Phosducin Facilitates Light-driven Transducin Translocation in Rod Photoreceptors

EVIDENCE FROM THE PHOSDUCIN KNOCKOUT MOUSE*

Received for publication, October 8, 2003, and in revised form, January 26, 2004
Published, JBC Papers in Press, February 18, 2004, DOI 10.1074/jbc.M311058200

Maxim Sokolov, Katherine J. Strissel, Ilya B. Leskov, Norman A. Michaud, Viktor I. Govardovskii, and Vadim Y. Arshavsky

From the Department of Ophthalmology, Harvard Medical School and the Massachusetts Eye and Ear Infirmary, Boston, Massachusetts 02114

Phosducin is a photoreceptor-specific protein known to interact with the βγ subunits of G proteins. In pursuit of the function of phosducin, we tested the hypothesis that it regulates the light-driven translocation of G protein transducin from the outer segments of rod photoreceptors to other compartments of the rod cell. Transducin translocation has been previously shown to contribute to rod adaptation to bright illumination, yet the molecular mechanisms underlying the translocation phenomenon remain unknown. In this study we provide two major lines of evidence in support of the role of phosducin in transducin translocation. First, we have demonstrated that transducin βγ subunits interact with phosducin along their entire intracellular translocation route, as evident from their co-precipitation in serial tangential sections from light-adapted but not dark-adapted retinas. Second, we generated a phosducin knockout mouse and found that the degree of light-driven transducin translocation in the rods of these mice was significantly reduced as compared with that observed in the rods of wild type animals. In knockout animals the translocation of transducin βγ subunits was affected to a larger degree than the translocation of the α subunit. We also found that the amount of phosducin in rods is sufficient to interact with practically all of the transducin present in these cells and that the subcellular distribution of phosducin is consistent with that of a soluble protein evenly distributed throughout the entire rod cytoplasm. Together, these data indicate that phosducin binding to transducin βγ subunits facilitates transducin translocation. We suggest that the mechanism of phosducin action is based on the reduction of transducin affinity to the membranes of rod outer segments, achieved by keeping the transducin βγ subunits apart from the α subunit. This increased solubility of transducin would make it more susceptible to translocation from the outer segments.

Vertebrate photoreceptors are highly specialized neurons responsible for the reception and primary processing of visual information. The outer segment compartment of the photoreceptor contains large amounts of the proteins involved in light detection and in the generation of the visual signal (for review, see Refs. 1–4). Photons entering the outer segment are absorbed by rhodopsin, which triggers sequential activation of many molecules of the photoreceptor-specific heterotrimeric G protein, transducin. Transducin activation consists of GTP binding to its α subunit followed by dissociation of the α subunit from the transducin βγ subunits and the subsequent stimulation of the downstream effector, cGMP phosphodiesterase. Signaling persists until GTP is hydrolyzed and transducin subunits re-associate into a heterotrimer. In dark-adapted rods most of the transducin is located in the outer segments. However, prolonged exposures to bright light cause massive transducin translocation from rod outer segments to other subcellular compartments of the rod (5–10). In a recent study with living rats (9), we determined that this process contributes to rod light adaptation by reducing the rate of transducin activation, which desensitizes rods under conditions of intensive background illumination. However, the molecular mechanisms that govern transducin translocation in rods remain to be elucidated.

In this study we tested the hypothesis that phosducin, an abundant photoreceptor-specific protein that interacts with transducin βγ subunits in vitro (11–15), is a facilitator of transducin translocation. We have found that upon illumination, transducin βγ subunits interact with phosducin along the entire path of their light-driven translocation through the rod cell, but almost no interaction takes place in dark-adapted photoreceptors. We next generated a phosducin knockout mouse and found that the degree of transducin translocation in the rods of knockout mice was lower than in those of control mice. The effect of the knockout was most pronounced with the transducin βγ subunits, consistent with these subunits being the partner in transducin-phosducin interaction. Finally, we found that rods contain approximately as much phosducin as transducin and that phosducin is present in all subcellular compartments of the rod, consistent with the distribution of a soluble cytoplasmic protein. These data indicate that the interaction of phosducin with transducin βγ subunits facilitates transducin translocation in light-adapted rod photoreceptors.

EXPERIMENTAL PROCEDURES

Antibodies—Sheep anti-phosducin serum was produced by Elmira Biological (Iowa City, IA) using recombinant rat phosducin as the antigen (a gift from A. Bohm, Boston Biomedical Research Institute). The antibody was purified from serum on an AminoLink Plus column (Pierce) with covalently attached recombinant phosducin. Antibodies

* This work was supported by National Institutes of Health Grant EY-10336 (to V. Y. A.), a grant from the Massachusetts Lions Eye Research Fund (to V. Y. A.), grants from Knights Templar Eye Foundation (to M. S. and K. J. S.), and United States Civilian Research and Development Foundation Grant RB1-217 (to V. I. G. and V. Y. A.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Permanent address: Institute of Evolutionary Physiology and Biochemistry, St. Petersburg, Russia 194223.

§ To whom correspondence should be addressed: Dept. of Ophthalmology, Harvard Medical School, Howe Labs/MEII, 243 Charles St., Boston MA 02114. Tel.: 617-573-4371; Fax: 617-573-4290; E-mail: vadim_arshavsky@meei.harvard.edu.

This paper is available on line at http://www.jbc.org
against cytochrome C (H-104), transducin, α subunit (P-19), transducin α subunit (K-20), 14-3-3 (H-8) and synaptophysin (SVP-38) were obtained from Santa Cruz Biotechnology. The antibody against cytochrome oxidase subunit IV (A-6431) was obtained from Molecular Probes. Monoclonal anti-phosphorylated antibody against the transducin β subunit (KT) was a gift from W. F. Simonides (NIDDK, National Institutes of Health); a monoclonal antibody against arrestin (F4C1) was a gift from L. A. Donoso (Wills Eye Hospital); a rabbit polyclonal antibody against phosducin-like protein was a gift from B. M. Willardson (Brigham Young University).

Immunohistochemistry—The eyes were enucleated from dark- or light-adapted rats and fixed for 1.5 h at room temperature in a fresh solution of 4% paraformaldehyde with 2.5% glutaraldehyde in 0.1 M cacodylate buffer containing 25 mM Tris, 192 mM glycine, 15% (v/v) methanol for 18 h. The eyecups were washed four times with PBS, incubated overnight in tissue freezing compound (Fisher) at room temperature, and then quickly frozen in the same compound using liquid nitrogen-chilled isopentane. 8-μm cross-sections were collected on Superfrost Plus slides (Fisher), thawed, air-dried for 30 min at room temperature, rinsed in PBS, permeabilized 20 min with PBS containing 0.1% Triton X-100, washed 3 times with PBS, and incubated for 1 h with blocking solution (3% normal goat serum in PBS) in a humid chamber. Sections were then incubated for 15 h with anti-phosducin or control pre-immune serum. The sections were washed 3 times with PBS, incubated for 1 h with anti-sheep-Cy3 secondary antibody, washed, mounted with Gelmount (Fisher) under glass cover slips, and viewed using the Olympus IX70 fluorescent microscope equipped with a digital camera.

Preparation of Plastic-embedded Cross-sections of the Retina—Eyes were enucleated, cleaned of outside tissue, and fixed for 1 h in freshly prepared 2% paraformaldehyde with 2.5% glutaraldehyde in 0.1 M cacodylate buffer containing 2.5 mM CaCl₂ (pH 7.4). The eye globe was then hemi-sectioned along the vertical meridian and allowed to fix overnight at the same buffer. The eyecup was washed with excess 0.1 M cacodylate buffer (pH 7.4), placed into 2% osmium tetroxide for 1.5 h, and rinsed twice with water. The eye cup was gradually dehydrated in an increasing ethanol series (25–100%) and embedded in Epon. 1-μm cross-sections were obtained and stained with alcoholic toluidine blue for light microscopy.

Serial Sectioning with Western Blotting—This method was used as previously described (9) with several optimizations. A mouse or rat eye was enucleated and dissected in Ringer’s solution containing 130 mM NaCl, 3.6 mM KCl, 2.4 mM MgCl₂, 1.2 mM CaCl₂, 0.02 mM EDTA, 10 mM HEPES-NaOH (pH 7.4) with the osmolality adjusted to 313 mosmol. 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-μm thick tissue section (for mice) or 3-μm thick tissue (rats) was used to punch a central disc through the entire posterior eye cup. The retina was gently pulled away from the eyecup disc, transferred onto a polycylinidene difluoride membrane with the photoreceptors facing up, and positioned on a polycylinidene tray. The tray was flat-mounted between two glass slides using the Olympus IX70 fluorescent microscope equipped with a digital camera.

Western Blotting—Western blotting was performed using the Criterion™ system (Bio-Rad). The sample was dissolved in 50 μl of SDS-PAGE sample buffer. For each tested protein, 10-μl aliquots were separated on a 26-well 10–20% Tris-HCl gel for 1 h at 150 V. For rhodopsin detection, 50-fold diluted aliquots were used. The transfer to Immun-Blot™ polyvinylidene difluoride membranes (Bio-Rad) was carried out in Towbin buffer containing 25 mM Tris, 192 mM glycine, 15% (v/v) methanol for 2 h at 30 mA. The antibody of interest was probed with specific and visualized using either the SuperSignal® West Pico ECL detection system (Pierce) or the ECF detection system (Amersham Biosciences). For ECL analysis, the densities of immunobound bands were quantified using the Personal Densitometer SI (Molecular Dynamics) with ImageQuant software. In ECL analysis the fluorescence of the bands was quantified by using Storm 860 Gel and Blot Imaging System (Molecular Dynamics) equipped with the same software. We found that the ECL detection system was more sensitive than the ECF system but that ECF provided better linearity of the signal. For this reason, the ECF system was used in all quantitative experiments.

Immunoprecipitation—In affinity-purified polyclonal antibodies against the transducin γ subunit were conjugated to protein A-agarose beads (Sigma) as follows. 200 μg of antibodies were bound to 0.5 ml of beads in 5 ml of PBS buffer (pH 7.4) for 1 h at room temperature. The beads were washed with 10 ml of 0.2 M Tris-HCl (pH 8.7) and resuspended in 5 ml of freshly prepared 1 mg/ml solution of the BS3 cross-linking reagent (Pierce) in the same buffer. Cross-linking was allowed to proceed for 30 min with constant shaking at room temperature. To neutralize the remaining cross-linker, beads were washed with 10 ml of 0.2 M Tris-HCl (pH 8.0) and left in the same buffer for 2 h. Non-covalently bound antibodies were removed from the beads by a 3-min wash containing 10 ml of 0.2 mM glycine-HCl (pH 2.5) followed by repeated PBS washes until the pH of the suspension reached 7.4. Beads were stored in PBS buffer containing 0.1% NaN₃ at 4°C until used.

10-μm-thick sections from rat retinas were obtained as described above. Enucleated tissue was washed in 0.1 ml of the PBS buffer supplemented with 1.85% IGEPA CA-630 (formerly known as the Nonidet P-40 detergent), 0.2 mM phenylmethylsulfonyl fluoride, and 1 μM Microcystin LR (all from Sigma). Samples were vortexed, and insoluble material was precipitated by centrifugation at 20,000 × g for 3 min. 50-μl aliquots of the supernatants were incubated with 20 μl of beads in Hande’s Mini-Spin Columns (Pierce) with constant shaking for 30 min at room temperature. The beads were washed three times for 3 min with 200 μl of the same buffer, and bound proteins were eluted by 50 μl of SDS-PAGE sample buffer.

Light Adaptation of Animals and Determination of Rhodopsin Bleaching Levels—Before light exposure, animals were anesthetized by intraperitoneal injection of a ketamine (100 mg/kg for rats and 200 and 50/50 mg/kg for rats and mice, respectively) and 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-W 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 on the eye surface was measured by a calibrated photodiode in which the spectral sensitivity closely matched that of rhodopsin. The photodiode was attached to a Fiberoptic fiber-optic guide from a photodiode amplifier (TTI Inc., Oriskany, NY). Mice were typically subjected to a 30-min exposure at 38 scotopic candela m⁻², which resulted in the bleaching of ~25% rhodopsin in their retinas. In some experiments we used the luminance of 400 scotopic candela m⁻², resulting in the bleaching of 90% rhodopsin.

Rhodopsin concentration was determined by difference spectroscopy using the molar extinction coefficient of 40,500 (16). 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 (9) was used to determine rhodopsin regeneration. A single retina (extracted from a mouse) was flattened from underneath the glass filter, removing the entire solution from the flattening chamber. The retina, still attached to the polycylinidene difluoride membrane, was then flat-mounted between two glass slides separated by 0.5-mm-thick spacers and frozen. The cover glass and the 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 specimen holder of the microtome and allowed to freeze. It was then sectioned through to create a flat surface large enough to accommodate the base slide holding the frozen retina. The retina was then mounted to the freezing compound by the addition of water drops to 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 pre-cooled 0.5-ml Eppendorf tube and stored at −80°C until used.

Generation of the Phosducin Knockout Mouse—The phosducin knockout mouse was generated by inGenious Targeting Laboratory (Stony Brook, NY) on a commercial basis. The phosducin gene was cloned from a 129sv genomic library (Stratagene) and was used to construct a
Cornea and the electrode was achieved through GonakTM (Akorn) that completely covering the mouse pupil. Electrical contact between the enclosed inside plastic armatures terminated with white contact lenses and 0.25% tropicamide. The recording electrodes were silver wires coated with a mixture of 1% cyclopentolate-HCl, 2.5% phenylephrine, simultaneously from both eyes using the Espion electrophysiology system such a gene in both human and mouse genomes. (17); however, this is essentially ruled out by the presence of only one CAC), which amplified a 1.3-kilobase product-containing fragment of GCCACTTGTGTAGC) and d (5’-TATTCATCAGCCTGTATTATCAG-3’). The targeted allele contained a deletion of phosducin gene promoter, and Exon 1. IT2 embryonic stem cells were transfected with the targeting vector and incubated in media containing G418. Surviving colonies were analyzed by PCR analysis to identify homologous recombinants. One correctly targeted embryonic stem cell clone was microinjected into C57BL/6J host blastocysts to generate chimeric mice, which were bred with C57BL/6J mice to obtain heterozygous offspring. Homozygous knockout animals and wild type littermates were obtained by sibling mating among the heterozygotes. Mouse genotyping was performed with genomic DNA isolated from tail tips using the PUREGENE™ DNA isolation kit (Gentra Systems). The wild type phosducin allele was identified by PCR using primers a (5’-TTCAAAAGGGTTCAGCGAG) and b (5’-TTCCACGACATATTGTATAACTC) which amplified a 1-kilobase product. The targeted allele was identified by PCR using primers c (5’-TCCGAGCGCCAGGACCATTGTGTAGG) and d (5’-TATTCATCAGCCTGTATTATCAGCAG), which amplified a 1.3-kilobase product-containing fragment of the neomycin resistance cassette. It should be mentioned that a possibility for the existence of two phosducin genes was previously discussed (17); however, this is essentially ruled out by the presence of only one such a gene in both mouse and mouse genomes.

Electroretinographic (ERG) Measurements—ERGs were recorded simultaneously from both eyes using the Espion electrophysiology system (Diagnostics LCC, Littleton, MA) according to published methods (18). In brief, dark-adapted mice were anesthetized, and their pupils were dilated with a mixture of 1% cyclopentolate-HCl, 2.5% phenylephrine, and 0.25% tropicamide. The recording electrodes were silver wires enclosed inside plastic armatures terminated with white contact lenses completely covering the mouse pupil. Electrical contact between the cornea and the electrode was achieved through Gonak™ (Akorn) that also protected the eyes from drying during the experiment. The reference electrode was a silver wire covered with cotton wetted in a 0.9% NaCl solution placed in the mouth. Mouse body temperature was maintained at 37 °C using a homeothermic blanket connected to its control unit (Harvard Apparatus). To calculate the number of photoisomerization events, $\Phi$, produced by test flashes of various light intensity we measured directly the amount of rhodopsin bleaching caused by a series of flashes of the maximal intensity and corrected the bleaching level for each flash intensity used. The total number of rhodopsin molecules present in a mouse rod outer segment was taken as $10^8$ (1).

RESULTS

Subcellular Distribution of Phosducin in Rat Rods—Crucial for elucidating phosducin function is the knowledge of its subcellular localization in rods. The published data on phosducin subcellular distribution, all obtained using the immunohistochemical technique, are rather controversial. Most of the early studies attributed phosducin as a rod outer segment protein (11, 12). The subsequent studies indicated that the majority of phosducin is localized in rod inner segments (19–21) or that phosducin is found most abundantly near the rod synapse (22).

In order to overcome the limitations of the immunohistochemical approach, we analyzed the subcellular distribution of phosducin by the novel technique combining serial tangential sectioning of flat-mounted frozen retinas with the Western blot analysis of proteins of interest in each section (9). In this approach, the subcellular localization of proteins of interest in the rod is determined by comparing its distribution in the sections with the distribution of protein markers confined to specific subcellular compartments. Because Western blotting is based on the analysis of proteins completely unfolded by SDS, detached from their interacting partners, and separated according to their size, this approach does not suffer from the problem of epitope masking and permits quantitative analysis of individual protein bands regardless of antibody cross-reactivity with other protein bands on the same blot. Because cones comprise only ~3% of the total photoreceptor pool in the rodent retina (25) and they are smaller than rods, we considered that essentially all detectable phosducin in this analysis originates from rods.

A representative experiment using the serial sectioning/Western blotting technique is shown in Fig. 2. We compared the distribution of phosducin throughout the serial sections obtained from the retina of a dark-adapted rat with the distribution of three marker proteins, rhodopsin (rod outer segment marker), cytochrome c (mitochondrial marker located mostly in the ellipsoid part of the inner segment and near synaptic terminal), and synaptophysin (a marker for pre-synaptic membrane vesicles located between the rod nucleus and synaptic terminal). Our data indicate that phosducin is present in all sections from the photoreceptor layer, with some enrichment in the sections corresponding to the rod inner segment. This distribution is consistent with phosducin being a soluble protein present throughout the entire volume of rod cytoplasm. No phosducin immunostaining was found in any other section of the retina beyond the photoreceptor layer (data not shown).
consistent with immunohistochemical data obtained by others (19, 21, 22) and us (Fig. 1).

To calculate the fraction of phosducin present in rod outer segments we used the approach originally developed for calculating the outer segment fraction of transducin (9). We first measured the density of the immunostained phosducin band in each section and plotted it as the percent of the total density of all bands (Fig. 3). We next identified those outer segment (rhodopsin-containing) sections that were not contaminated by the inner segment, as judged by the absence of cytochrome c. We then determined the fractions of phosducin and rhodopsin present in these sections and calculated the phosducin amount in the entire outer segment by taking their ratio. Based on such an analysis conducted with 5 dark-adapted rats we determined that the rod outer segment contains 12.5 ± 2.7% (S.E.) of the total phosducin present in the rods. No evidence of light-dependent phosducin re-distribution throughout the cell was obtained by the analysis of six rats subjected to light exposures saturating for transducin translocation (see Fig. 3 for a representative experiment). The outer segment content of phosducin in this case was 13.0 ± 2.2% of the total.

Phosducin Quantification in Rat Rods—In the next series of experiments we measured phosducin content in rat rods based on the consideration that the amount of phosducin in rods is practically equal to its total amount in the retina. This is because phosducin is present exclusively in rods, and cones and rods constitute over 97% of all photoreceptors in the rat retina (25). Retinas were extracted from dark-adapted rats under dim red illumination and disrupted by sonication in water. One aliquot was used to determine phosducin concentration by quantitative Western blotting using a calibration curve obtained with a set of recombinant rat phosducin standards (Fig. 4). Phosducin concentrations in the standards were determined spectrophotometrically using a calculated molar extinction coefficient of 16,530 at 280 nm. Another aliquot was used to determine rhodopsin concentration by difference spectroscopy. The amount of phosducin in the retinas, calculated as a molar ratio with rhodopsin, was 1:13.7 ± 1.6 (S.E., n = 5).

The amount of phosducin in rods is very close to that of transducin, present in rodents at a ~1:12 molar ratio with rhodopsin (26). Therefore, phosducin is one of the most abundant proteins in the rod, and its quantity is sufficient to potentially interact with all transducin βγ subunits in the same cell. The ~1:14 phosducin/rhodopsin molar ratio determined in this study is similar to the ~1:16 ratio reported for bovine retinas (27) and reasonably close to another estimate of ~1:21, also obtained using bovine retinas (21). The latter measurements, however, could have been underestimated because rat phosducin standard and anti-rat phosducin antibodies were used for quantification of bovine phosducin, a protein homologous but not identical to the rat protein.

Co-Immunoprecipitation of Phosducin with Transducin By Subunits in Serial Tangential Sections of Rat Retina—We next studied the formation of phosducin complex with transducin βγ subunits in individual subcellular compartments of dark- and light-adapted rods. This was achieved by monitoring phosducin co-immunoprecipitation with transducin βγ subunits by antibodies against the transducin γ subunit in 10-μm thick serial tangential sections from flat-mounted frozen rat retinas. One-half of the material from each section was used for detecting the distributions of phosducin and transducin subunits by Western blotting, and the other half was used for immunopre-
This reduction was likely to be directly caused by the knockout of the transducin subunit, as indicated by densitometric analysis of its content in 5 knockout and 5 wild-type mice, although a systematic examination of the phosducin gene promoter and exon 1 with a neomycin resistance cassette (see Experimental Procedures) indicated that knockout animals had no noticeable morphological alterations of their retina, at least up to 6 months of age.

The overall normal functioning of the rods from phosducin knockout mice was confirmed by electroretinographic recordings of their dark-adapted flash responses conducted by the technique of electroretinography (Fig. 6E). No difference was observed in kinetics and amplitude of the ERG α-waves that originate from the light-dependent suppression of the rod-circuit current. The values of the amplification constant, A, reflecting signal amplification in the phototransduction cascade (for review, see Refs. 4 and 28) were 6.1 ± 0.6 s⁻¹ (S.E., n = 8) in wild type mice and 6.8 ± 0.6 s⁻¹ (S.E., n = 8) in knockout animals. Taken together, the observations presented in Fig. 6 indicate that retinas of phosducin knockout mice lack any abnormality that would preclude this animal model from being useful in studies of light-driven transducin translocation.

**Generation and Characterization of the Phosducin Knockout Mouse**—To directly assess the role of phosducin in transducin translocation, we generated a phosducin knockout mouse and studied transducin translocation in its rod photoreceptors. The knockout was introduced by replacing the genomic fragment containing the phosducin gene promoter and exon 1 with a neomycin resistance cassette (see “Experimental Procedures”). Light microscopy of plastic-embedded retina cross-sections indicated that knockout animals had no noticeable morphological alterations of their retinas, at least up to 6 months of age (Fig. 6A). At this age, rhodopsin content in their retinas was 0.55 ± 0.01 nmol per retina (S.E., n = 3), which is essentially the same as in their wild type homozygous littermates (0.59 ± 0.01 nmol of rhodopsin per retina; S.E., n = 3). Western blot analysis confirmed that phosducin was completely absent from knockout retinas (Fig. 6B). No reliable reduction in phosducin content was observed in heterozygous animals. The amount of transducin α subunit in knockout was normal (Fig. 6C) and equal to 96 ± 10% (S.E., n = 5) of the α subunit content in the wild type animals. The amount of transducin β subunit was also close to that in wild type mice, although a systematic densitometric analysis of its content in 5 knockout and 5 wild type retinas revealed a reduction of 28 ± 4%. Although small, this reduction was likely to be directly caused by the knockout since our systematic analysis of transducin β subunit content in the parent mouse strains (129Sv and C57Bl/6J) has not revealed any noticeable difference between the strains and individual animals (data not shown). We also found no reliable change in the knockout retinal contents of phosducin-like protein, arrestin, and 14-3-3 protein (data not shown).

The overall normal functioning of the rods from phosducin knockout mice was confirmed by electrophysiological recordings of their dark-adapted flash responses conducted by the technique of electroretinography (Fig. 6E). No difference was observed in kinetics and amplitude of the ERG α-waves that originate from the light-dependent suppression of the rod-circuit current. The values of the amplification constant, A, reflecting signal amplification in the phototransduction cascade (for review, see Refs. 4 and 28) were 6.1 ± 0.6 s⁻¹ (S.E., n = 8) in wild type mice and 6.8 ± 0.6 s⁻¹ (S.E., n = 8) in knockout animals. Taken together, the observations presented in Fig. 6 indicate that retinas of phosducin knockout mice lack any abnormality that would preclude this animal model from being useful in studies of light-driven transducin translocation.

**Light-dependent Transducin Translocation in Phosducin Knockout Mice**—Light-dependent transducin translocation was measured in anesthetized mice subjected to controlled lighting conditions, as described in detail under “Experimental Procedures.” The subcellular distribution of both α and β subunits of transducin throughout the rod photoreceptor layer was assessed by serial sectioning with Western blotting. In a typical experiment shown in Fig. 7, we analyzed transducin distribution in the rods of wild type and phosducin knockout mice that were either dark-adapted or subjected to 30 min of illumination that bleached ~25% of rhodopsin. Although light caused transducin translocation in both animal types, the degree of this translocation in phosducin knockout mice was significantly reduced. The outer segments of dark-adapted knockout animals contained a smaller fraction of the total transducin than the outer segment of wild type mice, whereas the outer segments of light-adapted knockout mice contained a larger transducin fraction than the wild types.

The quantification of transducin subunit content in rod outer segments was conducted as described above for phosducin. As evident from the data summarized in Table I, smaller amounts of each transducin subunit were translocated from the outer segments of knockout mice than from the outer segments of their wild type littermates. The effect of phosducin knockout was most pronounced in the case of the transducin β subunit; whereas the difference between the transducin β subunit content in dark- and light-adapted wild type rod outer segments was ~4.3-fold, the corresponding difference in the knockout was only ~1.6-fold. The effect of phosducin knockout on the translocation of transducin α subunit was also detected, but it was smaller (compare ~3.0-fold translocation in wild type mice with ~1.9-fold translocation in the knockouts).
Control experiments showed that extending the duration of dark adaptation to over a week did not increase the fraction of transducin present in rod outer segments of either mouse type. Other controls showed that a 10-fold increase in the intensity of conditioning light did not affect the degree of transducin translocation. Importantly, the reduced transducin translocation in phosducin knockout mice was not paralleled by a reduction in arrestin translocation, as assessed in wild type and knockout mice.

**FIG. 6.** Characterization of the retinas from phosducin knockout mice. A, inactivation of the phosducin gene. Top, partial genomic structure of the phosducin gene. Restriction sites: RI, EcoRI; RV, EcoRV; N, NcoI. Middle, targeting construct. Bottom, altered phosducin locus. The primers a, b, c, and d, used for mouse genotyping, are described under “Experimental Procedures.” B, cross-sections of plastic-embedded retinas from a 50-day-old phosducin knockout mouse (−/−) and its control wild type littermate (+/+). See the Fig. 1 legend for abbreviations of the retina layers. C, Western blot analysis of phosducin from the retinas of wild type (+/+) and phosducin knockout (−/−) mice. Each sample used for Western blotting in B and C contained 1 pmol of rhodopsin. The data in panels C and D are taken from at least three independent experiments. D, Western blot analysis of individual transducin subunits in the retinas of wild type (+/+) and phosducin knockout (−/−) mice. Each sample used for Western blotting in B and C contained 1 pmol of rhodopsin. The data in panels C and D are taken from at least three independent experiments. E, normalized ERG a-waves of the dark-adapted mice. ERGs were recorded from 3-month-old wild-type (gray) and phosducin knockout (black) mice. The number of rhodopsin photoisomerizations in each rod (Φ) induced by each flash was determined as described under “Experimental Procedures” and is indicated in the graph. The traces recorded after isomerizing 890 and 8900 rhodopsins per rod are averaged from 15 individual recordings. The traces produced after isomerizing 890,000 rhodopsins per rod are averaged from 2 individual recordings. The data are normalized to the maximal a-wave amplitude recorded after saturating flashes (Φ = 890,000). The absolute amplitudes of the a-waves at this flash intensity were 0.546 and 0.528 mV for the phosducin knockout and wild type mouse, respectively. The data are taken from one of six similar experiments. OS, outer segments; IS, inner segments; ONL, outer nuclear layer; OPL, outer plexiform layer.

**FIG. 7.** The distribution of transducin subunits in the rods of dark- and light-adapted mice. Proteins in 5-μm serial sections were analyzed by Western blotting and quantified by densitometry, as described under “Results.” Blue symbols represent the transducin α subunit, green symbols represent the transducin β subunit, and red symbols represent rhodopsin. Cytochrome oxidase data are not shown, but the asterisk on each graph indicates the first section where it was detected. Drawings illustrating the corresponding anatomy of the rod cell are shown in the lower panel with abbreviations explained in the Fig. 2 legend. The data for each condition are taken from one of four similar experiments with dark-adapted mice and five experiments with light-adapted mice. OS, outer segments; IS, inner segments; ONL, outer nuclear layer; OPL, outer plexiform layer.
mice by serial sectioning with Western blotting. In both cases, the rod outer segment content of arrestin in completely dark-adapted mice was below the detectability of this technique of −3%. In contrast to transducin translocation, saturating light caused the same maximal amount of arrestin translocation, 76 ± 13 and 80 ± 7% (S.E., n = 3) of the total arrestin content in the rods of phosducin knockout and the wild type mice, respectively. This result indicates that the effect of phosducin knockout is specific to transducin translocation. Finally, we have shown that our standard illumination conditions did not cause a change in the total amount of transducin β subunit in rods of either animal type. In wild type mice, the amount of the β subunit measured immediately after illumination was 100 ± 9% (S.E.; n = 3) of the dark-adapted level. In phosducin knockout mice, the corresponding number was 107 ± 12% (S.E.; n = 4). Interestingly, the 3–4-fold maximal extent of transducin translocation in wild type mice is lower than the translocation previously observed in wild type rats (5-fold for the β and 10-fold for the α subunits from Sokolov et al. (9)). We believe this difference reflects variations between the two species rather than any difference in experimental conditions or protocols.

These data indicate that phosducin facilitates light-driven transducin translocation from rod outer segments. The larger effect of the knockout on the translocation of transducin β than on that of the α subunit is consistent with transducin βγ being the primary target of phosducin interaction.

**DISCUSSION**

**Phosducin Facilitates Light-driven Transducin Translocation in Rod Photoreceptors**—The central observation obtained in this study is that phosducin, a protein interacting with transducin βγ subunits, facilitates light-dependent transducin translocation from rod outer segments to other compartments of the rod cell. Any hypothesis detailing the mechanism of phosducin action should take into account that phosducin (as well as its complex with βγ) is a soluble protein and that phosducin knockout affects the translocation of both βγ and α subunits of transducin. Based on these considerations we favor the hypothesis that phosducin acts by enhancing the mobility of the transducin subunits through a reduction in their affinity to photoreceptor disc membranes.

Transducin is a peripheral membrane protein kept on the surface of photoreceptor disc membranes by the heterogeneous fatty acylation of the α subunit (29, 30) and farnesylation of the γ subunit (31). Importantly, the membrane affinity of the αβγ heterotrimer is much higher than the affinities of individual α and βγ subunits (32, 33). Transducin activation by photoreacted rhodopsin causes dissociation of the subunits from one another, thus reducing their membrane affinity. However, the completion of the transducin activation cycle upon the hydrolysis of GTP on the α subunit rapidly restores the heterotrimeric state that is tightly anchored to the membranes. Phosducin can interfere with this cycle by binding to the transducin βγ subunit and preventing its re-association with the α subunit, thus keeping both α and βγ subunits apart for a prolonged period of time. During this time, transducin subunits would remain more mobile.

This hypothesis is supported by a number of published experimental observations. First, transducin α and βγ subunits exit rod outer segments apart from one another, as evident from the different rates of their translocation (9). Second, phosducin directly competes with the transducin α subunit for βγ subunit binding (15, 34). Third, the transducin βγ subunit complex with phosducin has a lower affinity to membranes than the free βγ subunit (13, 35).

The role of phosducin as a facilitator of transducin subunit solubility is consistent with our observation that transducin translocation in the phosducin knockout mouse is significantly reduced but not abolished. This is because the primary role in breaking transducin subunits apart belongs to photoexcited rhodopsin, whereas phosducin acts by keeping them apart beyond the normal duration of transducin activation/inactivation cycle. The idea that transducin translocation requires the separation of its subunits is also consistent with a recent report that transducin βγ subunits are distributed throughout the entire rod cell in transducin α subunit knockout mice regardless of illumination (36). Although the authors favor the idea that the α subunit is required for the delivery of βγ subunits to rod outer segments, this result is also consistent with the βγ subunit lacking sufficient membrane affinity to accumulate on the discs of the outer segments on its own. If βγ could not be delivered to the outer segment without α, then βγ would have been expected to be completely absent from the outer segments of these mice.

There are two alternative mechanisms responsible for the translocation of transducin subunits from the outer segment after their dissociation from the disc membranes, diffusion or active transport by molecular motor systems. Sufficient evidence is not currently available to favor either of these mechanisms, and a more detailed discussion of this topic can be found in recent publications (9, 36). If transducin translocation is based on diffusion, then phosducin could additionally facilitate the rate of transducin βγ subunit translocation by keeping it more soluble through the “hiding” of the farnesyl moiety inside an intramolecular cleft (37). This might explain why the translocation of the transducin βγ subunit in the phosducin knockout is affected more than the translocation of the α subunit. If transducin translocation requires the action of molecular motors, transducin would still need to be removed from the membranes before its transport, although it could also be suggested that the phosducin complex with βγ is a preferential cargo for its corresponding molecular motor system. It could be also suggested that phosducin facilitates transducin movement by providing binding sites for it in the inner segment. However, since the phosducin complex with transducin βγ is soluble, this mechanism could not shift the final equilibrium state.

**Alternative Hypotheses on Phosducin Function in Photoreceptors**—Over the past decade phosducin remained one of the least understood photoreceptor-specific proteins, with a range of hypotheses proposed for its physiological role. The demonstration that phosducin facilitates light-dependent transducin translocation does not exclude it from having other, additional functions in photoreceptors. The results of our study allow the evaluation of several existing ideas regarding phosducin function. The historical first hypothesis suggested that phosducin regulates photoreceptor light sensitivity by binding to transducin βγ subunits in the outer segment upon continuous illumination, thus reducing the amount of transducin heterotrimer available for the activation by rhodopsin (13, 14, 35, 38). It was
Phosducin Facilitates Transducin Translocation

Further assumed that phosducin binding to transducin βγ is regulated through the cycle of phosducin phosphorylation and dephosphorylation. The light-dependent phosducin dephosphorylation in rod outer segments was thought to make it competent for assisting translocation. For example, phosducin phosphorylation in the dark was thought to release a protein called 14-3-3 (22). Phosducin interaction with 14-3-3 synaptic terminals and that this process may be modulated by a critical reading of the manuscript and T. Li for advice in generating the phosducin knockout mouse.

Acknowledgments—We are grateful to Drs. K. M. Martemyanov for critical reading of the manuscript and T. Li for advice in generating the phosducin knockout mouse.

REFERENCES

1. Pugh, E. N., Jr., and Lamb, T. D. (2000) in Handbook of Biological Physics: Molecular Mechanisms in Visual Transduction (Stavenga, D. G., DeGrip, W. J., and Pugh, E. N., Jr., ed) pp. 183–225, Elsevier Science Publishers B. V., Amsterdam
2. Burns, M. K., and Baylor, D. A. (2001) Annu. Rev. Neurosci. 24, 779–805
3. Pain, G. L., Matthews, H. H., Cornwall, M. C., and Koutalos, Y. (2001) Physiol. Rev. 81, 117–151
4. Arshavsky, V. Y., Lamb, T. D., and Pugh, E. N., Jr. (2002) Annu. Rev. Physiol. 64, 153–187
5. Brann, M. R., and Cohen, L. V. (1987) Science 235, 585–587
6. Phlip, N. J., Chang, W., and Long, K. (1987) FEBS Lett. 225, 127–132
7. Whelan, J. P., and McGinnis, J. F. (1988) J. Neurosci. Res. 20, 263–270
8. Organisac, D. T., Xie, A., Wang, H.-M., Jiang, Y.-L., Darrow, R. M., and Donoso, L. A. (1991) Exp. Eye Res. 53, 773–779
9. Sokolov, M., Lyubarsky, A. L., Strissel, K. J., Sarczenko, A. B., Govorovskiy, V. I., Pugh, E. N., Jr., and Wolfrum, U. (2002) Neuron 34, 95–106
10. Pulvermüller, A., Giesl, A., Heck, M., Wettrech, R., Schmitt, A., Ernst, O. P., Choe, H. W., Hofmann, K. P., and Wolfrum, U. (2002) Mol. Biol. Cell. 12, 2202–2203
11. Lee, R. H., Brown, B. M., and Lolley, R. N. (1984) Biochemistry 23, 1972–1977
12. Lee, R. H., Lieberman, B. S., and Lolley, R. N. (1987) Biochemistry 26, 3983–3990
13. Yoshida, T., Willardson, B. M., Wilkins, J. F., Jensen, G. J., Thornton, B. D., Thomas, J., Yang, X., and Choe, H. W., Hofmann, K. P., and Wolfrum, U. (2002) Mol. Biol. Cell. 13, 504–517
14. Nishida, T., Willardson, B. M., Wilkins, J. F., Jensen, G. J., Thornton, B. D., and Bitesen, M. W. (1984) J. Biol. Chem. 259, 24050–24057
15. Willardson, B. M., Wilkins, J. F., Yoshida, T., and Bitesen, M. W. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1474–1479
16. Gaudet, R., Bohm, A., and Sigler, P. B. (1996) Cell 87, 577–588
17. Bownds, D., Gordon-Walker, A., Gudheuguenin, A.-C., and Robinson, W. (1997) J. Gen. Physiol. 110, 525–547
18. Abe, T., Kikuchi, T., Chang, T., and Shinohara, T. (1993) Gene (Amst.) 133, 179–186
19. Lyubarsky, A. L., Falsini, B., Pessini, M. E., Valenti, P., and Pugh, E. N., Jr. (1999) J. Neurosci. 19, 442–455
20. Lee, R. H., Whelan, J. P., Lolley, R. N., and McGinnis, J. F. (1988) Exp. Eye Res. 46, 829–840
21. Gropp, K. E., Huang, J. C., and Aguird, G. D. (1997) Exp. Eye Res. 64, 857–866
22. Thulin, C. D., Howes, K., Driscoll, C. D., Savage, J. R., Rand, T. A., Baehr, W., and Willardson, B. M. (1999) Molec. Vis. 5:40
23. Nakano, K., Chen, J., Tall, G. E., Yoshida, T., Yohn, J. M., and Bitesen, M. W. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 4693–4698
24. Kuo, C.-H., and Miki, N. (1986) Neurosci. Lett. 103, 8–10
25. Ehrt, S. D., and Heth, C. A. (1987) Biochemistry 26, 7950–7957
26. Carter-Dawson, L. D., and LaVail, M. M. (1979) J. Comp. Neurol. 188, 245–262
27. Tsang, S. H., Burns, M. E., Calvert, P. D., Gouras, P., Baylor, D. A., Goff, S. P., and Arshavsky, V. Y. (1998) Science 282, 117–121
28. Margulis, A., Dang, L., Pulukuri, S., Lee, R., and Sitararamayya, A. (2002) Mol. Vis. 8, 477–482
29. Lamb, T. D., and Pugh, E. N., Jr. (1992) J. Physiol. (Lond.) 449, 719–758
30. Neuert, T. A., Johnson, R. S., Hung, W. J., and Walsh, K. A. (1992) J. Biol. Chem. 267, 18274–18277
31. Kosaka, K., Fukada, Y., Yoshizawa, T., Takas, I., and Shimonishi, Y. (1992) Nature 359, 749–752
32. Fukada, Y., Takao, T., Ohguro, H., Yoshizawa, T., Akino, T., and Shimonishi, Y. (1990) Nature 348, 458–460
33. Bigay, J., Faurobert, E., Franco, M., and Chabre, M. (1994) Biochemistry 33, 14081–14090
34. Seitz, K. R., Kistler, J., and Seelig, A. (1999) Biochemistry 38, 7980–7986
35. Ishikawa, T., Tung, T. D., and Lee, R. H. (2002) Methods Enzymol. 344, 96–109
36. Ho Lee, R. T., and Kistler, J., and Seelig, A. (1999) Biochemistry 38, 7980–7986
37. Ho Lee, R. T., and Kistler, J., and Seelig, A. (1999) Biochemistry 38, 7980–7986
38. Ho Lee, R. T., and Kistler, J., and Seelig, A. (1999) Biochemistry 38, 7980–7986
39. Ho Lee, R. T., and Kistler, J., and Seelig, A. (1999) Biochemistry 38, 7980–7986
40. Ho Lee, R. T., and Kistler, J., and Seelig, A. (1999) Biochemistry 38, 7980–7986
41. Ho Lee, R. T., and Kistler, J., and Seelig, A. (1999) Biochemistry 38, 7980–7986
42. Ho Lee, R. T., and Kistler, J., and Seelig, A. (1999) Biochemistry 38, 7980–7986
43. Ho Lee, R. T., and Kistler, J., and Seelig, A. (1999) Biochemistry 38, 7980–7986
44. Ho Lee, R. T., and Kistler, J., and Seelig, A. (1999) Biochemistry 38, 7980–7986
45. Ho Lee, R. T., and Kistler, J., and Seelig, A. (1999) Biochemistry 38, 7980–7986
