

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FIGURE 10 Membranes and microtubules formed in microtubule polymerization buffer. (a) Vesicles formed by warming the detergent-free scallop membrane-matrix extract alone. Bar, 100 nm. × 63,000. (b) Microtubules and vesicles formed by "copolymerization" of the membrane extract and skate brain tubulin. Bar, 100 nm. × 40,000.
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