Tubulin in bovine retinal rod outer segments

DIANE F. MATESIC*, NANCY J. PHILP, JOHN M. MURRAY and PAUL A. LIEBMAN†

Department of Anatomy, 143 Anatomy/Chemistry G3, School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA
*Present address: Department of Pediatrics/Human Development, B240 Life Sciences, Michigan State University, East Lansing, MI 48824, USA
†Author for correspondence

Summary

Bovine rod outer segment (ROS) preparations contain a major 58 kDa protein doublet that was identified by immunoblot as tubulin. Quantification by gel densitometry showed that the total amount of tubulin was 5- to 10-fold higher than that attributable to the rod axoneme, suggesting additional role(s) for tubulin in photoreceptor cells. Approximately 20% of this non-axonemal tubulin (15% of total tubulin) is tightly associated with outer segment membranes. This fraction remains membrane-associated after extensive low- or high-salt washing, requiring detergents or protein denaturants for release from ROS membranes. Unlike ROS soluble tubulin it associates tightly with liposomes upon detergent solubilization and reconstitution. The ROS membrane-associated tubulin is highly enriched in isolated ROS plasma membrane fractions compared to the total outer segment membrane pool and can be localized to the plasma membrane but not to disks by immunofluorescent staining, suggesting a possible role in the structure or electrophysiology of the rod outer segment plasma membrane.

Key words: membrane, tubulin, photoreceptor.

Introduction

Tubulins are a multigene family of closely related proteins that are expressed according to cell type and developmental stage (Cleveland and Sullivan, 1985). Tubulin heterogeneity is further increased by posttranslational modification (Barra et al. 1974; Cleveland and Sullivan, 1985; Edde et al. 1990; Field et al. 1984). A membrane-associated form of tubulin may exist in several functionally specialized tissues including scallop gill ciliary membranes (Stephens, 1977), olfactory ciliary membranes (Chen and Lancet, 1984), neuronal tissue (Bhattacharyya and Wolf, 1975, 1976), isolated synaptic vesicle membranes (Feit et al. 1971; Zisapel et al. 1980) and synaptic junctions (Kelley and Cotman, 1978; Walters and Matus, 1975). The membrane-associated tubulin (MAT) is distinguished from soluble tubulin in these studies by its co-purification with isolated membrane fractions (Feit et al. 1971; Stephens, 1977; Zisapel et al. 1980), resistance to removal from membranes by high- or low-salt washes in the absence of detergents (Kelley and Cotman, 1978; Stephens, 1983) and re-association with liposomes upon reconstitution (Feit and Shay, 1980; Stephens, 1983; Stephens, 1986). Despite extensive studies in a variety of systems and several reports of chemical differences between MAT and soluble tubulin (Nath and Flavin, 1978; Stephens, 1981; Stephens, 1986), the existence of a distinct MAT form has been controversial. The controversy is due primarily to the difficulty in ruling out non-specific membrane association. Assignment of a functional role for tubulin in membranes has been slow, although proposals have been offered (Stephens, 1986).

In this paper, we present evidence for a plasma membrane-associated fraction of tubulin in rod photoreceptor outer segments (ROS) isolated from bovine retinas. The ROS system offers several unique experimental features which allow us to overcome some of the previous difficulties associated with MAT identification and characterization. These include the ability to obtain large quantities of pure ROS, the ability to isolate the ROS plasma membrane from disk membranes and the abundance of information available on ROS structure and biochemistry.

Materials and methods

Rod outer segment membrane preparation

Rod outer segments (ROS) were prepared from bovine retinas by differential centrifugation as previously described (Sitaramayya and Liebman, 1983). All procedures were carried out at 4°C in room light unless otherwise indicated. Briefly, isolated fresh retinas were vortexed for 30-45 s in 45% sucrose (w/v) in buffer A (20 mM MOPS, 100 mM KCl, 0.5 mM MgCl₂, 1 mM DTT, pH 7.0 at 23°C) at 12 retinas/10 ml buffer. Vortexed retinas were diluted to 30 ml/10 retinas with...
Fig. 1. Preparation of ROS membranes. Vortex agitation of retinas in sucrose or Ficoll medium shears rods at the connecting cilium, respectively, to yield sheared rod outer segment (ROS) fragments with open stacks of disk membranes and associated plasma membrane (sucrose) or osmotically intact whole ROS (Ficoll). Hypotonic, low Mg²⁺ washing of ROS disrupts axonemes, removes peripheral proteins and disrupts stacks of disks, resulting in vesicles (sRM) with diameters of 0.2±0.09. sRM contain both disk (>90% of total membrane surface area) and plasma membrane (<10%) fractions. The plasma membrane fraction is isolated from whole ROS by binding carbohydrate groups of plasma membrane extracellular surface to immobilized Con A. Disks and axonemes are removed by subsequent hypotonic, low Mg²⁺ lysis leaving the Con A-bound membrane to be released by α-methylmannoside/octylglucoside washing.

45% sucrose buffer, layered with 5 ml buffer A and centrifuged for 30 min at 27,000 g. ROS collected at the sucrose/buffer interface were further purified on another step gradient containing 38% sucrose. ROS collected at the interface were washed and resuspended in buffer A at 5-10 mg/ml protein. Peripheral proteins were removed by washing 3-5 times in 10 mM Tris-HCl, pH 7.5, at 4°C containing 50 μM GTP in the first wash. Membranes were forced through an 18 gauge syringe needle between washes and pelleted by centrifugation at 45,000 g for 40 min. Stripped rod membranes (sRM) thus prepared were stored as frozen suspensions (10-15 mg/ml protein) in hypotonic Tris buffer. For high-salt washing, sRM or reconstituted liposomes were diluted 10- to 20-fold with 1.8 M KCl in 10 mM Tris-HCl buffer, pH 7.5, incubated for 20 min and pelleted as for sRM preparation. Urea washing was performed by addition of 4 M urea in 10 mM Tris-HCl, pH 7.5, to membranes which were then forced through an 18 gauge needle 2-3 times, incubation for 30 min at 4°C followed by ultracentrifugation at 140,000 g for 1 h. Alkali extraction (Fujiki et al. 1982) was performed by incubation of membranes with 0.1 M sodium carbonate, pH 11.5, for 30 min, followed by centrifugation as for urea washing. For some experiments, ROS with an unbroken plasma membrane were prepared in Ficoll according to Schnetkamp et al. (1979). A schematic diagram of the preparation of different ROS membrane fractions is shown in Fig. 1.

**Plasma membrane preparation**

The presence of carbohydrate groups that bind Concanavalin A (Con A) on the extracellular face of ROS (Adams et al. 1987) and not on the extravesicular face of disks allowed isolation of plasma membrane-enriched membrane fractions (Matesic and Liebman, 1987). ROS with intact plasma membranes prepared as described by Schnetkamp et al. (1979) were incubated for 30 min at 4°C in buffer A containing Con A-Sepharose beads washed in buffer A at ≤1 mg protein per ml of gel. Bound ROS were hypotonically ruptured to release disks by 20-fold dilution into 10 mM Tris-HCl, pH 7.5 (4°C), buffer. Con A-Sepharose beads were sedimented at 500 g and washed in Tris buffer. The two Tris buffer supernatants containing predominantly disk membranes were pooled and concentrated by centrifugation. A column formed from the sedimented Con A beads was further washed with 5 column volumes of hypotonic Tris buffer, followed by 0.25 M alpha-methylmannoside in buffer A containing 1% octylglucoside to solubilize bound membranes and remove Con A-bound and plasma membrane-associated proteins. This plasma membrane protein fraction was concentrated by Centricon 10 concentrator units (Amicon, Danvers, MA) and stored frozen at −20°C.

**Protein electrophoresis**

SDS-polyacrylamide slab gels were composed of 14% acrylamide, 0.12% bis-acrylamide, 0.38 M Tris-base, pH 8.8-9.0, 0.1% SDS, 25-40 mM KCl, 1.67 μl/ml of 10% ammonium persulfate and 0.4 μl/ml TEMED (N,N',N′,N′-tetramethyl-ethylenediamine). In some experiments, running gel solutions were pre-mixed 2-3 weeks before use. This resulted in better resolution of alpha and beta tubulin subunits, possibly due to partial pre-polymerization of the bis-acrylamide (modified bis-acrylamide). This modification allowed resolution of tubulin subunits while maintaining the relative migration of...
most other ROS proteins. Stacking gels were composed of 5.0% acrylamide, 0.1% bis-acrylamide, 0.1% SDS, 0.125 M Tris-base, pH 8.8-9.0, 25-40 mM KCl, 4.0 \mu M amonium persulfate and 1.0 \mu M TEMED. Samples were solubilized in an equal volume of sample buffer (16% glycerol (v/v), 0.1 M Tris-HCl, 3.5% SDS (w/v), 0.25 M DTT, 0.1 g/ml bromo- 
mol blue, pH 6.8) before electrophoresis. Gels were run at 10 
\text{mA} for 14-16 h and fixed and stained with Coomassie blue as 
described by Laemmli (1970). Silver staining was done 
according to Merrill et al. (1981). Coomassie-stained gels were 
scanned in spatial integration mode on a Shimadzu (Colum- 
bia, MD) CS-930 densitometer at 560 nm. Amounts of 
dividual protein bands were determined by Gaussian curve 
resolving and integration software together with protein-
specific calibration curves. Preparative gels were soaked in 4.0 
M sodium acetate after electrophoresis (Higgins and Dahmus, 
1979) to make protein bands visible against a dark back-
ground. Bands were cut out and electroeluted from the gel 
using a Biorad protein eluter apparatus.

Western blots 
Proteins were transferred from SDS/polyacrylamide gels to 
nitrocellulose paper in 20% methanol, 0.3% Tris-base (w/v) 
1.43% glycine (w/v) at 0.3 \text{mA} for 4-5 h and washed overnight 
at 4°C in blot wash buffer (0.05 M Tris-base, 0.15 M NaCl, 
0.1% bovine serum albumin (BSA), 0.05% NP-40, pH 8.0 at 
23°C). Nitrocellulose blots were incubated with primary 
anitbody diluted 1:500-1:5000 in blot wash buffer for 8-10 h 
at 4°C, then washed in 3-4 changes of buffer. Peroxidase-
conjugated secondary antibody diluted 1:2000 was incubated 
with blots for 60 min at room temperature, washed 2-3 times 
with blot wash buffer, twice with 50 mM Tris-HCl, pH 7.6 and 
developed with diaminobenzidine (DAB) or 4-chloro-
apthol. Immunoblots were scanned in reflectance mode on a 
Shimadzu densitometer at 600 nm.

Dot immunobinding assay 
Quantitative determination of anti-tubulin antibody binding 
to purified ROS membrane tubulin and bovine brain tubulin 
was carried out according to the method of Jahn et al. (1984) 
with the following modifications: samples were diluted in 
buffer A containing 1% cholate and 0.7% Triton X-100 before 
blotting onto nitrocellulose; blocking solution contained 50 
mM Tris-base, 150 mM NaCl, 0.1% BSA, 0.02% Nonidet P-
40, pH 8.0; nitrocellulose sheets were incubated with primary 
anitibodies diluted 1:500 in blocking buffer for 10-15 h at 4°C; 
125I-labelled secondary antibodies were used instead of 125I-
labelled protein A.

Purification of ROS membrane-associated tubulin 
Membrane tubulin-enriched protein fractions were obtained 
by the method of Cook et al. (1987), formerly developed for 
the purification of cGMP-dependent cation channel com-
ponents. Briefly, hypotonically washed sRM from 40-60 
bovine retinas were solubilized in 20 mM CHAPS in 20 mM 
MOPS, 0.15 M KCl, 10 mM CaCl$_2$, 1.0 mM DTT, 1.0 mg/ml 
PC (phosphatidyl choline), protease inhibitors (1.0 \mu M each 
of aprotinin, leupeptin, pepstatin and 1.0 mM PMSF), pH 
7.4, at 4°C and the high-speed (130,000 \text{g}) supernatant 
was applied to a DEAE-Sepharose 0.9 cm x 11.0 cm column equilibrated in 
buffer B (0.15 M KCl, 20 mM MOPS, 10 mM CaCl$_2$, 1.0 
mg/ml PC, 1 mM DTT, 15 mM CHAPS and protease 
inhibitors). The column was washed with buffer B until the 
UV absorbance (280 nm) returned to baseline. Proteins 
eluted with a step gradient of buffer B containing 0.75 M KCl 
were then applied to a 0.9 cm x 5.0 cm AF-Red Fractogel 
column (Merck) equilibrated in the 0.75 M KCl buffer.

Proteins which remained bound to the column after extensive 
washing with 0.75 M KCl buffer were eluted with 1.8 M KCl in 
buffer B. Fractions were desalted and concentrated in buffer 
B to 0.2-0.5 mg/ml protein using Amicon Centricon-30 
concentrator units.

Preparation of ROS soluble tubulin 
Soluble tubulin from ROS was prepared by washing ROS with 
hypotonic Tris buffer as described above for sRM prep-
aration, omitting the GTP. The supernatant from the first 
wash was further centrifuged at 130,000 \text{g} for 60 min to 
remove possible contaminating sRM and concentrated by 
Centricon-30 units to 5 mg/ml protein. This soluble tubulin 
fraction thus contained several other ROS proteins including 
cGMP phosphodiesterase (PDE) and 48 kDa protein, but 
their presence did not interfere with the ability to quantitate, 
immunoblot or use the soluble tubulin in liposome reconstitut-
ion experiments described below.

Liposome-tubulin reconstitution 
Desalted, 58 kDa protein-enriched (MAT) fractions from Red 
Fractogel with cholate buffer (100 mM KCl, 20 mM MOPS, 0.5 
mm MgCl$_2$, 0.5 mM DTT, 1.0 mg/ml soybean phosphatidyl 
choline (PC), 1.0% sodium cholate, pH 7.0), followed by 
concentration using centricon-30 units, repeated 3 times. The 
ROS soluble tubulin fraction was also diluted with the cholate 
buffer. Protein concentrations were adjusted to 0.1-0.2 
mg/ml, PC and CaCl$_2$ were added to 5.0 mg/ml and 10 mM, 
respectively. Samples were dialyzed for 20-24 h at 4°C against 
50 mM KCl, 10 mM MOPS, 2.0 mM MgCl$_2$, 0.5 mM DTT, pH 
7.0. Membranes were pelleted by centrifugation at 45,000 \text{g} for 30 min. In relevant cases, reconstituted vesicles were 
resuspended by sonication to improve exposure to dialysis buffer 
or washed with 1.8 M KCl in 10 mM Tris-HCl, pH 7.5 at 4°C 
and centrifuged in a microcentrifuge for 30 min. Membrane 
pellets and concentrated supernatants were desalted when 
necessary and solubilized in sample buffer for SDS/gel 
electrophoresis.

Cyanogen bromide cleavage 
Electrophoretically purified 58 kDa protein or bovine brain 
tubulin samples were dialyzed against 0.5% SDS; 1.5 g CNBr 
was dissolved in 2.5 ml 98% formic acid and 0.8 ml was added 
to 0.2 ml samples containing 20-100 \mu g total protein. Samples 
capped under nitrogen were incubated for 24-28 h at room 
temperature in the dark. Samples were diluted with 4 volumes 
of distilled water or 1.0 M urea, then dried under a stream of 
nitrogen. Dried samples were redissolved in gel sample buffer 
and fragments separated on 10% to 18% acrylamide SDS/gels 
containing 6.0 M urea as described by Hashimoto et al. 
(1985).

Immunohistochemical staining of ROS tubulin 
ROS isolated according to Schnetkamp et al. (1979) were 
fixed for 30 min at room temperature by dilution of 1.0 ml 
ROS suspension directly from the Ficoll gradient with 2.0 ml 
0.1% glutaraldehyde, 3.7% paraformaldehyde in 100 mM 
Pipes, pH 6.8, 3% sucrose, 1 mM MgCl$_2$, then washed 2-3 
times in 3% sucrose in PBS, pH 7.4, by centrifugation at 5000 
\text{g}. Fixed ROS were resuspended in 3% sucrose/PBS and 
adsorbed to gelatin-coated slides for 15 min, excess buffer 
drawn off and dried 30 min at room temperature. ROS were 
incubated for 30 s with fixation buffer, washed 2 times with 
3% sucrose/PBS and permeabilized with -20°C acetone for 
30 s, followed by 2 washes with PBS. ROS were then 
icubated for 15 min in PBS containing 2 mg/ml sodium
borohydride, washed 2 times with PBS, then blocked for 1 h at room temperature in 5% normal goat serum in PBS containing 0.05% Tween-20. Primary antibodies were diluted 1:100 in PBS containing 1% BSA and 0.05% Tween-20 and incubated with ROS for 2 h at room temperature, followed by 3 washes in the antibody dilution buffer. Fluorescein- or Texas red-conjugated secondary antibodies were diluted 1:200 and incubated with ROS for 1-2 h at room temperature, followed by 3 washes in antibody dilution buffer. Fluorescein or Texas red-conjugated secondary antibodies were excited by the 476 nm line of a krypton laser and viewed through a 490-540 nm band-pass filter, and Texas red conjugates were excited by the 568 nm line of the krypton laser and viewed with a 625 nm long-pass filter.

**Materials**

Octylglucoside and CHAPS were purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN), sodium cholate, vinblastin and colchicine from Sigma Chemical Co. (St. Louis, MO.), AF Red Fractogel from Merck (Munich, FGR), and CNBr from Aldrich Chemical Co. (Milwaukee, WI). Anti-sea urchin tubulin polyclonal antiserum (IgG fraction) was purchased from Polysciences, Inc. (Warrington, PA), polyclonal anti-chick embryo tubulin from Sigma Chem. Co., and (anti-chick brain tubulin) monoclonal antibodies specific for the alpha and beta subunits were from Amersham (Arlington Hts, IL) or ICN (Costa Mesa, CA). 125I-labelled secondary antibodies were from Jackson Labs (Bar Harbor, ME) and Texas red-conjugated antibodies from Molecular Probes, Inc. (Eugene, OR). Reagents for electrophoresis were purchased from Biorad (Melville, NY), and Con A-Sepharose from Pharmacia (Piscataway, NJ). Monoclonal antibodies specific for the C terminus of rhodopsin were generously provided by Dr. Paul Hargrave, and polyclonal antibodies specific for the N terminus of rhodopsin were a gift from Dr. David Papermaster. Polyclonal antiserum specific for kidney Na+/K+-ATPase was obtained from Dr. James Nelson.

**Results**

**Identification of tubulin in rod outer segments**

Isolated bovine rod outer segment (ROS) preparations (Fig. 1) consist of partially intact stacked disk membranes and associated plasma membrane, peripherally bound proteins and various amounts of soluble proteins. These ROS preparations contain protein that reacts with tubulin antiserum. Fig. 2 shows Western blots of ROS proteins using polyclonal antisera specific for sea urchin tubulin (lane c) or chick brain tubulin (lane d). The immunoreactive protein can be resolved on SDS/polyacrylamide gels into 2 components near 58 kDa, which co-migrate with the alpha and beta subunits of tubulin isolated from bovine brain (Murray, 1984; Shelanski et al. 1973) (lanes e and f). Isolated IgG fractions from polyclonal antisera to purified tubulin (Polysciences) and a mixture of monoclonal antibodies specific for tubulin alpha or beta subunits (Amersham or ICN) also cross-reacted with the two subunits of ROS tubulin (not shown). Gel densitometry of Coomassie blue-stained ROS proteins shows total tubulin to be 4-5% of the rhodopsin content (Table 1). Preparation of ROS by methods designed to assure osmotic integrity (Schnetkamp et al. 1979) did not alter the tubulin fraction of total protein (normalized to rhodopsin, Table 1).

The amount of ROS tubulin contributed by axonemes was determined by isolating the axonemal fraction from Triton X-100-solubilized ROS preparations by sucrose gradient centrifugation according to the method of Fleishman (Fleishman and Denisevich, 1979; Fleishman et al. 1980). Isolated axonemes were visualized by immunofluorescence microscopy using tubulin polyclonal antibodies (Polysciences) and rhodamine-conjugated secondary antibodies. SDS/polyacrylamide gel electrophoresis and densitometry of proteins present in the isolated axonemal fraction revealed a protein composition similar to that reported by Fleishman et al. (1980). Axonemal tubulin in this fraction was found to

**Table 1. Quantification of tubulin in ROS membrane preparations**

<table>
<thead>
<tr>
<th></th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact ROS</td>
<td>4.59±0.39 (6)</td>
</tr>
<tr>
<td>ROS</td>
<td>4.79±1.41 (4)</td>
</tr>
<tr>
<td>Washed ROS (sRM)</td>
<td>0.74±0.22 (7)</td>
</tr>
<tr>
<td>Plasma membrane</td>
<td>12.03±3.40 (3)</td>
</tr>
<tr>
<td>Disks</td>
<td>0.38±0.01 (2)</td>
</tr>
</tbody>
</table>

Rhodopsin (%) in each fraction is determined by gel densitometry and is given ± standard deviation of (n) determinations.
be 0.47% of the total ROS protein (0.72% of rhodopsin). The amount of axonemal tubulin in ROS was also calculated from the known axoneme dimensions in ROS, tubulin packing (Snyder and McIntosh, 1976), and rhodopsin content per rod (Papamaster and Dreyer, 1974). Calculated and experimentally determined ratio of axonemal tubulin to rhodopsin agreed within a factor of 2. The total amount of measured ROS tubulin is thus 5-10 times greater than that attributable to axonemal tubulin.

**Soluble and membrane-associated tubulin in ROS**

About 85% of the tubulin present in isolated ROS could be removed by hypotonically rupturing ROS and washing the membranes in hypotonic buffer (sRM). Some tubulin, however, remained tightly associated with the sRM (Fig. 3). Further hypotonic washing only slightly reduced (<5% after 2 additional washes) this fraction in sRM. This tubulin remained membrane-associated when sRM were sonicated in high Ca²⁺ (5 mM) buffer or high salt (1.0 M KCl), conditions expected to release soluble proteins that might be trapped within the vesicles. Gel densitometry of Coomassie-stained sRM indicates that the membrane-associated tubulin fraction is about 0.74% of the rhodopsin (Table 1). Such membrane-associated tubulin was probably not due to axonemal contamination in the membrane pellets, since an “axonemal” fraction isolated from sRM by gradient centrifugation exactly as for ROS described above, showed no detectable axonemes, by immunofluorescence microscopy. Quantitation of the amount of tubulin in this sRM “axonemal” fraction by gel densitometry indicated that axonemal contamination in sRM contributed only 0.05-0.1% of the rhodopsin and cannot account for the 0.74% of rhodopsin contributed by the membrane-associated tubulin fraction. Thus, our hypotonic rupture/wash procedure removes ROS axonemes. Low Mg²⁺ conditions have previously been shown to destabilize rod axonemes (Fleishman et al. 1980). Membrane-associated tubulin in sRM was detergent-soluble in both Triton X-100 during the axoneme isolation procedure, and CHAPS (see below), while ROS axonemes are detergent-stable (Fleishman and Denisievich, 1979; Fleishman et al. 1980). Electron micrographs of negatively stained sRDM as well as OsO₄-stained, fixed and thin-sectioned sRM also showed no evidence of axonemes or basal bodies (data not shown). These structures are, however, detectable by electron microscopy in whole-rod preparations (Fleishman and Denisievich, 1979; Fleishman et al. 1980; Kaplan et al. 1987).

Polyclonal antiserum raised against kidney Na⁺/K⁺-ATPase immunohistochemically labels bovine rod photoreceptor Na⁺/K⁺-ATPase, which is localized exclusively in the inner segment plasma membrane and cross-reacts with the rod Na⁺/K⁺-ATPase on Western blots. The membrane-associated tubulin fraction was not derived from inner segment contamination of ROS preparations, however, since: (1) the Na⁺/K⁺-ATPase ratio seen on Western blots varied widely from preparation to preparation, and (2) at least one sRM preparation containing the usual amount of MAT contained no Na⁺/K⁺-ATPase, as measured by Western blotting (not shown).

Ficoll- or sucrose-purified ROS membranes contained ~7-fold more anti-tubulin immunoreactive protein than hypotonically washed membranes (Fig. 3, lanes d and e, and Table 1). Thus, about 85% of the tubulin in ROS is soluble plus axonemal and ~15% is associated tightly with the membranes.

Removal of the membrane-associated tubulin from sRM could be achieved by urea washing or alkali extraction (see Materials and methods). Two successive urea washes released 60-80% of the MAT, while two successive extractions with sodium carbonate, pH 11.5, removed 50-75% of the MAT. Urea denatures protein structure and therefore disrupts specifically and tightly bound membrane peripheral proteins (Tanford, 1961). As expected on the basis of its membrane topography, rhodopsin was completely resistant to extraction by urea or alkali. Taken together, these results argue that ROS membrane tubulin does not span the bilayer, but binding to one or more ROS
D. F. Matesic and others

Fig. 4. (A) Purification of ROS membrane-associated tubulin by DEAE ion exchange/red dye affinity chromatography. Lane (a) CHAPS solubilized sRDM. (b) Proteins eluted from DEAE-Sepharose at 0.15 M KCl and (c) corresponding anti-tubulin (Polysciences) immunoblot showing lack of immunoreactive proteins. (d,e) Membrane-associated tubulin purified by red dye affinity column chromatography eluted with 1.8 M KCl from two separate experiments. Lane (d) shows resolution of alpha and beta subunits. Lane (f) is an immunoblot corresponding to preparation in lane (e). Nitrocellulose blots were stained with antisera specific for sea urchin tubulin and gels were stained with Coomassie blue except lane (e) (silver stain). (B) Dot immunobinding assay showing binding of tubulin antibodies to purified ROS membrane-associated (●) or bovine brain (○) tubulin. Blots were incubated with an isolated IgG fraction of antisera to sea urchin tubulin (Polysciences), followed by 125I-labelled secondary antibodies. Data points are the average of 2 determinations for each protein concentration.

integral membrane proteins remains a possibility to be further addressed below.

Detergent solubilization of membrane-associated tubulin from ROS membranes

A fraction enriched in membrane-associated tubulin was obtained by solubilization of sRM using CHAPS detergent, followed by a two-step column fractionation procedure. This method was previously used to isolate components which yield enhanced cGMP-dependent Ca$^{2+}$ flux upon reconstitution into liposomes (Cook et al. 1987). We have also found this fraction to enhance Ca$^{2+}$ flux in liposomes compared to native membranes (Matesic, 1988).

In the first step, CHAPS-solubilized sRM proteins (Fig. 3, lane a) containing membrane-associated tubulin are fractionated on a DEAE-Sepharose column in isotonic KCl buffer containing CHAPS. All tubulin immunoreactive protein remained bound to DEAE under these conditions. Tubulin, an acidic protein, has been shown to bind tightly to DEAE ion-exchange resins at isotonic salt concentrations and physiological pH. Protein fractions which did not bind to the DEAE column under these conditions did not cross-react with tubulin antisera (Fig. 4A, lanes b and c). Fractions eluted subsequently by a 0.75 M KCl step gradient were enriched 5- to 8-fold in 58 kDa protein compared to starting material.

Further enrichment of the 58 kDa protein doublet resulted from fractionation on a red dye affinity resin. Dye affinity resins have been shown to have a high affinity for a variety of proteins containing nucleotide binding sites (Scawen et al. 1982; Wescott et al. 1979). Tubulin contains a GTP binding site on each alpha and beta subunit (Maccioni and Seeds, 1977; Shelanski et al. 1973). ROS proteins which remained bound to the red dye resin at 0.75 M KCl included the tubulin immunoreactive 58 kDa doublet, which could then be eluted with 1.8 M KCl buffer (Fig. 4A, lanes d-f). We found purified bovine brain tubulin to bind and elute from the red dye resin under identical conditions. Bovine brain tubulin also migrated at an apparent molecular mass of 58 kDa on our gels. Other ROS proteins in the 1.8 M KCl eluted fraction included a ~39 kDa protein present in near stoichiometric amounts with the 58 kDa protein and 2 high molecular mass proteins at ~220 and ~260 kDa. None of these cross-reacted with anti-tubulin antibodies on western blots. The 58 kDa protein doublet comprised 25-50 weight % of total protein in the 1.8 M KCl eluate as determined by gel densitometry of Coomassie-stained SDS/gels.

The enriched 58 kDa protein in these preparations cross-reacted with both polyclonal and monoclonal antibodies to tubulin. Quantitative dot immunobinding assays showed that the amount of antibody that bound to purified MAT was similar to the amount that bound to bovine brain tubulin within the linear range of protein concentrations shown in Fig. 4B. Comparable binding specificity for brain versus ROS membrane tubulin was also found using a mixture of monoclonal antibodies specific for alpha and beta tubulin subunits (Amersham) in dot immunobinding assays (not shown).

The 58 kDa protein was further purified to >95% homogeneity by preparative gel electrophoresis for one-dimensional peptide mapping. The major CNBr fragments of the 58 kDa protein are comparable to those of electrophoretically purified bovine brain tubulin (Fig. 5). These results further confirm the identity of the 58 kDa protein doublet as tubulin.

Tubulin reconstitution into liposomes

The membrane-associated tubulin (58 kDa protein)
Fig. 5. Peptide map of ROS membrane-associated tubulin. CNBr cleavage fragments separated on 10% to 18% acrylamide gradient gels of electrophoretically purified 58 kDa protein (a) or bovine brain tubulin (b). Peptides were stained with Coomassie blue.

Fig. 6. Fractions of ROS proteins partitioning with membrane (P) vs. soluble phase (S) in liposome reconstitution experiments. Values are mean±s.d. of 4 determinations for tubulin samples, 2 for 48 kDa protein, and 3 for KCl washed membranes.

purified by DEAE/red dye affinity chromatography partitioned with phosphatidyl choline (PC) vesicles reconstituted by detergent dialysis. Greater than 90% of added MAT was recovered in the liposome fraction (Fig. 6) and only ~5% was recovered in the soluble phase (high speed supernatant) at physiological salt concentrations. In similar reconstitution experiments, over 90% of rhodopsin, an integral membrane protein partitioned into the membrane phase. In contrast, the soluble tubulin fraction obtained from hypotonic extracts of ROS partitioned predominantly (>90%) with the aqueous phase. Another known soluble protein of ROS hypotonic extracts, 48 kDa protein (S-antigen), also partitioned with the aqueous phase. After reconstitution, the 58 kDa protein could not be extracted from the liposomes with high-salt (1.8 M KCl; Fig. 6) or low-salt washing (not shown) with or without sonication to disrupt vesicles. This suggests that the 58 kDa protein is tightly associated with the bilayer or with an integrally imbedded co-purificant, possibly the 39 kDa protein or either of the two high molecular mass proteins present in the DEAE/red dye affinity-purified MAT preparation.

Enrichment of membrane-associated tubulin in the ROS plasma membrane

Fractions enriched in plasma membrane were found to contain greatly increased proportions of the 58 kDa protein doublet (Fig. 7, lane c). The ratio of 58 kDa protein to rhodopsin was 5- to 10-fold higher in such membrane fractions than in sRM, which contain both disk and plasma membranes (Table 1). Isolated disks in turn showed reduced ratios of 58 kDa protein to rhodopsin (Table 1). Fig. 7 (lanes c-f) shows that the 58 kDa protein present in the plasma membrane preparation co-migrated with authentic tubulin and cross-reacted with tubulin antisera. The plasma membrane isolation procedure included low Mg2⁺, hypotonic rupture and wash steps, which would be expected to remove soluble tubulin from the membranes and disrupt axonemes as was the case for sRM. Failure of such procedures to remove tubulin from the plasma membrane-enriched fraction implies that tubulin is predominantly membrane-associated in this fraction.

Immunohistochemical localization of tubulin in ROS membranes

Further evidence for specific association of tubulin with the ROS plasma membrane was obtained by immunofluorescent staining of isolated, intact ROS (Fig. 8). Incubation of ROS with a mixture of monoclonal antibodies specific for alpha and beta tubulin (ICN)
Fig. 8. Localization of tubulin to ROS plasma membranes by confocal immunofluorescence microscopy. Fixed ROS isolated according to Schnetkamp et al. (1987) were double-labelled with monoclonal antibodies (ICN, mixture of clones 0ml-A and 0ml-B) to tubulin (1:200) and polyclonal N-terminal rhodopsin antibodies (1:500), followed by Texas red-conjugated anti-mouse and fluorescein-conjugated anti-rabbit secondary antibodies (1:200 each), and viewed under a 625 nm long-pass filter to visualize Texas red (A,C) or a 490-540 band-pass filter to visualize fluorescein (B). (C) No primary antibody. (D) ROS-labelled with C-terminal rhodopsin monoclonal antibodies and linked to rhodamine-conjugated secondary antibodies showing labelling of disk membranes. Bar, 10 μm.

followed by Texas red-conjugated secondary antibodies labelled the periphery of ROS (Fig. 8A), as well as the axonemes and diffuse cytosolic contents. These monoclonal antibodies reacted exclusively with the 58 kDa protein doublet on Western blots of ROS proteins. As a plasma membrane marker, the extracellularly exposed N-terminal residues of rhodopsin were co-labelled using a polyclonal antibody to the carbohydrate moiety (Polans et al. 1986) and a fluorescein-conjugated secondary antibody. Under the permeabilization conditions used (see Materials and methods), only plasma membrane rhodopsin, not disk rhodopsin, was labelled (Fig. 8B), probably due to inaccessibility of antibodies to the intradiskal space. C-terminal labelling of rhodopsin in disks, however, showed accessibility of antibodies to the disk surface (Fig. 8D). In control experiments (Fig. 8C) omitting primary antibody, no plasma membrane labelling was seen. ROS plasma membrane labelling was also absent in experiments using antisera specific for Na+/K+-ATPase, which labels rod inner segments (not shown).

Discussion

Immunocytochemical staining of rod photoreceptors shows tubulin distributed diffusely throughout the outer segment as well as in the axoneme of the connecting cilium (Kaplan et al. 1987; Sale et al. 1988). Quantitation of ROS tubulin, reported for the first time in our study, indicates that ROS contain 5-10 times more tubulin than can be accounted for by the axoneme. Approximately 20% of this non-axonemal tubulin is tightly associated with ROS plasma membranes while little or none is found with the 20-fold more prevalent disk membranes. This argues strongly against a non-specific mode of membrane association of MAT.

Ninety per cent of our isolated ROS MAT partitions into liposomes on reconstitution while ROS soluble tubulin stays with the aqueous phase. Release of MAT from the plasma membrane required protein-denaturing urea or alkali washes, suggesting that ROS membrane tubulin may be tightly associated with one or more integral membrane proteins. Binding to integral membrane proteins was previously proposed by Stephens to account for the associations of MAT with cellular membranes (Stephens, 1986). Our finding that MAT copurifies with other ROS membrane proteins (39 kDa, 110 kDa, 220 kDa, 260 kDa) on DEAE/red dye affinity chromatography (Fig. 4A) might similarly account for the association of MAT with liposomes, since all of these proteins were present and incorporated from the reconstitution mixture. Conversely, absence of one or more of such proteins in the soluble tubulin fraction might explain the dramatic failure of this tubulin fraction to partition with liposomes (Fig. 6). Thus, a chemically distinct form of tubulin might not be required to account for the membrane affinity of MAT in this system.

One of the principal proteins that coelutes with MAT upon solubilization in CHAPS and purification by ion exchange/red dye affinity chromatography is the ROS cGMP-dependent cation channel. Copurification of the voltage-dependent sodium channel and its stoichiometric association with the cellular structural proteins, ankaryn and spectrin, has been reported (Srinivasan et al. 1988). More recently, spectrin has been reported to copurify with the ROS cGMP-sensitive channel (Moladay et al. 1990). Stephens has also reported evidence for a detergent-stable tubulin-containing lipid-protein structural complex in mechanosensory ciliary membranes (Stephens, 1985). MAT has been identified as a
major component of vertebrate olfactory, but not respiratory, ciliary membranes, suggesting a role in olfactory transduction (Chen and Lancell, 1984).

Our work is consistent with these findings and similarly suggests that MAT may function in confining specific proteins to a specialized sensory membrane. Rods thus provide a well-defined system for determining the role of membrane-associated tubulin in a signal transducing cell.

This research was supported by U.S.P.H.S. grants EY00012, EY01583, T32EY07035-09, RR-01412, S10RR05088, and NSFDCCB8519677. We thank Dr. Vivianne Nachmius for helpful advice and for use of her Zeiss microscope.

References


(Received 12 February 1992 - Accepted, in revised form, 9 June 1992)