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Recoverin Undergoes Light-dependent Intracellular Translocation in Rod Photoreceptors*

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Photoreceptor cells have a remarkable capacity to adapt the sensitivity and speed of their responses to ever changing conditions of ambient illumination. Recent studies have revealed that a major contributor to this adaptation is the phenomenon of light-driven translocation of key signaling proteins into and out of the photoreceptor outer segment, the cellular compartment where phototransduction takes place. So far, only two such proteins, transducin and arrestin, have been established to be involved in this mechanism. To investigate the extent of this phenomenon we examined additional photoreceptor proteins that might undergo light-driven translocation, focusing on three Ca^{2+}-binding proteins, recoverin and guanylate cyclase activating proteins 1 (GCAP1) and GCAP2. The changes in the subcellular distribution of each protein were assessed quantitatively using a recently developed technique combining serial tangential sectioning of mouse retinas with Western blot analysis of the proteins in the individual sections. Our major finding is that light causes a significant reduction of recoverin in rod outer segments, accompanied by its redistribution toward rod synaptic terminals. In both cases the majority of recoverin was found in rod inner segments, with ~12% present in the outer segments in the dark and less than 2% remaining in that compartment in the light. We suggest that recoverin translocation is adaptive because it may reduce the inhibitory constraint that recoverin imposes on rhodopsin kinase, an enzyme responsible for quenching the photoexcited rhodopsin during the photoresponse. To the contrary, no translocation of rhodopsin kinase itself or either GCAP was identified.

The phototransduction cascade of vertebrate photoreceptors has served as a "benchmark system" where many of the basic molecular principles of G protein-coupled receptor signal transduction have been discovered and elaborated. Vision begins with the excitation of a prototypic G protein-coupled receptor, rhodopsin, which leads to the activation of multiple molecules of the G protein, transducin. Activated transducin stimulates the activity of its effector, cGMP phosphodiesterase, which leads to a decrease in cellular cGMP, closure of cGMP-gated channels in the plasma membrane, and ultimately development of the electrical signal known as the photoresponse (see Refs. 1–4 for recent reviews on phototransduction). Inactivation of phototransduction is required to prevent photoreceptors from saturating when exposed to constant illumination. Temporal resolution of vision also requires rapid inactivation of the components of the phototransduction cascade so that the cell can rapidly respond to the next light event. Inactivation of rhodopsin is achieved by its phosphorylation by rhodopsin kinase followed by binding of arrestin to completely prevent any further transducin activation (5). The termination of cGMP hydrolysis is achieved by the GTPase activity of transducin, a reaction greatly accelerated by the GTPase activating protein, RGS9 (4, 6). Finally, restoration of cGMP to its dark level to reopen cGMP-gated channels is accomplished by guanylate cyclase (7).

Photoreceptors have the ability to adjust the speed and sensitivity of their photoresponses to ever changing conditions of ambient illumination. The illumination at the earth's surface varies by nearly 11 orders of magnitude during the normal day-night cycle, and two types of photoreceptors, rods and cones, cover this entire range of light intensities (8). It is well established that this property called light adaptation is underlaid by many individual molecular mechanisms (9). Many of these mechanisms act in response to the decrease in intracellular Ca^{2+} that takes place during the photoresponse. Ca^{2+} decrease causes an enhanced synthesis of cGMP, an increase in cGMP sensitivity of the channels, and shortening of the lifetime of photoactivated rhodopsin. These effects of Ca^{2+} are mediated by several Ca^{2+}-binding proteins, among which the most characterized are recoverin (10) and guanylate cyclase activating proteins (GCAPs)[1] (11, 12). Ca^{2+}-bound recoverin interacts with rhodopsin kinase and inhibits its ability to phosphorylate rhodopsin. The reduction in Ca^{2+} caused by light releases the kinase from the complex, thus enhancing rhodopsin phosphorylation and signal turnoff (13–16). GCAP1 and GCAP2 interact with guanylate cyclase and either inhibit its activity at high Ca^{2+} or stimulate it as Ca^{2+} decreases (17–20). As such, the light-adapted photoreceptor has enhanced cyclase activity that allows it to efficiently counter the depletion in cGMP caused by continuous phosphodiesterase activation. Each of these Ca^{2+}-bindng proteins, recoverin, GCAP1, and GCAP2, is acylated at its N terminus with a medium chain length (C12-C14) fatty acyl residue. In the case of recoverin, Ca^{2+} binding increases exposure of the myristoyl residue, which in turn increases the affinity of recoverin for membranes

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[1] The abbreviations used are: GCAP1 and GCAP2, guanylate cyclase activating proteins 1 and 2.

(reviewed in Ref. 21). In contrast, binding of Ca^{2+} to GCAPs does not cause increased membrane binding (22, 23).

An entirely novel type of photoreceptor light adaptation, based on massive reversible translocation of signaling proteins between the rod or cone outer segment, where phototransduction takes place, and the rest of the cell has been revealed in recent studies. This translocation has been documented in a broad range of animal species from flies to mammals (see Refs. 24–27 for recent reviews). So far, only two signaling proteins, arrestin (originally described in Ref. 28) and transducin (independently described in Refs. 29–31), have been shown to undergo light-driven translocation in mammalian rods. Upon illumination, transducin moves from rod outer segments, whereas arrestin moves in the opposite direction. It is thought this mechanism allows the photoreceptor to optimize the protein composition of its outer segment as the ambient lighting conditions change during the normal diurnal cycle. Evidence in support of this function has been obtained in both vertebrates (32) and invertebrates (33, 34).

Here we searched for additional proteins undergoing light-driven translocation in rods by focusing on the Ca^{2+}-binding proteins, recoverin and GCAPs. The subcellular distribution of each of these proteins was measured using a quantitative approach originally developed to study transducin translocation (32). The method is based on serial sectioning through the photoreceptor layer of a flat-mounted retina followed by protein detection in each section using Western blotting. Our major finding is that light causes a 5–10-fold reduction of recoverin content in rod outer segments, accompanied by its redistribution toward rod synaptic terminals. To the contrary, the subcellular distribution of rhodopsin kinase and both GCAP1 and GCAP2 remained unchanged in dark- and light-adapted rods.

EXPERIMENTAL PROCEDURES

Antibodies—Western blotting of proteins was performed using the following antibodies: anti-recoverin and anti-GCAP1 and -GCAP2 rabbit polyclonal antibodies (a gift from A. M. Dizhoor, Pennsylvania College of Optometry), polyclonal antibodies against cytochrome oxidase subunit IV (A-6431 from Molecular Probes), monoclonal antibodies against rhodopsin kinase (G-8 from Santa Cruz Biotechnology, Inc.), and monoclonal anti-rhodopsin antibodies 4D2 (a gift from R. S. Molday, University of British Columbia).

Serial Sectioning with Western Blotting—The method was used essentially as previously described (32, 35). A mouse eye was enucleated and dissected in Ringer's solution containing 130 mM NaCl, 3.6 mM KCl, 2.4 mM $MgCl_2$, 1.2 mM $CaCl_2$, 0.02 mM EDTA, 10 mM HEPES-NaOH (pH 7.4), with the osmolarity adjusted to 313 mOSM. All tissue manipulations were conducted in Ringer's solution. The anterior portion of the eye was cut away, and the lens was removed. A 2-mm trephine was used to punch a central disc through the entire posterior eye cup. The retina was pulled away from the eyecup disc, transferred onto a polyvinylidene difluoride membrane with the photoreceptors facing up, and positioned on a porous glass filter. The retina was flattened by applying gentle suction from underneath the glass filter, removing the entire solution from the flattening chamber. The retina, still attached to the polyvinylidene difluoride membrane, was then flat-mounted between two glass slides separated by 0.5-mm thick spacers and frozen. The cover glass and spacers were subsequently removed, and the retina was sectioned in a cryomicrotome. The alignment of the retina surface with the cutting plane of the microtome knife was performed as follows. Tissue-freezing compound was applied to the microtome specimen holder and allowed to freeze. A flat surface, large enough to accommodate the base slide holding the frozen retina, was made by sectioning through to a sufficient depth in the freezing compound. The base slide was mounted to the freezing compound by addition of water drops along the sides of the glass base. The retina was trimmed to remove any folded edges, and the remaining flat surface was cut in 5-μm serial sections. Each section was collected in a precooled 0.5-ml Eppendorf tube and stored at −80 °C until used.

Western blotting was performed using the Criterion™ system (Bio-Rad). Each frozen section was dissolved in 50 μl of SDS-PAGE sample buffer. For each tested protein, 10-μl aliquots were separated on 26-well 10–20% Tris-HCl gel during 1 h at 150 V. For rhodopsin detection, 20-fold diluted aliquots were used. For the detection of GCAPs 10 mM EGTA was added to the samples. The transfer to Immun-Blot™ polyvinylidene difluoride membrane (Bio-Rad) was carried out in Towbin buffer containing 25 mM Tris, 192 mM glycine, 15% (v/v) methanol for 2 h at 0.2 A. Proteins of interest were probed with specific antibodies against the Ca^{2+}-binding proteins and intracellular markers (rhodopsin and cytochrome oxidase) and visualized using the ECF detection system (Amersham Biosciences). The fluorescence of the bands was quantified by using the Storm 860 gel and blot imaging system (Molecular Dynamics) equipped with the same software. We found that the ECF detection system provides better linearity of the signal than the more ubiquitously used ECL system, which makes it more suitable for quantitative experiments.

To calculate the fraction of each protein of interest present in rod outer segments, we used the approach originally developed for calculating the outer segment fraction of transducin (32). The density of the immunostained bands in each section was plotted as percent of the total density of all bands. Those sections that contained rhodopsin but not cytochrome oxidase we considered as sections containing non-contaminated rod outer segment material. The fractions of the Ca^{2+}-binding proteins of interest and rhodopsin present in these sections were calculated, and the amount of each protein in the entire outer segment was derived by taking their ratios.

Light Adaptation of Animals and Determination of Rhodopsin Bleaching Levels—Prior to light exposure, mice were dark-adapted for at least 12 h and anesthetized with an intraperitoneal injection of a ketamine/xylazine mixture (75/10 mg/kg, respectively). The pupils were dilated with a mixture of 1% cyclopentolate-HCl, 2.5% phenylephrine, and 0.25% tropicamide. To prevent cataract formation in anesthetized animals, Gonak™ (Akorn) was applied on the cornea several times during the course of light exposure. Light was delivered to the eyes by fiber optic guides from an adjustable light source equipped with a 100-watt halogen bulb. Even illumination throughout the entire retina was achieved by positioning a white screen between the light guide and the eye, just above the cornea. The light intensity at the eye surface was measured by a calibrated photodiode, the spectral sensitivity of which closely matched that of rhodopsin. The photodiode was attached to a PDA-700 amplifier (TTI Inc., Oriskany, NY). Mice were typically subjected to a 30-min exposure at 60 scotopic candela m^{-2}, which resulted in the bleaching of ~40% rhodopsin in their retinas at the end of the illumination period.

Rhodopsin concentration was determined by difference spectroscopy using the molar extinction coefficient of 40,500 (36). The degree of rhodopsin bleaching in the retinas of light-adapted animals was determined by measuring the amount of rhodopsin before and after its regeneration with 11-*cis*-retinal. A protocol modified from Ref. 32 was used as follows. A single retina was extracted from an eye under dim red illumination and sonicated in 250 μl of water. A 100-μl aliquot was mixed with 20 μl of 200 mM hydroxylamine (titrated to pH 7.5 by NaOH) containing 10% n-octyl-β-D-glucopyranoside. The sample was centrifuged in a tabletop microcentrifuge, and rhodopsin concentration in the supernatant was measured by difference spectrometry. The rest of the sonicated sample was mixed with 0.5 μl of 4 mM 11-*cis*-retinal solubilized in ethanol, sonicated again, and incubated at 37 °C for 40 min in complete darkness. Upon the completion of regeneration, rhodopsin concentration was determined in a 100-μl aliquot, as in the non-regenerated sample. The extent of rhodopsin bleaching in the retina was calculated as the difference between the measured rhodopsin concentration in regenerated and non-regenerated samples.

Preparation of Mouse Recoverin Standard—The cDNA for mouse recoverin was generated by RT-PCR from mouse retinal mRNA (isolated from strain C57BL/6). This cDNA was cloned into the pTriex2 vector (Novagen). Myristoylated recoverin was generated in *Escherichia coli* and purified over phenyl-Sepharose as previously described (37). Purified myristoylated recoverin was >90% pure as judged by SDS-PAGE. The concentration of the purified protein was determined by amino acid analysis (AAA Laboratories, Mercer Island, WA).

RESULTS

Subcellular Distribution of Recoverin in Dark- and Light-adapted Retinas—Previous reports addressing the distribution of recoverin in the retina indicated that it is localized primarily in rods and cones with a very small fraction also present in some of the cone bipolar cells (10, 38, 39). In all of these studies, recoverin immunostaining was observed throughout the entire cellular volumes of rods and cones. However, immunostaining

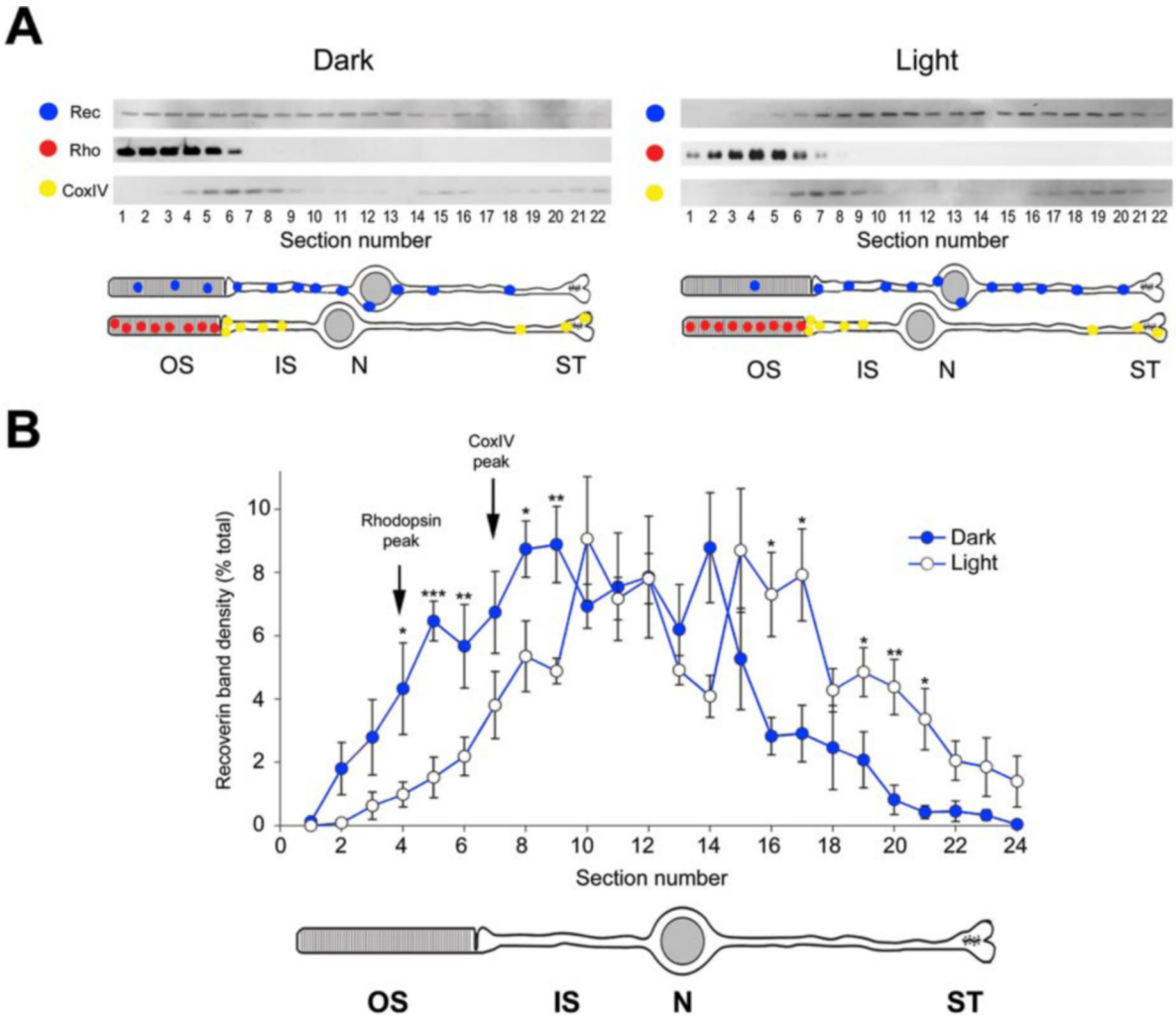

FIG. 1. **Recoverin undergoes light-dependent translocation in rods.** *A*, recoverin content in serial 5-μm sections obtained from the retinas of dark- and light-adapted mice was determined by Western blotting and compared with the contents of two intracellular markers, rhodopsin (a marker for outer segments) and cytochrome oxidase (a mitochondrial marker). Below are shown schematic drawings of rods illustrating the distribution of recoverin (*green*), rhodopsin (*red*), and cytochrome oxidase (*yellow*). *OS*, outer segment; *IS*, inner segment; *N*, nucleus; *ST*, synaptic terminal. *B*, the subcellular distribution of recoverin averaged from four dark and five light experiments with *error bars* representing S.E. The density of protein bands in each experiment was measured and plotted as a percentage of the total protein present in all sections. The averaging was performed after aligning the data among individual experiments by the section containing the maximal amount of rhodopsin. The position of this peak and the peak of cytochrome oxidase after the alignment are indicated by *arrows*.

is not an optimal technique to quantify the distribution of recoverin between the outer segment and the other cellular compartments. This is because the efficiency of antigen-antibody recognition can vary significantly in different cellular compartments and different antibodies or tissue fixation techniques often yield variable quantitative results. Collectively these phenomena are known as epitope masking (*cf.* Ref. 40).

A quantitative approach for analyzing the subcellular distribution of photoreceptor-specific proteins was introduced in our study of light-driven transducin translocation in rods (32). This method exploits the highly layered structure of the vertebrate retina by combining serial tangential sectioning of flat-mounted frozen retinas with the Western blot analysis of proteins in each section. The subcellular localization of any given protein is then determined by comparing its distribution in the sections with the distribution of protein markers confined to specific subcellular compartments of the rods. Because in this analysis proteins are completely unfolded by SDS, detached from their intracellular environments and interacting partners, and separated from one another on the gel, it does not suffer from epitope masking and allows quantitative analysis of individual protein bands on Western blots.

We previously used serial sectioning to determine the distribution of recoverin in dark-adapted mouse rods (41). We extended this analysis to quantify the subcellular distribution of recoverin in rods of dark- and light-adapted mice (Fig. 1*A*). Most of the recoverin in rods is localized in the inner segments. Remarkably, light caused a significant redistribution of recoverin from the outer segments toward the synaptic terminals. To quantify the extent to which recoverin is lost from the outer segments upon illumination, we averaged recoverin distribution profiles obtained from four dark and five light experiments and calculated the average recoverin content as described under "Experimental Procedures." We found that the recoverin content in the outer segments of dark-adapted mice was 12 $\pm$ 3% (S.E.) of its entire cellular pool whereas light caused its reduction to only 1.8 $\pm$ 0.5% (S.E.). When we then conducted a formal calculation of the amount of recoverin translocating between the proximal and distal halves of the cell (with the dividing line designated as section 12) the total extent of translocation was 24%. This amount is about twice as large than the recoverin content in the outer segments in the dark, which indicates that recoverin from both outer and inner segments moves toward the synaptic terminals upon illumination.

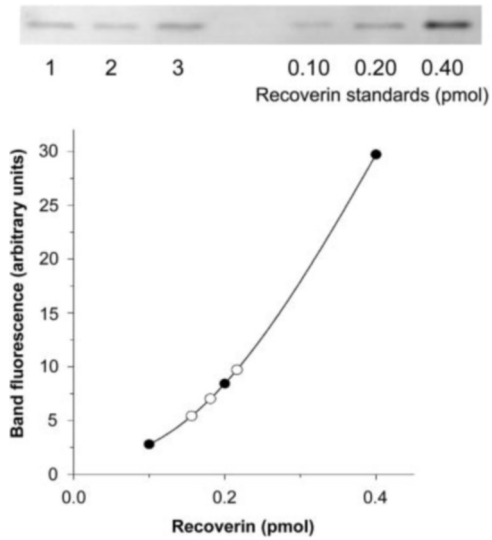

FIG. 2. **Quantification of recoverin in mouse retinas.** Three retina samples (samples *1–3*), each containing 2 pmol rhodopsin, were analyzed by Western blotting alongside three samples containing various amounts of mouse recoverin standard. To minimize possible artifacts originating from the presence of multiple proteins in crude retina homogenates, we matched the composition of all samples by mixing the standards with retina homogenates from recoverin knock-out mice containing 2 pmol rhodopsin. The calibration curve was obtained by plotting the band density (*closed circles*) against the amount of recoverin in the corresponding standard. The amounts of recoverin in samples 1–3 (*open circles*) were determined to be 156, 180, 216 fmol. The data are taken from one of two independent experiments, with the total amount of retina samples analyzed being six.

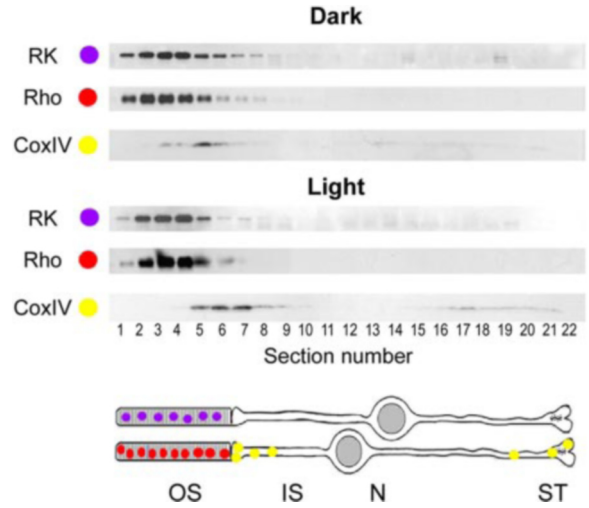

FIG. 3. **Rhodopsin kinase localization in retinas of dark- and light-adapted mice.** Serial sectioning/Western blotting analysis was performed as described in the Fig. 1 legend.

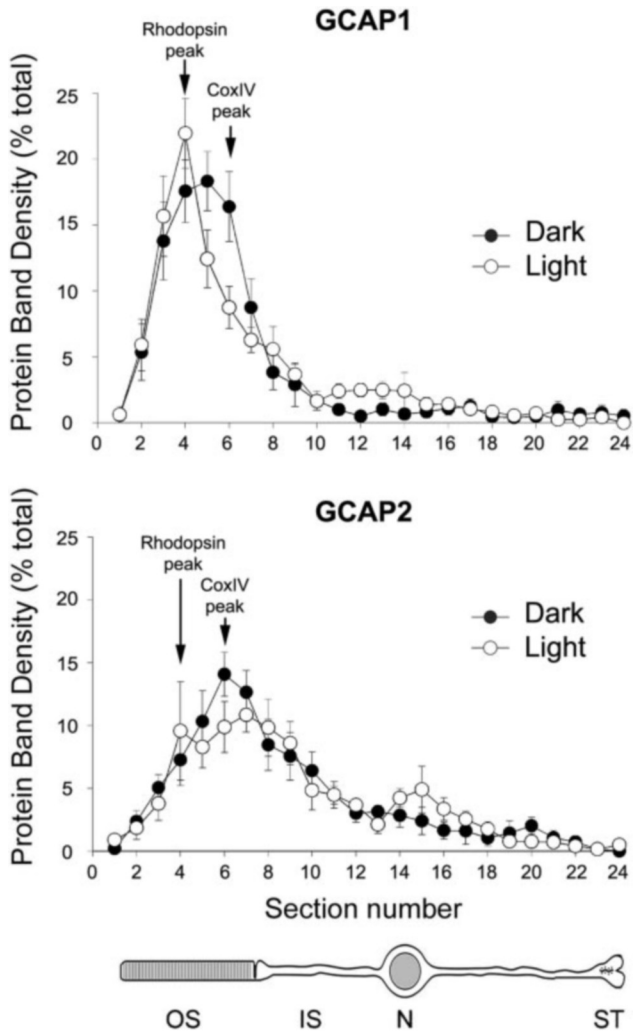

FIG. 4. **GCAP1 and GCAP2 localization in retinas of dark- and light-adapted mice.** Serial sectioning, Western blotting, densitometry, and alignment of data from multiple experiments were performed as described in the Fig. 1 legend.

The Amount of Recoverin in the Entire Rod Cell Is Comparable with Transducin—A previous report using isolated bovine rod outer segments estimated the molar ratio of recoverin to rhodopsin to be 1:174 (15). However, our observation that even in the dark ~90% recoverin in rods is localized outside the outer segments calls for re-evaluation of its total cellular amount. The molar ratio of recoverin to rhodopsin was determined by quantitative immunoblotting of mouse retina homogenates. Because cones comprise only ~3% of the total photoreceptor pool in the rodent retina (42) and recoverin immunostaining in bipolar cells is truly minor (39), we assumed that the total recoverin amount in the retina would be very close to its amount in rods. We isolated whole retinas from dark-adapted mice, disrupted them by sonication, and quantified rhodopsin by difference spectroscopy (see "Experimental Procedures"). Recoverin was analyzed in the same extracts by quantitative immunoblotting, using recombinant mouse recoverin as the standard (Fig. 2). As expected, the molar ratio of recoverin to rhodopsin determined by this approach was much higher than previously reported and equal to 0.080 ± 0.010 (S.E., $n = 6$). This is approximately equimolar with transducin in mouse rods (0.084 ± 0.004 molar ratio with rhodopsin from Ref. 43).

Translocation of Recoverin Is Not Accompanied by Translocation of Rhodopsin Kinase—Because the most well known function of recoverin is to regulate the activity of rhodopsin kinase, it was interesting to test whether rhodopsin kinase also undergoes light-driven translocation. However, the data illustrated in Fig. 3 indicate that rhodopsin kinase remains in the same outer segment sections as rhodopsin, regardless of the conditions of illumination.

Subcellular Distribution of GCAPs in the Dark- and Light-adapted Retinas—We next conducted similar analyses for determining the distributions of two other photoreceptor-specific Ca^{2+}-binding proteins, GCAP1 and GCAP2 (Fig. 4). Although the distribution profiles of GCAP1 and GCAP2 are different from one another, neither one undergoes a statistically significant translocation upon illumination. The rod outer segment content of GCAP1 was calculated to be 70.3 ± 5.5% (S.E., $n =$

4) in the dark and 70.2 ± 5.8% (S.E., $n = 4$) in the light. In fact, the actual number might be somewhat higher because GCAP1 is enriched in cones that are located at the base of rod outer segments. This cone-localized GCAP1 may be counted toward the rod inner segment sections in our analysis. An observable difference in the GCAP1 amounts in section 6 of the dark and light profiles is not statistically significant ($p = 0.15$) and is paralleled by a slightly (and also statistically insignificant) difference in the average rhodopsin content in section 6. The rod outer segment content of GCAP2 was calculated to be 25.7 ± 3.8% (S.E., $n = 4$) in the dark and 22.1 ± 1.3% (S.E., $n = 4$) in the light. Based on this analysis, we conclude that unlike recoverin neither GCAP undergoes light-dependent translocation from their relative positions in the cell.

The overall distribution of each GCAP throughout the serial sections is generally consistent with previous immunolocalization analysis of GCAP in photoreceptors. In those studies, GCAP1 was shown to be present predominantly in the outer segments of rods and cones, whereas GCAP2 was shown to be present throughout the entire photoreceptor cell (12, 44–47).

DISCUSSION

The central observation obtained in this study is that recoverin undergoes light-driven translocation from rod outer segments toward the rod synaptic terminals. The significance of this finding is that recoverin is only the third signaling protein shown to translocate in this manner in vertebrate rods. Signal-dependent protein translocation occurs in other types of cells as well (48, 49), but the combination of a high degree of subcellular compartmentalization of the rod photoreceptor and its extremely well characterized function makes it a unique model for studying cell polarization and signal-dependent redistribution of intracellular proteins.

What could be the physiological role and the underlying cellular mechanisms of recoverin translocation? As the answers to these questions are as yet unresolved we present the following analysis based on the available literature.

What is the Functional Role of Recoverin Translocation?—The function of recoverin in phototransduction has been a subject of intensive investigation. *In vitro*, recoverin regulates the lifetime of activated rhodopsin by sequestering rhodopsin kinase in a Ca^{2+}-dependent manner (13–15, 50). This suggested a mechanism where the light-induced Ca^{2+} decrease in the outer segments is accompanied by kinase deinhibition of recoverin, enhanced rhodopsin phosphorylation, and ultimately in a shortened photoresponse that is characteristic of the light-adapted photoreceptor. However, this putative mechanism was challenged by the lack of Ca^{2+} effect on rhodopsin phosphorylation in rods permeabilized by α-toxin (51). In addition, the Ca^{2+} dependence of rhodopsin phosphorylation *in vitro* (13–15) and in dialyzed rod outer segments (52) did not match the physiological range of cytoplasmic Ca^{2+} changes (53–55). The latter was explained by Bownds and co-workers (15) who argued that this range is dependent on the concentration of reacting components. Key to their analysis was the observation that the membrane association of recoverin increases its affinity to Ca^{2+} (56) along with the fact that rod outer segments are tightly packed by the membranes of the photoreceptor discs.

This apparent controversy was recently resolved by the demonstration that rods of the recoverin knock-out mice have shortened photoresponses, consistent with the role of recoverin in the regulation of rhodopsin kinase activity (16, 41). What could be the role of recoverin translocation in this context? One possibility is that recoverin departure from the outer segments works in concert with the reduction in Ca^{2+} concentration in deinhibition of rhodopsin kinase. Thus, recoverin translocation may serve as a cellular mechanism contributing to the shortening of the photoresponses in light-adapted rods.

Another aspect of recoverin translocation revealed in our study is its light-dependent elevation in synaptic terminals. Given that the majority of recoverin in rods resides outside the outer segments it seems likely that it has an additional role(s) in the cell. Indeed, a recent report (41) indicates that recoverin knock out shortens the light responses of rod bipolar and ganglion cells. This shortening occurs at a time in the photoresponse before the effect of recoverin is detectable. The increase of recoverin in synaptic terminals upon light adaptation may further modify the transmission of visual information downstream from photoreceptors.

What are the Cellular Mechanisms of Recoverin Translocation?—Recoverin undergoes a light-dependent translocation in the same direction as transducin (29–31). The relationship between these movements is unclear because there is no established interaction between recoverin and transducin. The movement of recoverin could be mediated either by diffusion through the photoreceptor cytoplasm or by active transport involving molecular motors. Both types of mechanism for the light-driven translocation of signaling proteins in photoreceptors have been considered in a recent review (27).

We find the diffusion hypothesis particularly appealing in the case of recoverin. The Ca^{2+}-bound form of recoverin, with its high affinity for membranes, may be attracted to the high membrane content of the outer segment in darkness when intracellular free Ca^{2+} concentrations are high. In light, when intracellular free Ca^{2+} levels fall, recoverin dissociates from membranes and either redistributes itself throughout the rod or binds preferentially to a target within the rod cell body. A similar scenario may exist in all other subcellular compartments where the amount of membrane-associated recoverin is determined by the balance between Ca^{2+} concentration and the corresponding membrane density. Consistent with this, GCAP1 and GCAP2, which do not translocate between the cytosol and membranes in a Ca^{2+}-sensitive manner, also do not redistribute themselves differently between the outer segment and cell body in light and dark.

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