Heterologous Expression of Bovine Rhodopsin in Drosophila Photoreceptor Cells

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PURPOSE. Vertebrate and invertebrate visual pigments are similar in amino acid sequence, structural organization, spectral properties, and mechanism of action, but possess different chromophores and trigger phototransduction through distinct biochemical pathways. The bovine opsin gene (Rho) was expressed in Drosophila, to examine the properties of a vertebrate opsin within invertebrate photoreceptor cells.

METHODS. Transgenic Drosophila expressing the bovine opsin gene (Rho) in photoreceptors were created. Protein expression and cellular location of bovine rhodopsin was assessed by protein blots and immunofluorescence. The glycosylation state was determined by mobility profiles in SDS-PAGE before and after treatment with endoglycosidase. The rhodopsin chromophore was determined by HPLC-mass spectroscopy (MS) and the spectral properties by spectroscopy. The ability of the bovine rhodopsin to couple to Drosophila phototransduction components was assessed by electoretinography and to couple to vertebrate transducin by light-mediated GTPγS-binding assays.

RESULTS. Rho shown stable expression even in the absence of endogenous Rh1 opsin and chromophore. It was correctly targeted to the rhabdomeric membranes. Rho remained glycosylated during the maturation process and possessed a distinct glycosylation pattern from that of native Rho. The Drosophila-expressed Rho associated with the 3-hydroxyretinal chromophore but failed to evoke an electoretinogram response from fly photoreceptors. However, the Drosophila-expressed Rho activated transducin in a light-dependent manner.

CONCLUSIONS. Drosophila photoreceptors express a vertebrate rhodopsin as a functional visual pigment, but the expression does not activate the Drosophila phototransduction pathway. The system allows the characterization and comparison of vertebrate and invertebrate visual pigment properties in a common cell type. (Invest Ophthalmol Vis Sci. 2006;47:3722–3728) DOI:10.1167/iovs.06-0281

Visual pigments, or rhodopsins, are central to the process of vision. They are composed of an opsin apoprotein and a covalently linked vitamin A–derived chromophore. Vertebrates, like most animals, use retinal as the chromophore, whereas Drosophila and some other insects use 3-hydroxyretinal. After exposure to light, vertebrate visual pigments rapidly bleach into opsin and all-trans-retinal. The regeneration of the visual pigment in vertebrates after bleaching takes place by a series of enzymatic reactions that convert all-trans-retinal back to 11-cis-retinal before it reattaches itself to the opsin via Schiff’s base linkage. In contrast, invertebrate visual pigments are thermally stable. Regeneration of the invertebrate visual pigment is a light-dependent process wherein the activated rhodopsin’s chromophore is converted back to the 11-cis form, thereby reconstituting the visual pigment.

The major difference in the vertebrate and invertebrate phototransduction cascade mechanism is that different classes of G-proteins interact with activated rhodopsin and consequently use different downstream effector molecules. In vertebrates, transducin (Gt) serves as the heterotrimeric G-protein that activates a phosphodiesterase catalyzing the hydrolysis of cGMP to 5’-GMP, leading to the closure of cGMP-gated ion channels and resulting in the hyperpolarization of the photoreceptor cell. However, in Drosophila, Gq serves as the G-protein that activates the norpA-encoded phospholipase C, catalyzing the hydrolysis of PIP2 to DAG and IP3, leading to the opening of cation channels TRP and TRPL on the photoreceptor cell membranes and resulting in the depolarization of the photoreceptor cells.

The Drosophila compound eye is composed of approximately 800 units referred to as ommatidia. Each ommatidium contains eight photoreceptor cells, six of which (R1-6) express the major rhodopsin Rh1. Rhodopsin is localized to the rhabdomere, a highly compact microvillus organelle composed of infoldings of the plasma membrane of the photoreceptor cells. Rhabdomeres are the functional equivalent of the membrane discs in the outer segments of the vertebrate photoreceptor cells. In vertebrate rod photoreceptor cells, rhodopsin is present in the stacks of membranous discs packing the outer segment. The outer segment is connected via connecting cilium to the inner segment, which houses all the components of cell sustenance. Rhabdomeres and outer segments are the adaptations in photoreceptor cells that generate an elaborate network of membranes to accommodate large amounts of rhodopsin and, therefore, increase the efficiency of the response to light.

The molecular basis for the differences between the vertebrate and invertebrate visual pigments remains largely uncharacterized. Vertebrate opsins have been expressed in standard cell expression systems; however, attempts to express invertebrate opsin in similar expression systems have failed. This report documents the successful expression of a vertebrate (bovine) opsin within the R1-6 photoreceptor cells of the Drosophila visual system. This approach allows evaluation of the characteristics of vertebrate and invertebrate visual pigments in a common cell type.
METHODS

The pH1-Rho Expression Vector

The bovine rhodopsin cDNA, Rho, was directionally cloned using EcoRI and NotI restriction sites into a modified pCaSpeR4 expression vector,12 in which the polylinker region was replaced with a 3.0-kb HindIII-BamHI fragment corresponding to the ninaE promoter. EcoRI and NotI restriction enzyme sites for the cloning of the Rho cDNA, and a 0.7 kb PsiI-HindIII region corresponding to the 3’ untranslated region of the ninaE gene. Correct construction was confirmed by DNA sequencing, and the element introduced into w1118 fly strain using standard P element transformation techniques.13 The transformant line containing the transgene was recombined with the ninaE17 strain to obtain the fly strain expressing Rho in an Rh1 null background (<pRh1-Rho> Rh1-).

Fly Strains and Media

Flies expressing Rho in ninaE265 and ninaG2350 mutant background were generated by using standard Drosophila crossing schemes to obtain ninaE265/ninaG2350, <pRh1-Rho> ninaE17/TM3, and <pRh1-Rho> ninaE17/ninaG2350/ninaG2350 fly strains. Chromophore requirement experiments were conducted by rearing flies on vitamin A-deficient medium for at least one life cycle (egg to adult stage). Vitamin A-deficient medium was prepared by the method described by Nichols and Pak.14 Fly strains used in electrophysiology experiments were generated by crossing the flies containing the <pRh1-Rho> ninaE17 element into w; norpA; <pRh1-norpA>/TM3 to obtain a w; norpA; <pRh1-norpA>/<pRh1-Rho> ninaE17 strain. Spectral work was performed with white-eyed flies to prevent interference from eye pigment by making a cn bu/cn br; <pRh1-Rho> ninaE17/<pRh1-Rho> ninaE17 strain by standard genetic crosses.

Quantification of Rho Expression Levels in Transgenic Flies

Rho expression in Drosophila was detected by homogenizing two to three fly heads in Laemmli SDS sample buffer. Typically, one fly head equivalent was loaded on a 10% SDS-PAGE gel and centrifuged for 5 minutes at 500 g. The resulting suspension was dissolved in Laemmli SDS sample buffer containing 100 mM NaCl, 4 mM MgSO4, and 1 mM GTP. Samples were then applied to nitrocellulose membranes prepared as previously described.19

Immunopurification and Spectral Properties of Rho in Drosophila

Rho was detected by homogenizing two to three fly heads and centrifuging for 5 minutes each in 1× PBS (pH 7.5). The stained head sections were mounted in antifade mounting medium (Vectorshield; Vector Laboratories, Burlingame, CA) and viewed by fluorescence microscope (Microphor; Nikon, Tokyo, Japan) equipped with a digital camera (Spot; Diagnostic Instruments, Sterling Heights, MI).

Characterization of Rho Glycosylation in Drosophila

Cleared fly head homogenates (5 minutes, 500g) from the wild-type and Rho-expressing flies or bovine ROS membranes (200 ng Rho) were treated with 5 milliunits of N-glycosidase F or 5 units of endoglycosidase H (Calbiochem, Palo Alto, CA) for 3 hours at 37°C according to the manufacturer’s recommendations. The proteins were separated by 12% SDS-PAGE and probed by protein blotting using 1D4 monoclonal and anti-Rh1 polyclonal antibodies.

Retinoid extraction and HPLC-UV/VIS-MS analyses were performed as previously described.16 Electrophysiological analysis was performed by recording the Electoretinogram (ERG) of the appropriate fly strains at 3 days after eclosion, as previously described.17

GTPγS-Binding Assay

Bovine rod transducin (Gt) was purified from cattle retina as described elsewhere.18 Light-dependent activation of Gt by Rho expressed in Drosophila was measured using cleared fly head homogenate (5 minutes, 500g) equivalent to two heads per 100 μL of buffer A. Gt (3 μM) and [35S]GTPγS (20 μM, 2 Ci/mmole) were added to the homogenates in the dark, and the homogenates were kept in the dark or exposed to light at the indicated time points, aliquots were removed from the reactions and mixed with 1 mL of 50 mM Tris-HCl (pH 7.5) buffer containing 100 mM NaCl, 4 mM MgSO4, and 1 mM GTP. Samples were then applied to nitrocellulose filters (Whatman; Eastman Kodak, Rochester, NY), washed three times with 3 mL of the same buffer without GTP, and counted in a liquid scintillation counter. The rates of GTPγS are given as the mean ± SE of three independent measurements. To compare the activities of Rho from flies and native Rho, we performed similar GTPγS binding assays using bovine urea-stripped ROS (uROS) membranes prepared as previously described.19

Immunopurification and Spectral Properties of Rho from Drosophila

Rho in Drosophila photoreceptor cells to express and use a vertebrate visual pigment, a transgenic fly strain carrying the bovine Rho gene was created. The transgene consisted of the Rho open reading frame under control of the Rh1 gene promoter to drive expression in the R1-6 photoreceptor cells. Protein blot analysis experiments showed that these flies express Rho (Fig. 1A, upper panel). The ninaE17 allele was present in the genetic background of these flies to eliminate the expression of the Drosophila Rh1 rhodopsin allele (Fig. 1A, lower panel). Thus, the Rho visual pigment is the only visual pigment being expressed in the R1-6 cells of these flies. Figure 1B shows that Rho reaches a steady state level by 5 days,
of age that remains constant through at least 10 days of age. The protein blot analysis using bovine Rho from ROS as a standard (Fig. 1C, upper panel) demonstrated that the pRh1-Rho transgenic flies express ~50 ng Rho per fly head (~6 × 10⁷ molecules/photoreceptor cell; Fig. 1C lower panel), which is comparable with the estimates of Rh1 levels (~6 × 10⁷ molecules/photoreceptor cell) in the wild-type flies.²¹,²²

Immunofluorescence was used to investigate the localization of Rho within the *Drosophila* retina. Rho localization (Fig. 2A) mimics that of Rh1 rhodopsin (Fig. 2B). These results show that Rho correctly localized to the rhabdomeres, the light-sensitive organelles of the R1-6 photoreceptor cells. There was no observable staining in the rhabdomere of the R7 cell (Figs. 2A, 2B, arrow), an internal control, documenting the specificity of the Rho and Rh1 immunofluorescence reagents.

**Glycosylation of Rho in *Drosophila***

The protein blot analysis indicated that Rho expressed in flies had a slightly slower mobility (~34 kDa) than did Rh1 (~33 kDa) even though the Rho primary sequence is composed of fewer amino acid residues than Rh1: 348 versus 373 residues respectively (Fig. 1A). However, Rho expressed in flies had slightly faster mobility than Rho (~38 kDa) from bovine ROS membranes (Fig. 3; left, middle). To test whether the difference in mobility of the recombinant and native opsins is due to different N-glycosylation forms, fly Rho and Rho from ROS

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**Figure 1.** Expression of Rho in *Drosophila* photoreceptor cells. (A) Protein blot of head homogenates of flies carrying the <pRh1-Rho> transgene results in the expression of a protein of expected size (top), even in the absence of expression of endogenous Rh1 (bottom). (B) Protein blot of head homogenates of Rho-expressing flies over a time course showed that the Rho protein reached steady state level in 3-day-old flies and was maintained until at least 10 days of age. (C) Protein blots of known amounts of Rho from rod outer segments (RhoROS; top) compared with Rho expressed in flies (bottom) showed that ~50 ng of Rho was expressed per fly head.

**Figure 2.** Localization of Rho in *Drosophila* photoreceptor cells. (A) Immunostaining of LRW-embedded retinal cross-sections of Rho-expressing flies in the Rh1 null genetic background showed the localization of the protein in outer R1-6 photoreceptor cells. The R7 cell (arrow) lacked staining because the Rh1 promoter is active only in R1-6 cells. (B) Identical staining pattern of Rh1 in wild-type flies compared with Rho confirms the localization of Rho in R1-6 photoreceptor cells.
Identity of Chromophore Associated with Rho in Drosophila

Rho usually accommodates retinal as the chromophore, whereas Drosophila Rh1 binds 3-hydroxyretinal.\textsuperscript{1} To identify the chromophore associated with the Rho when expressed in flies, HPLC and mass spectrometry profiles\textsuperscript{10} of the chromophore extracted from wild-type and Rho-expressing flies were compared with profiles of retinal and 3-hydroxyretinal standards. The retinoid profiles at 374 nm from both wild-type (Fig. 5A) and Rho-expressing flies (Fig. 5B) contain prominent chromatographic peaks eluting at 3.8 (Fig. 5B, arrow) and 4.1 (Fig. 5B, arrowhead) minutes. Electrospray mass spectrum in the positive ion mode of these peaks showed a base peak at m/z 301 corresponding to the protonated forms of all-trans and cis isomers of 3-hydroxyretinal, respectively (data not shown). The identification of the 3.8- and 4.1-minute peaks as isomers of 3-hydroxyretinal was confirmed by HPLC-mass spectrometric analysis of the 3-hydroxyretinal standard. The chromatographic profile of the 3-hydroxyretinal standard shows a dominant peak at 3.8 minutes (Fig. 5C, arrow) and a minor peak at 4.1 minute (Fig. 5C, arrowhead), representing all-trans and cis isomers of 3-hydroxyretinal, respectively (Fig. 5C). Since Rho is associated with retinal as the chromophore in bovine retina, it is possible that Rho in flies is also associated with retinal. To determine this, the elution profiles of retinoids extracted from Rho-expressing flies were compared to the all-trans retinal standard. The all-trans retinal profile showed a prominent peak at 13.2 minutes (Fig. 5D). However, the retinoid profile from flies expressing Rho did not show a peak at 13.2 minutes, thereby indicating that flies expressing Rho do not contain detectable amounts of retinal.

Absorption Spectra of Affinity-Purified Rho from Drosophila

No spectroscopically functional pigment could be detected after the 1D4 resin purification procedure involving solubilization of membranes with 1% n-dodecyl-β-D-maltoside (data not shown). Subsequent experiments demonstrated that the solubilization treatment severely reduces the ability of Rho expressed in flies to activate transducin, suggesting that the 3-hydroxyretinal-containing pigment is unstable in detergent. In contrast, the ability of Rho to activate transducin was preserved when the fly head homogenates were preincubated with 11-cis retinal before membrane solubilization with n-dodecyl-β-D-maltoside (data not shown). The immunopurified pigment was then obtained with the typical λ\textsubscript{max} of 500 nm. On
illumination for 10 minutes, Rho yielded a $\lambda_{\text{max}}$ of 380 nm (Fig. 6).

**Rho Coupling with Gq in Flies**

Electroretinography was used to examine the ability of Rho to induce a light response in *Drosophila* R1-6 cells. In this experiment, light-dependent activity of only the R1-6 cells was measured. The light response from R7 and R8 cells was eliminated by expressing the *norpA*-encoded phospholipase C, the effector for Gq, only in R1-6 cells under the control of the Rh1 promoter. Light-induced current by Rh1 rhodopsin and Rh1 null in R1-6 cells served as the control for the ERG recordings. Expression of Rh1 generated a robust ERG response (Fig. 7, top trace), and Rh1-null flies failed to generate any light response (Fig. 7, middle trace). In Rho-expressing flies, no light response was observed. The trace is identical with the Rh1 null flies (Fig. 7, bottom trace). These results show that Rho does not evoke a light-induced electrophysiological response, probably due to the inability to activate *Drosophila* Gq.

**FIGURE 5.** Characterization of visual pigment chromophore associated with Rho in *Drosophila* photoreceptor cells. HPLC elution profiles at 374 nm of retinoids extracted from Rho flies (A), wild-type flies (B), 3-hydroxyretinal standard (C), and retinal standard (D) are shown. The comparison of elution profile between the retinoid standards and fly extracts show that both wild-type and Rho-expressing flies possessed all-trans-3-hydroxyretinal and 11-cis-3-hydroxyretinal, eluting at 3.8 (arrow) and 4.1 (arrowhead) minutes, respectively. Rho-expressing and wild-type flies did not contain any detectable amount of retinal, as shown by the absence of the 13.2 minutes chromatographic peak representing retinal (D).

**FIGURE 6.** Spectral characterization of Rho purified from *Drosophila* heads. Rho, reconstituted with 11-cis-retinal and solubilized with 1% n-dodecyl-β-D-maltoside, was purified by immunoaffinity chromatography on 1D4-Sepharose. Absorption spectra (superimposed) were obtained before (dark) and after 10 minutes of bleaching (light).

**FIGURE 7.** Characterization of electrophysiological response by Rho in *Drosophila* photoreceptor cells. ERG recordings displaying light response elicited in R1-6 cells by Rh1 (top trace), Rh1 null (middle trace), and Rho (bottom trace) when exposed to 5-second pulses of orange (O = 580 nm) and blue (B = 480 nm) light in the order depicted in the event marker. The flies expressed *norpA*-encoded phospholipase C only in R1-6 cells under the Rh1 promoter, thereby limiting the light response to these cells only. Detailed genotypes of the flies shown are: Rh1: w *norpA*/Y; <pRh1-norpA> ninaE<sup>117</sup>/+, Rh1 null: w *norpA*/Y; <pRh1-norpA> ninaE<sup>117</sup>/pRh1-norpA> ninaE<sup>117</sup>, and Rho: w *norpA*/Y; <pRh1-norpA> ninaE<sup>117</sup>/pRh1-Rho> ninaE<sup>117</sup>. 

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Rho Activation of Transducin

To test the ability of Rho synthesized in flies to activate transducin, transducin activation capability was compared to native Rho extracted from ROS membranes. The GTPγS-binding assay using fly head homogenates reconstituted with purified bovine Gt showed potent light-dependent activation of transducin by the fly-expressed Rho (Fig. 8A). Moreover, bovine ROS membranes containing equivalent concentrations of native Rho null flies under light (■) and dark (▲) conditions. Two homogenized fly heads were used in 100 μl assays, and Rho isolated from bovine rod outer segments was present at 25 nM. The k_{app} values (minutes^{-1}) calculated from slopes are: Rho (light, ■) = -0.074 ± 0.002; (dark, ▲) = -0.004; Rh1 (light, ▲) = 0.005, (dark, ▲) = -0.003; uROS (light, ■) = -0.067 ± 0.002; (dark, ▲) = -0.003; null (light, ▲) = -0.005, (dark, ▲) = -0.002.

DISCUSSION

This report documents the expression of vertebrate opsin in the Drosophila photoreceptor cells. This effort enabled direct comparison of the molecular properties of vertebrate and invertebrate visual pigments in the context of expression, maturation, trafficking, and G-protein binding specificity. Previous attempts to develop a common expression system, in cell-based expression systems, were not successful. Vertebrate opsins could be expressed in cell culture systems. However, expression of an invertebrate visual pigment in both vertebrate (COS1) and invertebrate (Sf9) cell culture expression systems were not successful.

The failure to express invertebrate opsins in cell culture systems has been attributed to the requirement of certain invertebrate photoreceptor cell-specific factors. Several specific requirements have been described for the ninaE-encoded major opsin, Rh1, expressed in R1-6 photoreceptor cells in Drosophila. Chromophore availability is essential for Rh1 production. Lack of the 3-hydroxyretinal chromophore, either by dietary deprivation or mutations in vitamin A–processing enzymes, such as NinaB dioxygenase and NinaG oxidoreductase, leads to very low levels of detectable opsin in photoreceptor cells. The ninaA-encoded chaperone is also required in the endoplasmic reticulum of the photoreceptor cells for the proper folding of the Rh1 rhodopsin.

The ability of Drosophila photoreceptor cells to stably express Rho stably and to traffic it to the rhodopsinomes shows that vertebrate opsin expression in Drosophila does not require any vertebrate-specific components. The requirement of the invertebrate components for the expression of vertebrate rhodopsin was also evaluated. Unlike Drosophila Rh1 opsin, Rho does not depend on the availability of chromophore. The expression of Rho is also independent of ninaA and ninaG activity. Thus, the results are consistent with the heterologous expression in cell culture systems showing the ease of expression of the vertebrate opsins relative to the invertebrate opsins.

N-linked glycosylation is believed to be essential for the folding and stability of Rho and other GPCRs. Bovine Rho from ROS is modified at Asn2 and Asn15 by the hexasaccharide moieties Man₆GlcNac₆. Rho can be fully deglycosylated with PNGaseF. Treatment with Endo H trims only the mannose residues and results in partial deglycosylation of native Rho. N-glycosylation of Rho expressed in flies appears to be different. Endo H is capable of full deglycosylation of Rho from flies, indicating that the sugar modifications are not converted to a complex form in the Golgi apparatus of fly photoreceptor cells. A similar Endo H sensitive glycosylation of Rho was reported for Rho expressed in yeast Saccharomyces cerevisiae. Significant efforts have been extended to achieve expression of Rho and its mutants with limited and homogeneous N-glycosylation for potential structural studies. However, the heterogeneity of N-glycosylation of Rho in cultured mammalian cells limits the use of these systems, particularly, for crystallographic applications. The protein yields and the lack of complex glycosylation of Rho expressed in the Drosophila eye indicate potential utility of transgenic flies as an expression system for Rho and other GPCRs.

Rho expressed in Drosophila contains 3-hydroxyretinal, instead of the native retinal, as the chromophore. Flies lack detectable stores of retinal, presumably due to the efficient conversion of retinal to 3-hydroxyretinal. Rho coupled to 3-hydroxyretinal forms a functional visual pigment, as the Drosophila Rho is capable of robust activation of transducin in a light-dependent manner. The study was not able to determine whether Rho containing the 3-hydroxyretinal chromophore has different spectral properties than Rho containing retinal, because extraction conditions suitable for spectral analysis were not established.

Drosophila Rh1 rhodopsin is incapable of coupling with transducin. Moreover, despite utilization of Drosophila visual pigment chromophore, Rho in flies cannot activate the Drosophila G-protein, Gq. These findings indicate that productive interaction for activation of transducin and Gq is confined to the corresponding visual pigment. Rho and Rh1 have extensive amino acid sequence variation in the cytoplasmic regions re-

FIGURE 8. Light-dependent transducin activation. (A) Time courses of [³⁵S]GTPγS binding to transducin (3 μM) in the presence of Drosophila-expressed Rho under light (●) and dark conditions (▲) and in the absence of Drosophila Rho1 in light (■) and dark conditions (▲). Two homogenized fly heads were used in 100 μl assays. (B) Control experiments using Rho from urea-stripped bovine rod outer segments under light (●) and dark (▲) conditions and extracts of Rh1 null flies under light (■) and dark (▲) conditions. Two homogenized fly heads were used in 100 μl assays, and Rho isolated from bovine rod outer segments was present at 25 nM. The k_{app} values (minutes^{-1}) calculated from slopes are: Rho (light, ●) = -0.074 ± 0.002; (dark, ▲) = -0.004; Rh1 (light, ▲) = 0.005, (dark, ▲) = -0.003; uROS (light, ●) = -0.067 ± 0.002; (dark, ▲) = -0.003; null (light, ▲) = -0.005, (dark, ▲) = -0.002.
sponsible for G-protein interaction, and some of these differences must specify the ability to interact only with the correct G-protein. It should now be possible, by analyzing chimeric visual pigments in the experimental system introduced herein, to determine the distinct motifs, as well as common structural regions, of the vertebrate and invertebrate visual pigments.

**Acknowledgments**

The authors thank the Center for Environmental Science and Technology at the University of Notre Dame for allowing generous access to the HPLC system and mass spectrometer, Bill Boggess and Michelle Joyce for assistance with HPLC analysis, and Kathleen Mitchell for assistance with *Drosophila* genetics.

**References**


