Direct binding of visual arrestin to microtubules determines the differential subcellular localization of its splice variants in rod photoreceptors.

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Running Title: Interaction of arrestin with microtubules
Summary.

Proper function of visual arrestin is indispensable for rapid signal shut-off in rod photoreceptors. Dramatic light-dependent changes in its subcellular localization are believed to play an important role in light adaptation of photoreceptor cells. Here we show that visual arrestin binds microtubules. The truncated splice variant of visual arrestin, p44, demonstrates dramatically higher affinity for microtubules than the full-length protein (p48). Enhanced microtubule binding of p44 underlies its earlier reported preferential localization to detergent-resistant membranes, where it is anchored via membrane-associated microtubules in a rhodopsin-independent fashion. Experiments with purified proteins demonstrate that arrestin interaction with microtubules is direct and does not require any additional protein partners. Importantly, arrestin interactions with microtubules and light-activated phosphorylated rhodopsin (P-Rh*) are mutually exclusive, suggesting that microtubule interaction may play a role in keeping p44 arrestin away from rhodopsin in dark-adapted photoreceptors.
Introduction

In response to stimulation by extracellular stimuli, G protein-coupled receptors (GPCRs) assume a distinct conformation (R*), which activates G proteins and initiates a downstream signaling cascade. Signaling by most GPCRs is rapidly quenched by a conserved mechanism. First, G protein-coupled receptor kinases (GRKs) recognize and phosphorylate the activated receptor. Next, the phosphorylated active receptor binds the cytosolic protein, arrestin, which prevents further receptor-G protein interaction. For most GPCRs, arrestin binding also initiates receptor internalization (1,2). In retinal rods, visual arrestin specifically binds to activated and phosphorylated rhodopsin (P-Rh*), thereby blocking the activation of transducin (Gt), and terminating phototransduction (3-5). In vitro studies suggest that arrestin also inhibits dephosphorylation of P-Rh*, preventing the reentry of rhodopsin into the phototransduction pathway (6). Release of arrestin can be facilitated by conversion of P-Rh* to phosphoopsin as all-trans-retinal dissociates and is reduced to all-trans-retinol (7). Thus, timely binding and dissociation of arrestin are equally important for proper function of rod photoreceptors. In mice lacking visual arrestin (8), rhodopsin kinase (9), or rhodopsin kinase phosphorylation sites on the rhodopsin C-terminus (10,11), rod photoresponse does not shut off normally. Excessive signaling in all these models eventually results in light dependent retinal degeneration (8-11).
The X-ray crystal structure of visual arrestin (12) yielded important insights into the molecular mechanism underlying the functional cycle of arrestin. Arrestin is an elongated two-domain molecule. Its basal conformation (inactive state) is maintained by the interactions among five charged residues in the fulcrum of the molecule (polar core) and by the interaction of its C tail with the N-domain. The initial binding of arrestin to phosphorylated receptor destabilizes the polar core (13) and releases the arrestin C tail (14). The disruption of these constraining intramolecular interactions allows arrestin transition into a high-affinity receptor-binding state involving the movement of the two arrestin domains relative to each other (15).

Photoreceptors have at least two forms of arrestin that lack the C-tail. First, in some species they express a splice variant of arrestin, termed p44, which is identical to the full-length arrestin (p48) except that the last exon encoding the C-terminal 35 amino acids is replaced by an alternative exon encoding a single alanine residue (16). Second, calpain can cleave off the last 27 amino acids from receptor-bound arrestin (17). Truncated forms of arrestin are present in bovine and human photoreceptors at a level of less than 10% of the amount of full-length arrestin (16,17). However, several lines of evidence indicate that they may play a distinct role in the regulation of phototransduction. The activation energy required for binding of p44 to P-Rh* is reduced in half compared to full length arrestin (18). The lack of the C-terminus also reduces arrestin specificity for P-Rh* so that the truncated forms can bind to light-activated unphosphorylated
rhodopsin (Rh*), as well as to dark phosphorylated rhodopsin (P-Rh) (17-19). This is important because small amounts of P-Rh are known to be present in photoreceptors even at low levels of light (20,21). This may be attributable to the relatively high basal activity of rhodopsin kinase (22) and its ability to be activated by a single Rh* to phosphorylate hundreds of inactive rhodopsin molecules (23,24).

Using time-resolved measurements of phosphodiesterase activity in a calorimetric assay, Langlois and co-workers compared the ability of p44 and full length arrestin to inhibit effector activity in response to dim flashes. Both forms of arrestin were able to shorten the responses, but p44 was ten times more potent than full length arrestin (25). Another reported difference between full-length and p44 arrestin is that most p44 is anchored to the membrane, unlike full length arrestin, which is a soluble protein translocating to the membrane only upon receptor phosphorylation (3-5). In addition, the two forms differ in their distribution between the inner and outer segments of the rod cell. In dark-adapted rods, full-length arrestin is localized to the inner segment and moves to the outer segment upon light exposure (26-30). In contrast, p44 was reported to be constitutively present in the outer segments (16). Schroder et. al. have proposed that membrane anchoring of p44 is due to its ability to bind dark P-Rh and that this interaction prevents p44 from binding to activated rhodopsin prior to its phosphorylation.
(31). Recently we found that arrestin p44 is localized to the detergent-resistant portion of the outer segment (OS) membrane (“lipid rafts”), whereas membrane-bound full length arrestin is present in the detergent-soluble fraction (32). Since rhodopsin is present in both microdomains, the reason for the specific localization of these two very similar arrestins was unclear. Here we investigate the molecular mechanisms responsible for the differential localization of full-length and truncated arrestin in photoreceptor cells.
Materials and Methods:

Materials.  Frozen bovine retinas were from Lawson, Co, Inc (Lincoln, NE). Recombinant arrestins were produced in *E.coli* as described earlier (33). Tubulin and taxol were from Cytoskeleton Inc, Heparin-Agarose was from Sigma. Monoclonal antibody to arrestin raised against amino acid residues 301-320 (SCT-128) was generously provided by Clay Smith (University of Florida); anti-opsin antibody (1D4) was a gift from Dr. R. Molday (University of British Columbia), and anti-tubulin antibodies were from Sigma. 35S-labeled methionine was from NEN, and the TNT T3-coupled *in vitro* translation reticulocyte system was from Promega. Protease inhibitor cocktail was from Boeringher.

Preparation of rod outer segment membranes: Rod outer segments (ROS) were prepared under dim red light using sucrose gradient centrifugation as described earlier (29). To obtain the membranes, ROS were passed through a 21-guage needle 5 times and the suspension was centrifuged in a table-top centrifuge at 14,000 RPM for 15 min. The pellet was resuspended in ROS buffer (10 mM HEPES pH 7.5, 100 mM KCl, 5 mM β-mercaptoethanol) and stored at 70C.
Preparation of detergent-resistant membranes (DRM): ROS membranes were resuspended in 0.5 ml of Buffer B (20 mM Tris pH 7.5, 150 mM NaCl, protease inhibitors) containing 0.5% Triton X100 to a final rhodopsin concentration of 25 µM and incubated for 5 minutes on ice. The lysate was mixed with an equal amount of 80% sucrose solution made in Buffer B. The mixture was transferred to the bottom of a centrifuge tube and layered successively with 1 ml of 30% sucrose and 0.5 ml of 5% sucrose. Following centrifugation at 35,000 RPM for 16 hrs, a light-scattering band of DRM formed at the 5-30% sucrose interface. Fractions (0.2 ml) were collected starting from the top of the gradient, and the pellet at the bottom of tube was solubilized in 0.25 ml SDS-PAGE sample buffer. Caveolin-enriched DRM were found in fractions 3-5 of the total 12 fractions. The fractions from the gradient were subjected to Western blot analysis for protein detection.

Analysis of in vitro translated arrestin: In vitro translation was carried out using the TNT kit (Promega) in accordance with the manufacturer’s instructions. 35S-labeled products were analyzed by 10% SDS-PAGE followed by autoradiography. To study the interactions of 35S-labeled arrestins with ROS membranes, radiolabeled arrestin (50 fmol) was incubated with the membrane (25 µM rhodopsin) in 50 µl buffer B for 10 min at room temperature in the dark or with illumination. The membranes were then
washed with ROS Buffer B to remove unbound arrestin and subjected to DRM fractionation as described above.

**Preparation of immobilized arrestin and tubulin.** E.coli-expressed, purified arrestin 1-378 or tubulin (Cytoskeleton, Inc.) were coupled to Aminolink Plus coupling gel (Pierce Biotechnology) according to the manufacturer's instructions. Proteins were suspended in coupling buffer (0.1M sodium phosphate, 0.15 M NaCl pH 7.2) at a concentration of 2 mg/ml and incubated with 0.1 ml of Aminolink Plus coupling gel overnight in a total volume of 0.3 ml. The beads were then washed with coupling buffer, and incubated with 1M Tris-HCl pH7.4 to block remaining active sites for 30 min at room temperature. The matrix was then washed with 1M NaCl and stored in the coupling buffer at 4°C.

**Affinity chromatography.** 100 µl of the affinity beads (tubulin or arrestin 1-378 coupled to Aminolink) was equilibrated with RIPA buffer (20 mM Tris pH7.4, 150 mM sodium chloride, 2 mM magnesium chloride, 0.5% sodium cholate, 0.1% SDS, 1% Triton X-100 supplemented with protease inhibitors). ROS DRM were lysed in RIPA buffer and added to the beads, which were then incubated at room temperature for 1h. The resins were then washed with 10 ml of RIPA buffer, the bound protein was eluted with 0.1 ml of 0.1 M glycine-HCl pH 2.9. The eluate was neutralized with 5 µl of 1M Tris pH 8.8,
concentrated by lyophilization and analyzed by Western blot.

**Analysis of rhodopsin phosphorylation by liquid chromatography coupled to mass-spectrometry.** Phosphorylation of rhodopsin in ROS homogenates was performed in vitro as described in the figure legend. The phosphorylation state of rhodopsin in DRM and total ROS membranes was determined by liquid chromatography/mass-spectrometry, as described (20,21). DRM or total ROS membranes were harvested by centrifugation at 54,000 RPM for 20 min. in a Beckman Optima ultracentrifuge. The pellet was washed twice with 10 mM Tris-HCl, pH 7.5, and then incubated at 30°C for 4 hours in 10 mM Tris-HCl, pH 7.5 containing 10 ng/µL endoproteinase aspN (Roche, Indianapolis, IN). Soluble peptides were recovered in the supernatant after centrifugation at 54,000 RPM. The supernatants (approximately 50 µl) were acidified with 5 µL of 5% acetic acid and stored at 70°C. Samples were then analyzed using an autosampler injecting 10 µL aliquots onto a 260 µm x 5 cm capillary column packed with Vydac C18 resin at a flow rate of approximately 1 µL/min. Samples were loaded in 0.08% heptafluorobutyric acid and eluted with a gradient of acetonitrile containing 0.08% heptafluorobutyric acid. The sample was then passed over a second column (Vydac C18 resin, 75 µm x 8 cm) packed in the needle used to deliver the peptides into the mass spectrometer (New Objective, Inc., Woborn, MA). The exact concentration of acetonitrile used to elute the peptides varied with flow rate and the length of the columns, but generally ranged from 11%-15%.
acetonitrile. Sample was delivered to the mass-spectrometer by microspray through a 15 µm needle (New Objective, Inc. Woborn, MA). For all experiments, we used a Finnegan LCQ deca ion trap mass spectrometer in positive ion mode. Data from the mass spectrometer was filtered through narrow mass windows (1.5-2 Daltons) centered on the predicted masses of the various phosphorylated forms of bovine rhodopsin (in their doubly charged state): 0P = 969.0; 1P = 1009.0; 2P = 1049.0; 3P = 1089.0; 4P = 1129.0; 5P = 1169.0; 6P = 1209.0. We confirmed the identity of eluting peptides by collision-induced dissociation. To obtain relative amounts of the various phosphorylated species in a given sample we integrated the area under peaks confirmed by CID to be rhodopsin C-termini. The relative amount of each species was calculated as a fraction of the total amount of rhodopsin peptide after correction for the decreased efficiency of phosphopeptide detection (20). To map the major sites of phosphorylation, we performed collision induced dissociation on mono-phosphorylated peptides as described previously in detail (20, 21). Collision induced dissociation was performed with an activation amplitude of 20.5, activation Q of 0.25, activation time of 50 ms and isolation width of 2.5 m/z. Ions in the CID spectra were assigned with the aid of Sherpa lite 4.0 software.

**Analysis of protein complexes on linear sucrose gradients.** Isolated DRM were solubilized in 1 ml of Buffer B containing 2% Triton X-100 for 15 min on ice. The lysate was then layered on top of a 10-40% linear sucrose gradient (volume: 10 ml) and
centrifuged at 35,000 rpm for 16 h in a Beckman SW41 rotor. Eighteen 0.5 ml fractions were collected starting from the top of the tube. Fractions were analyzed by SDS-PAGE and Western blotting.

**Preparation of axoneme.** Axonemes were prepared from ROS as described by Fleischman et al. 0.5 ml of ROS (500µM rhodopsin) were solubilized in a buffer containing 10 mM Hepes pH 7.4, 100 mM KCl, 5 mM MgCl₂, 2% Triton X-100 and protease inhibitors for 1h on ice. The detergent-soluble material and the axoneme were separated by centrifugation on a discontinuous sucrose step gradient consisting of three steps (5 ml each) of 40%, 50% and 60% (W/V) sucrose in ROS buffer. The axoneme-enriched fraction which appears as a distinct band at the 50%:60% sucrose interface was collected. The axoneme was diluted approximately 5-fold and centrifuged in Beckman Airfuge at 20 psi and then solubilized in SDS sample buffer.

**Microtubule binding (spin-down) assay.** Binding of arrestin to microtubules was assayed using reagents supplied by Cytoskeleton Inc. Microtubules were prepared in vitro as follows: purified tubulin (> 99% pure) was incubated at a concentration of 5 mg/ml in PEM buffer (80 mM PIPES, 1 mM MgCl₂, 1 mM EGTA and 1 mM GTP, pH 6.9) at 35°C for 15 min. Taxol was added to a final concentration of 20 µM, and the sample was
incubated at room temperature for 15 min. This resulted in a microtubule population averaging 2 \( \mu \text{m} \) long. These microtubules (10 \( \mu \text{g} \)) were mixed with recombinant arrestin (2\( \mu \text{g} \)) in 50 ml of PEM buffer and layered over 200 \( \mu \text{l} \) of 50% glycerol solution supplied by the manufacturer. The tube was centrifuged in the Beckman Airfuge at 20 psi for 15 min at room temperature. The supernatant and pellet were collected and analyzed by Western blot.

**Separation of microtubules and membranes by gel filtration.**

Sephadex G25 columns (1 ml) were equilibrated with ROS buffer. ROS membranes were loaded on the column and eluted with 1 ml of ROS buffer, and 0.15 ml fractions were collected. Membranes absorbed at the top of the column were eluted with SDS-containing buffer. All fractions were then concentrated and analyzed by Western blotting with arrestin, tubulin and rhodopsin.

**Turbidimetric Evaluation of Microtubule Assembly:** The assembly/disassembly of tubulin was measured using a light scattering assay according to instructions provided by Cytoskeleton, Inc. For the microtubule assembly assays, tubulin (3mg/ml final concentration) in PEM buffer (80 mM PIPES pH 6.9, 2 mM MgCl\(_2\), 0.5 mM EGTA, 5% glycerol) containing 1 mM GTP at 4 °C was mixed with 1-378 arrestin (5\( \mu \text{M} \)), full-
length arrestin (5 µM), and MAP2 (2 µM). Microtubule assembly was initiated by raising the temperature to 37 °C, and the absorbance at 340 nm was monitored over 50 min in a Tecan microtiter plate reader.

**Fluorescence Analysis of Rhodamine-labeled Tubulin Disassembly:** MAP-free tubulin and rhodamine-labeled tubulin (Cytoskeleton Inc.) were premixed at a ratio of 4:1 (unlabeled/labeled) to a final concentration of 1 mg/ml in PEM buffer containing 1 mM GTP at 37 °C for 30 min to form microtubules. 1-378 arrestin (5 µM) alone, full-length arrestin (5 µM), and MAP (2 µM), or buffer was then added to the mixture and incubated at 37 °C for 10 min. Reactions (10 µl) were diluted in 50 µl of 60% glycerol and gently mixed. A 4-µl aliquot was then placed on a glass slide with a coverslip, and microtubule patterns were observed by fluorescence microscopy using a Nikon Eclipse TE300 inverted microscope.
Results

Localization of truncated arrestin to DRM.

Truncated (p44) arrestin is present in the detergent resistant membranes (DRM) of bovine ROS while full-length (p48) arrestin is virtually excluded from the DRMs (Fig. 1A). To elucidate the specific structural features of p44 and full-length arrestin required for their differential localization in membrane microdomains, we first established that recombinant arrestins behave similarly to their endogenous counterparts. We expressed 35S-labeled full-length arrestin (WT) and its shorter version (1-367) which is structurally and functionally similar to p44 (19), in rabbit reticulocyte lysate and reconstituted them with bovine ROS membranes. Both 1-404 (WT) and 1-367 arrestins bound to ROS membranes containing P-Rh*. Further analysis of their distribution in membrane domains showed that most of the full-length arrestin was present in the detergent-soluble membrane (DSM) fractions (fractions 7-12) whereas most of the 1-367 arrestin was found in the DRM (Fig. 1B). As expected, in the absence of P-Rh* (light in the absence of ATP), binding of full length arrestin to the membranes was negligible (Fig. 1C). In contrast, the p44-like 1-367 arrestin bound to the membranes in the absence of ATP, and it was concentrated in the DRM fractions.

To further understand what part of the arrestin molecule is responsible for its
differential localization, we expressed a series of arrestin deletion mutants 1-191, 1-376, 1-378, 1-380, and 1-394, and studied their binding to membranes containing P-Rh\(^*\). All of the arrestin mutants bound to the rhodopsin-containing membranes, but only arrestins 1-376, 1-378, and 1-380 were enriched in the DRM. Arrestins 1-394 and 1-191 were mostly present in the DSM (Fig 2), suggesting that the amino acid sequence between 191 and 367 is required for specific targeting of the molecule to the DRM fraction, and the failure of 1-394 arrestin to localize to the DRM is due to masking of the sequence required for DRM localization.

**Rhodopsin phosphorylation status in the DRM and DSM.**

To test whether selective localization of truncated arrestin to the DRM occurs due to a difference in rhodopsin phosphorylation, we illuminated ROS membranes in the presence of \(^{32}\text{P-ATP}\) and fractionated the membranes to compare rhodopsin radiolabeling in the DRM and DSM. As shown in Fig. 3A, there was no apparent difference in rhodopsin phosphate incorporation between the two fractions. Since rhodopsin can be phosphorylated at several sites, we also performed a mass-spectrometry analysis assessing the number of phosphates per mole of rhodopsin in the isolated DRM and total ROS membrane rhodopsin. Since over 80% of rhodopsin is present in the detergent-soluble fraction, the analysis of total membranes provided a good estimate of the
phosphorylation of DSM rhodopsin. As expected, rhodopsin was largely unphosphorylated in the dark in both the DRM and DSM (Fig. 3B). Upon light activation, the distribution of single-, double-, and triple-phosphorylated rhodopsin was identical in the two membrane pools, suggesting that there is no significant difference in rhodopsin phosphorylation between the DRM and DSM. The distribution of phosphorylation sites in monophosphorylated rhodopsin in DRM and DSM was also virtually identical (Fig. 3C). Thus, specific targeting of p44 to the DRM is independent of rhodopsin phosphorylation.

**Arrestin is anchored to the DRM via a peripheral membrane protein**

To find out whether truncated arrestin localization to the DRM requires rhodopsin activation, we incubated recombinant arrestin (1-367) with dark-adapted ROS membranes. As shown in Figure 4A, arrestin (1-367) bound to the membranes in the dark and partitioned to the DRM, similar to endogenous p44. Thus, targeting of the shorter variant of arrestin to the DRM is independent of either phosphorylation or activation of rhodopsin, suggesting that it is anchored via a rhodopsin-independent mechanism. This finding disagrees with our earlier report (32) where we p44 arrestin was present in lower amounts in DRM in the dark. However, subsequently we found that those initial observations were due to problems with sample fractionation in the dark, and we established that light does not affect DRM localization of p44.
Since arrestin does not have lipid modifications, we reasoned that its targeting to membrane domains might be mediated by a DRM-associated protein. To test whether this putative anchor is sensitive to treatments affecting peripheral membrane proteins, we extracted ROS membranes with urea or low pH and then reconstituted them with 1-367 arrestin. We found that 1-367 arrestin binds to the membrane, apparently through rhodopsin, but was excluded from the DRM (Fig. 4B), indicating that specific anchoring of truncated arrestin to the DRM requires a peripheral membrane protein(s).

**Truncated arrestin interacts with tubulin.**

To isolate the putative arrestin anchor, we solubilized the DRM in RIPA buffer and subjected the extract to affinity chromatography on immobilized purified truncated arrestin (1-378) (Fig. 5A). This truncated form is functionally similar to arrestin (1-367) (18, A) but is more stable and therefore expresses better in *E. coli* (B,30). The truncated arrestin specifically retained a protein with an electrophoretic mobility of 55 kDa. Because the mobility of tubulin is 55kDa, we performed a Western blot of the eluate with specific anti-tubulin antibodies and confirmed the identity of this protein as tubulin (Fig 5A). A reciprocal experiment in which ROS lysate was incubated with tubulin-agarose beads, showed specific retention of endogenous p44 with very low binding of full-length p48 arrestin (Fig. 5B).

To determine whether arrestin interacts with the polymerized form of tubulin, (i.e.
microtubules), we treated tubulin with taxol, then added arrestin, and performed the standard spin-down assay (Fig. 5C). We found that arrestin 1-378 sedimneted through the 50% glycerol cushion along with the microtubules, whereas only a small fraction of full-length arrestin was found in the pellet. Thus, while both truncated and full length arrestin bind microtubules, truncated arrestin binds with a much higher affinity.

**Native arrestin-microtubule complex in ROS.**

To verify that a p44-microtubule complex actually exists in ROS, we first confirmed that tubulin is present in the DRM fraction isolated from ROS (Fig. 6A). We then solubilized the isolated DRM (fractions 3-5) in 2% Triton X-100 and studied the distribution of p44 arrestin and microtubules on a 10-40% linear sucrose gradient. We found that most of the p44 arrestin sedimented to the bottom of the gradient along with the microtubules (Fig. 6B), while most p48 arrestin did not, suggesting that microtubules may anchor p44 to the DRM. This p44-enriched fraction (fraction 18) was then solubilized and further analyzed using affinity chromatography for arrestin on heparin-agarose (34,35). Heparin-agarose with bound p44 arrestin retained microtubules (Fig. 6B), whereas in the absence of p44, microtubules were not retained (not shown), indicating that a native complex between p44 arrestin and microtubules exists in ROS.

It is known that the bulk of polymerized tubulin in photoreceptors is present in the axoneme in the form of microtubule bundles (36). Using an established procedure (37),
we isolated the axoneme and probed it with anti-arrestin and anti-tubulin antibodies. We found that the axoneme is enriched in both tubulin and p44 arrestin while only trace amounts of full length arrestin were present (Fig.6C). Thus, truncated arrestin is enriched in two microtubule-containing compartments of the rod cells, the axoneme and the detergent-resistant membranes.

**Binding of truncated arrestin to rhodopsin and microtubules is mutually exclusive.**

Next we tested whether arrestin can bind rhodopsin and microtubules simultaneously or if its interactions with rhodopsin and microtubules are mutually exclusive. First, we obtained the microtubule-enriched cytoskeletal fraction containing endogenous p44, and tested if the addition of rhodopsin-containing membranes can release p44 from the cytoskeleton. By separating the membranes from the cytoskeletal pellet on a step glycerol gradient, we found that light-activated phosphorylated rhodopsin, but not dark phosphorylated rhodopsin, removed p44 from microtubules (Fig. 7A). In a reciprocal experiment, the addition of excess exogenous microtubules to ROS membranes prevented the localization of arrestin (1-367) to the DRM (Fig. 7B). We were also able to demonstrate competition between rhodopsin-containing membranes and microtubules for truncated arrestin using another approach. We noticed that when a suspension of ROS membranes passes through a Sephadex G-25 mini-column, the membranes are retained on the top layer of the gel while soluble proteins flow through. The membrane-
associated proteins, such as rhodopsin, can then be eluted from the gel by an SDS-containing buffer. Using this approach, we added purified arrestin (1-378) to urea-washed ROS membranes, illuminated them to form the P-Rh*-arrestin complex, then incubated these membranes in the presence or absence of microtubules, and passed the mixture through the column. In the absence of microtubules, 1-378 arrestin was retained on the gel along with rhodopsin-containing membranes. However, in the presence of microtubules, arrestin was eluted from the column along with the microtubules (Fig. 7C). Using the spin-down assay we confirmed that microtubules and truncated arrestin exist as a complex in this fraction. Taken together, these results clearly demonstrate that arrestin binding to microtubules and rhodopsin is mutually exclusive.

The arrestin C-terminus impedes its interaction with microtubules.

Even though full length arrestin contains the entire sequence present in the truncated forms, which obviously includes microtubule-binding elements, it demonstrates very low binding to microtubules. Therefore we hypothesized that the regulatory C-tail of arrestin that is involved in intramolecular interactions stabilizing arrestin’s basal (inactive) conformation (12,38), masks arrestin elements necessary for its interaction with the microtubules. To test this hypothesis, we used two “constitutively active” full length arrestin mutants in which the basal conformation is destabilized by either the polar core charge reversal mutation R175E, or by the triple alanine substitution of three hydrophobic
residues at positions 375-377 (14,35). In both mutants the C-tail is detached from the body of the molecule because anchoring intramolecular interactions that keep wild type arrestin in its inactive conformation are destroyed. The mutants were produced by \textit{in vitro} translation and tested for their association with microtubules in the spin-down assay (Fig. 8). Both the R175E and the 3A mutations significantly increased binding of the full-length arrestin to microtubules, supporting the idea that the arrestin C-tail masks the main microtubule binding site.

**Arrestin does not affect tubulin polymerization and microtubule bundling.**

Since arrestin binds microtubules, we tested whether it affects tubulin polymerization or microtubule bundling. Using a light scattering assay to observe tubulin polymerization we found that the rate of tubulin polymerization did not change in the presence of purified full-length arrestin or 1-378 truncated form (Fig. 9A). Fluorescence microscopy using rhodamine-labeled tubulin also showed that neither full length arrestin nor truncated arrestin(1-378) have any effect on microtubule bundling. This is in sharp contrast to MAP2, which caused a dramatic increase in bundles in the same assay (Fig. 9B). Thus, visual arrestin does not affect either tubulin polymerization or microtubule bundling, suggesting that arrestin binds pre-existing microtubules and that this interaction may serve to regulate the differential localization of its full length and truncated forms.
Discussion.

The presence of visual arrestin is needed to ensure survival and normal physiological responses of photoreceptor cells (8-11). Massive movement of visual arrestin to rod outer segments in the light and to the inner segments in the dark underscores the functional importance of its proper subcellular localization. Like most downstream players in the visual amplification cascade, arrestin is a soluble protein. In contrast to transducin, cGMP phosphodiesterase, and rhodopsin kinase, it has no lipid modifications that can anchor it in the appropriate cellular compartment. Yet its precise subcellular localization, which is dramatically different in light- and dark-adapted photoreceptors, suggests that arrestin also needs an anchoring mechanism. Our data suggest that direct arrestin binding to microtubules may serve to localize arrestin to the inner segments, which are incredibly rich in microtubules (39), and play a role in the differential localization of the two visual arrestin variants (16,17,28,29).

Microtubules serve as a membrane anchor for truncated arrestin.

Both truncated forms of visual arrestin, the naturally occurring splice variant (p44) and the calpain fragment, associate with membranes (17). The mechanism of this membrane association was unclear since visual arrestin has no known lipid modifications. We found that membrane anchoring of truncated arrestin is due to its binding to tubulin. Although most of the ROS tubulin is concentrated in the axoneme, a significant amount is found
outside the axoneme, and about 20% of this non-axonemal tubulin is tightly associated with the membrane (40). It has been demonstrated that direct membrane binding requires palmitoylation of tubulin and that the DRM favor palmitoylated proteins (41,42), consistent with our finding that tubulin is localized to the DRM. Although the biological function of membrane-associated microtubules is unclear, an intriguing hypothesis that it can specifically scaffold signaling proteins to the membrane thus altering their signaling properties has been proposed (43). Our finding that interaction with microtubules facilitates localization of truncated arrestin to the membrane in the dark lends support to this hypothesis.

**Microtubule binding and the specificity of truncated arrestin for the different functional forms of rhodopsin**

Previous *in vitro* studies demonstrated that truncated arrestin has a significantly reduced ability to discriminate between the different functional states of rhodopsin (24,44). In contrast to full-length arrestin, its truncated forms show significant binding to dark (inactive) phosphorylated rhodopsin (P-Rh), and to activated unphosphorylated rhodopsin (Rh*). The ability of p44 to bind Rh* suggests that it can quench signaling even before rhodopsin phosphorylation, thus blocking transducin activation prematurely. Hoffmann and colleagues proposed that in the dark, p44 is sequestered by inactive P-Rh, whereas upon light-induced rhodopsin phosphorylation p44 is transferred from dark
P-Rh to P-Rh*. According to this model, binding of p44 to phosphorylated inactivated rhodopsin also explains why p44 is attached to the membrane in the dark (31).

Our results suggest an alternative model, where membrane-associated microtubules both anchor p44 to the membrane and prevent p44 from interaction with Rh*. The addition of exogenous P-Rh*-containing membranes causes the release of p44 from its complex with microtubules (Fig. 7A), whereas the excess of exogenous microtubules can also compete with rhodopsin for arrestin binding (Fig. 7C). Thus, arrestin can dynamically associate with either rhodopsin or microtubules with the amount of available P-Rh* being the key factor regulating this balance. In the dark, when the amount of Rh* and P-Rh* is negligible, p44 is bound to microtubules. At the onset of light, the amount of Rh* greatly increases but p44 remains bound to microtubules until a certain threshold is reached thereby allowing Rh* to activate transducin. P44 has much higher affinity for P-Rh* than for Rh*, so once rhodopsin is phosphorylated, the equilibrium decisively shifts towards the arrestin-P-Rh* complex.

Potential role of arrestin-microtubule interaction in arrestin transport.

Full-length arrestin is present in the inner segment in the dark and migrates to the outer segment in response to light (26-30). In contrast, p44 is largely confined to ROS even in the dark (16). Its strong association with microtubules may explain the retention of p44 in the outer segments in the dark.
Although we found that full length arrestin does not bind microtubules as avidly as its truncated forms (Fig. 5C), it nonetheless demonstrates detectable binding (Figs.1,5,8). In photoreceptors, microtubules are concentrated in the axoneme, cilia, and the inner segment (39). Regardless of the mechanism responsible for the massive arrestin translocation to the outer segment in the light and back to the inner segment in the dark, the route of this movement in either direction passes through the cilia. We found that along with p44, a fraction of the full-length arrestin is clearly detected in the axoneme (Fig. 6C). Interestingly, it has been shown in *Xenopus* photoreceptors that a pool of arrestin is associated with the axoneme even in dark-adapted photoreceptors when most of the arrestin is present in the inner segment (45). Therefore it is tempting to speculate that direct interaction between arrestin and microtubules may play a role in arrestin transport across the cilia and/or in the localization of arrestin to the microtubule-rich inner segment (36) in dark.

In summary, here we describe a novel interaction between visual arrestin and microtubules. This interaction is significantly enhanced in the native truncated form (p44), recombinant forms lacking the C-tail, and in constitutively active full-length arrestin mutants. Our data suggest that interaction of arrestins with the cytoskeleton plays a role in arrestin-mediated regulation of phototransduction, arrestin transport, and in the differential subcellular localization of its splice variants.
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Figure Legends

Figure 1. Specific partitioning of the native bovine p44 splice variant and truncated recombinant arrestin to the detergent-resistant membrane (DRM) rafts.

(A) Light-exposed ROS were treated with 0.5% Triton X-100 and fractionated on a discontinuous sucrose gradient. Endogenous arrestin species were detected in the fractions by Western blot with anti-arrestin antibody. (B) and (C) ROS membranes were reconstituted with in vitro translated 35S-Met-labeled p48 arrestin (WT), truncated 1-367 arrestin, or their mixture (WT/1-367). After unbound proteins were washed away by centrifugation, the membranes were further fractionated to obtain the DRM and DSM. The fractions were analyzed for the presence of 35S-labeled arrestins by SDS-PAGE followed by transfer to a nitrocellulose filter and exposure to X ray film. In (B), to promote rhodopsin phosphorylation, ROS membranes were prepared in the presence of ATP and exposed to light. In (C), to avoid rhodopsin phosphorylation, ROS membranes were prepared in the absence of ATP.

Figure 2. Association of arrestin deletion mutants with the DRM.

ROS membranes containing phosphorylated rhodopsin were incubated with in vitro
translated 35S-labeled arrestin constructs of increasing size (amino acid length is indicated to the right). All of the tested constructs were verified for their ability to bind to rhodopsin (Rh* or P-Rh*). Following incubation with the membranes the unbound protein was washed away, the membranes were treated with 0.5% Triton X100, and fractionated on a sucrose gradient to detect the specific localization of the arrestin species to the DRM and DSM.

**Figure 3. Rhodopsin phosphorylation status is the same in DRM and DSM.**

A) ROS were incubated with γ32P-ATP, exposed to light, washed to remove excess ATP, treated with 0.5% Triton X100, and fractionated to separate the DRM and the detergent-soluble membrane (DSM). The fractions from the sucrose density gradient were analyzed by SDS-PAGE followed by Coomassie staining. Equal amounts of rhodopsin from the DRM and DSM fractions were loaded on the gel. The gels were then dried and exposed to X-ray film to assess the amount of 32P incorporated into rhodopsin.  
B) Mass spectrometric analysis of rhodopsin phosphorylation. ROS were incubated in the light or dark in the presence of ATP. The total ROS membrane or isolated DRMs were treated with 6M Urea and washed with distilled water. Proteolytic digestion of rhodopsin was performed as described in Methods. Liquid chromatography/mass-spectrometry was performed on rhodopsin C-terminal peptides from DRMs or from total
ROS membranes to determine the distribution of the phosphorylated forms of rhodopsin in each preparation. The various species of phosphorylated (1-3P) or unphosphorylated (0P) rhodopsin are expressed as a fraction of the total amount of rhodopsin C-terminal peptide detected in the sample.

C) The sites of monophosphorylated rhodopsin were analyzed in the DRM and total ROS membranes that were exposed to light in the presence of ATP. Monophosphorylated bovine rhodopsin peptides resolve into at least 3 species under our chromatography conditions. Each peak contains the rhodopsin C-terminal peptide phosphorylated at a different site: S343, S338 or S334 (shown above each peak). The sites of phosphorylation were determined by collision-induced dissociation.

**Figure 4. Binding of truncated arrestin (1-367) to the DRM is independent of rhodopsin.**

A) Dark-adapted or light-exposed ROS membranes were reconstituted with $^{35}$S-Met labeled 1-367 arrestin, washed, lysed and fractionated on a sucrose density gradient. The presence of arrestin in the fractions was detected by SDS-PAGE followed by autoradiography.

B) ROS membranes were washed with 4 M urea or 100 mM glycine-HCl pH 2.9 and then incubated with *in vitro* translated $^{35}$S Met-labeled arrestin (1-367). Free arrestin was washed off and the membranes were lysed and fractionated to obtain the DRM. The
fractions were resolved on SDS-PAGE, transferred to a nitrocellulose filter and exposed to X-ray film.

**Figure 5. Endogenous and recombinant purified truncated arrestin bind microtubules.**

A) Left panel: Arrestin 1-378 was expressed in *E.coli*, purified, and immobilized on Amino-link Sepharose beads. ROS lysate was incubated with the affinity matrix, which was then washed and eluted with glycine-HCl pH 2.9. The eluate was resolved on SDS-PAGE and visualized by silver stain. The arrow indicates the position of tubulin, which is enriched in the ROS cytoskeletal fraction (CSK). Right panel: the input (ROS extract), and fractions eluted from the control beads (no arrestin) or beads conjugated to arrestin 1-378 were analyzed by Western blot using anti-α tubulin antibody.

B) ROS lysate was incubated with tubulin conjugated to Amino-link Agarose. The bound and the unbound fractions were resolved on SDS-PAGE and probed with anti-arrestin antibody.

C) *E.coli*-expressed and purified full length or truncated (1-378) arrestin were incubated in the presence or absence of taxol-polymerized tubulin (microtubules, MT), then layered on 50% glycerol and centrifuged at 100,000g for 1 h. The supernatant (S) and the pellet (P) were collected, resolved on SDS-PAGE, and analyzed by Western blot with anti-arrestin antibody.
Figure 6. A microtubule-arrestin complex exists in rod photoreceptor cells.

A) ROS DRM and DSM were obtained using the standard protocol described in Methods. In addition, the pellet from the bottom of the tube was collected and analyzed along with the fractions from the sucrose gradient. The presence of tubulin in all the fractions was determined by probing by Western blot with anti-α-tubulin antibody.

B) Top panel: The isolated ROS DRM pool was solubilized with buffer containing 2% Triton X-100 and fractionated on a linear gradient of 10-45% sucrose. Bottom panel: fraction 18, containing both arrestin and tubulin, was subjected to affinity chromatography on heparin agarose. Bound and unbound fractions were analyzed by Western blot with anti-arrestin and anti-tubulin antibodies.

C) The axoneme fraction was isolated as described in the Methods. Total ROS lysate and the axoneme were analyzed by Western blot with anti-arrestin (left panel) and anti-tubulin (right panel) antibodies.

Figure 7. Binding of arrestin to rhodopsin and microtubules is mutually exclusive.

A). The cytoskeletal fraction containing microtubules and truncated arrestin was obtained from bovine ROS as described in Methods. ROS membranes containing phosphorylated rhodopsin (P-Rh*) were added to the resuspended cytoskeleton and either kept in the dark or exposed to light. Membranes and cytoskeleton were then
separated by centrifugation on 50% glycerol and the cytoskeletal fractions were analyzed for the presence of tubulin and arrestin by Western blot. The bar graph shows quantitative analysis by desitometry scanning of arrestin retained in the cytoskeleton fraction after the treatment with ROS membranes containing phosphorylated rhodopsin (P-Rh) or light-activated phosphorylated rhodopsin (P-Rh*). Data from three experiments.

B) ROS membranes were incubated with $^{35}$S-Met labeled arrestin (1-367) in the presence (+MT) or the absence of added microtubules. The membranes were then lysed and fractionated on a sucrose gradient to obtain the DRM. The fractions were tested for the presence of arrestin (1-367) using autoradiography. Fraction 13 represents the detergent-insoluble cytoskeletal fraction.

C). Top panels: Purified arrestin (1-378) was incubated with urea-washed ROS membranes and exposed to light to allow arrestin binding to rhodopsin. The membranes were then washed to remove unbound arrestin. Microtubules were added to the membrane suspension, and the mixture was passed through a 1 ml mini-column containing Sephadex G25 equilibrated with PBS. Six fractions of 0.15 ml were collected, resolved on SDS-PAGE, and analyzed by Western blot using anti-arrestin, anti-rhodopsin or anti-α tubulin antibodies. The membrane fraction (M) remaining on the Sephadex was eluted with SDS PAGE sample buffer; it contained residual endogenous
arrestin that was not released by pretreatment with urea.

Lower panels: The eluate containing the putative arrestin-microtubule complex (fraction 3) was layered on a 50% glycerol solution and centrifuged at 100,000g for 30 min. The resulting supernatant (S) and pellet (P) were analyzed by Western blot with anti-arrestin or anti-α tubulin antibodies.

**Figure 8. Binding of full length arrestin mutants to microtubules.**

*In vitro* translated full-length arrestin (WT) or the 3A and R175E mutants were incubated in the presence or absence of taxol-polymerized tubulin (microtubules, MT), layered on 50% glycerol and centrifuged at 100,000g for 1 h. The supernatant (S) and the pellet (P) were collected, resolved on SDS-PAGE, and subjected to autoradiography.

**Figure 9. Visual arrestin does not affect tubulin polymerization and microtubule bundling.**

A) Tubulin polymerization was studied by a light scattering as described in Methods. Tubulin polymerization was analyzed by measuring absorbance at 340 nm in real time using a microtiter plate reader. The rate of tubulin polymerization was compared in the presence of *E.coli*-produced purified arrestin (1-378) (black triangles), or control buffer (gray squares), and MAP2 (crosses). The effect of full-length arrestin was identical to that of truncated form.
B) Microtubule bundling was studied by fluorescent microscopy. Rhodamine-labeled tubulin was included in the tubulin solution and polymerization was initiated in the presence of purified arrestin (1-378), full length arrestin, or MAP2 (positive control). The polymerized tubulin was fixed and visualized by fluorescence microscopy.
Figure 2

Mutants

1-191

1-376

1-378

1-380

1-394

| DRM | DSM |
|-----|-----|
| 1   | 2   |
| 3   | 4   |
| 5   | 6   |
| 7   | 8   |
| 9   | 10  |
| 11  | 12  |
Figure 4

A.

Dark

Light

1 2 3 4 5 6 7 8 9 10 11 12

DRM DSM

B.

Control

4 M Urea

Glycine-HCl (pH2.9)

1 2 3 4 5 6 7 8 9 10 11 12

DRM DSM
Figure 5

A.

B.

C.

WT Arrestin

1-378 Arrestin
Figure 6

A.

B.

C.

- α-Tubulin
- p48
- p44
- p44 arrestin
- Heparin Agarose
- Bd. Unbd.
- ROS Axoneme
- p48 arrestin
- p44 arrestin
- α Tubulin
Figure 7

A. 

p44 arrestin

\[ \text{\textalpha-tubulin} \]

\[ \text{P-Rh} \quad \text{P-Rh}^* \]

B. 

\[ \begin{array}{c}
\text{DRM} \\
\text{DSM} \\
\text{1-367} \\
\end{array} \]

\[ \begin{array}{c}
\text{DRM} \\
\text{DSM} \\
\text{1-367 + MT} \\
\end{array} \]

C. 

\[ \begin{array}{c}
\text{1-378 Arrestin} \\
\text{\textalpha Tubulin} \\
\text{Eluate} \\
\text{S} \quad \text{P} \\
\end{array} \]
Figure 8
Figure 9

A.

![Graph showing Absorbance over Time for MAP2, Ctrl, and Arr 1-378.]

B.

![Images of WT arrestin, 1-378 arrestin, and MAP 2.]
Direct binding of visual arrestin to microtubules determines the differential subcellular localization of its splice variants in rod photoreceptors

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