Invertebrates such as Drosophila or Limulus assemble their visual pigment into the specialized rhodopsin membranes of photoreceptors where phototransduction occurs. We have investigated the biosynthesis of rhodopsin from the Limulus lateral eye with three cell culture expression systems: mammalian COS1 cells, insect Sf9 cells, and amphibian Xenopus oocytes. We extracted and affinity-purified epitope-tagged Limulus rhodopsin expressed from a cDNA or cRNA from these systems. We found that all three culture systems could efficiently synthesize the opsin polypeptide in quantities comparable with that found for bovine opsin. However, none of the systems expressed a protein that stably bound 11-cis-retinal. The protein expressed in COS1 and Sf9 cells appeared to be misfolded, improperly localized, and proteolytically degraded. Similarly, Xenopus oocytes injected with Limulus opsin cDNA did not evoke light-sensitive currents after incubation with 11-cis-retinal. However, injecting Xenopus oocytes with mRNA from Limulus lateral eyes yielded light-dependent conductance changes after incubation with 11-cis-retinal. Also, expressing Limulus opsin cDNA in the R1−R6 photoreceptors of transgenic Drosophila yielded a visual pigment that bound retinal, had normal spectral properties, and coupled to the endogenous phototransduction cascade. These results indicate that Limulus opsin may require one or more photoreceptor-specific proteins for correct folding and/or chromophore binding. This may be a general property of invertebrate opsins and may underlie some of the functional differences between invertebrate and vertebrate visual pigments.

In all animals, visual pigments consist of an apoprotein and bound chromophore, usually 11-cis-retinal (most animals), 3,4-didehydro-11-cis-retinal (some fish, amphibia, and reptiles), or 3-hydroxy-11-cis-retinal (some insects) (1–3). Typically, in both vertebrates and invertebrates the visual pigment is very stable in the dark, with thermal isomerization rates measured in years (4, 5). This stability contributes significantly to the overall limit of visual sensitivity. In addition, the mechanism of visual pigment activation is similar in vertebrates and invertebrates, in that absorption of a photon causes the isomerization of the chromophore from the cis to the trans conformation. This event initiates a series of conformational changes in the chromophore-protein complex that lead to the formation of an active state that interacts with G-proteins to regulate membrane conductance (6–8).

Despite these similarities, the invertebrate and vertebrate pigments differ in at least two significant ways. First, the active states have different thermal stabilities. In vertebrates, the active state, metarhodopsin II bleaches (or decomposes) rapidly into the approprotein and free all-trans-retinal. In contrast, invertebrate metarhodopsin (M) is stable under physiological conditions (for a review, see Ref. 9). Second, the regeneration of pigment containing the 11-cis-chromophore following exposure to light differs between vertebrates and invertebrates. Vertebrate opsins can bind 11-cis-retinal, synthesized enzymatically in the adjacent pigment epithelium, and spontaneously form the Schiff’s base linkage, apparently without any additional protein cofactors (2). In invertebrates, the pigment can be efficiently converted back to the dark-adapted state by light at ambient temperature, a process called photoregeneration, or by thermal isomerization of the chromophore (9). Photoregeneration is the main mechanism by which the rhodopsin concentration is maintained in bright light (10–12).

Studies to investigate the molecular bases for the differences between vertebrate and invertebrate pigments have been limited by the lack of a suitable expression system for invertebrate opsins that would be comparable with those available for vertebrate opsins (13–19). Here, we have evaluated a number of these systems for their suitability to express Limulus rhodopsin. We find that none of the common systems used for the expression of vertebrate opsin produce a Limulus opsin capable of binding retinal. In fact, our findings suggest that one or more protein cofactors in invertebrate photoreceptors are necessary for the functional expression of the Limulus visual pigment.

**EXPERIMENTAL PROCEDURES**

Nucleic Acids

COS1 Expression Vector—The Limulus rhodopsin cDNA, Lim Rh1 (20), was cloned into the mammalian expression vector, pMT3 (21). In order to facilitate detection and purification of the expressed protein, the carboxyl Up and Down amino acids of Limulus rhodopsin were replaced with 14 amino acids from bovine rhodopsin, thus introducing the epitope recognized by the 1D4 monoclonal antibody (22). The resulting cDNA,
Lim Rh1–1D4, contained 180 base pairs of 5′-untranslated sequence, the coding region for amino acids 1–362 (of 367 total) from Lim Rh1 (including an alanine at position 105, which is a polymorphic site in Limulus (20)), and the cDNA for amino acids 330–348 from bovine rhodopsin.

Sf9 Expression Vector—Lim Rh1–1D4 was transferred into the SmaI-NotI restriction sites of the baculovirus expression vector pH11032 (Pharmingen). To form the infecting virus, this construct was recombined with Baculogold DNA (Pharmingen) in Sf9 cells according to the manufacturer’s instructions.

Drosophila Expression Vector—An expression construct was generated in which either the Lim Rh1–1D4 cDNA (referred to as P(Rh1+LimMT) or wild type Lim Rh1 cDNA (referred to as P(LimRh1)) was cloned into an expression cassette containing 2.5 kb of Drosophila ninaE promoter sequence, the transcription initiation site, 33 bp of the 5′-untranslated region, a short polylinker, and 650 bp from the 3′-end of the ninaE gene that includes the polyadenylation signal (23). Both constructs were subcloned into the p′-marked P-element vector “C4” (24).

In Vitro Transcription—Lim Rh1–1D4 was cloned into the EcoRI-NotI restriction sites of pALTER1 (Invitrogen). Capped RNA was prepared by run-off transcription using NotI-linearized plasmid and T7 polymerase (either RibolMax or RiboMax kit from Promega or Ambion, respectively). Transcripts were polyadenylated in vitro, purified using oligo(dT) chromatography (PolyATtract, Promega, Inc.), and quantified using UV spectroscopy. Finally, RNA samples were suspended in water.

mRNA—Retinas from adult Limulus polyphemus (200 animals), collected in summer and fall near Woods Hole, Massachusetts, were isolated and used either immediately for RNA extraction or frozen in dry ice and stored at −70 °C until use. RNA was extracted using the TRIzol reagent (Invitrogen) and poly(A) RNA isolated using oligo(dT) chromatography (PolyATtract; Promega) and eluted in water at a concentration of ∼0.5 mg/ml.

Expression in COS1 Cells
COS1 cells were grown and transfected using the DEAE-dextran protocol (25). All Limulus rhodopsin transfection experiments were performed in parallel with bovine rhodopsin transfections as a positive control. Transfected cells were allowed to incubate 48–72 h post-transfection. Three methods were used for regeneration of visual pigment. In method 1 (26), reconstitution was carried out in the dark by incubation of cells suspended in Buffer Y (50 mM HEPES, pH 6.6, 140 mM NaCl, 3 mM MgCl₂, and 1 μg/ml protease inhibitors (aprotinin, pepstatin, leupeptin, and benzamidine)) with 5 μM 11-cis-retinal (in ethanol) for 3–18 h at 4 °C. In method 2, cell membranes were first isolated (27) and then resuspended in 67 mM NaPO₄, pH 7.0, containing protease inhibitors. Reconstitution was carried out with 2 μM 11-cis-retinal at 4 °C for 3 h. To remove retinal, membranes were washed with 4% BSA in 67 mM NaPO₄. In method 3, COS1 cells were grown in the dark in the presence of 5 μM 11-cis-retinal, beginning 24 h after DNA addition. The medium was changed and cells were then incubated twice prior to harvest of the cells. Pigment formation was determined by taking UV-visible spectra of protein purified using immunoaffinity chromatography (26) or by measuring light-dark difference visible spectra on solubilized membranes. Membranes were solubilized using 2% digitonin or 1% DM on ice for 20 min with occasional homogenization with a Teflon-glass homogenizer. Insoluble material was removed by centrifugation in a JA-20 (Beckman) rotor at 19,000 rpm for 45 min. The top 9/10 of the supernatant was removed and transferred to a new tube for spectral analysis.

Expression in Sf9 Cells
The insect cell line, Sf9, from Invitrogen was cultured in SF-900II SHG (Invitrogen) with 10 units/ml penicillin and 10 μg/ml streptomycin. The titer of the amplified Baculo/LimRh1–1D4 was about 10⁶ plaque-forming units as estimated by end-point assay. Sf9 cells at 5–6 x 10⁶ cells/ml were infected with the Baculo/LimRh1–1D4 by incubating cells, recombinant viruses and fresh medium, mixed in the ratio of 10:1:20 (v/v/v). Sf9 cells were harvested 1–4 days after infection. To reconstitute with chromophore, Sf9 cells were harvested 2 days after infection. The chromophore, 11-cis-retinal or all-trans-retinal, was added to a final concentration of 2 μM 1 day after infection and again at each of the following steps: cell harvesting, membrane solubilization, and immunoaffinity purification. Infected cells were harvested as previously described (28). Cell membranes were solubilized in buffer A (5 mM PIPES, pH 6.8, 10 mM EDTA containing protease inhibitors) with 1% DM on ice for 20 min. The following 14 protease inhibitors were used in each step of the purification: aprotinin, benzamidine, calpain, chymostatin, leupeptin, pepstatin A, phenanthroline, phenylalnine, phenylmethylsulfonyl fluoride, toy3 arginine methyl ester, tosyl lysine chloromethyl ketone, tosyl phenylethylchloromethyl ketone, antipain, and p-chloromercuriiphenyl sulfonic acid. All of these inhibitors were found in optimal concentration of 5 μg/ml, in the exception of phenylmethylsulfonyl fluoride (0.1 mM). The unsolubilized material was removed by centrifugation at 43,000 x g at 4 °C for 30 min. The expressed opsin was purified by immunoaffinity chromatography and used for spectral analysis.

Expression in Drosophila
P(Rh1 + LimRh1) and P(Rh1 + LimRh1–1D4) were injected into y w; sr ninaE77 mutant embryos, and multiple independent P-element-mediated germ line transformants were obtained, as previously described (24). Four homozygous lines containing P(Rh1 + Lim1) on the X (line 4), second (line 242), and third (lines 30 and 155) chromosomes were retained. Six lines of flies that contained P(Rh1 + Lim1–1D4) on the X (lines 63 and 89), second (lines 133 and 147), and third (lines 93 and 179) chromosomes were retained. The strains used in this study were constructed using visible markers and balancer chromosomes. The following mutant alleles were used: ninaE77, norpA74 and Q174.

Expression in Xenopus Oocytes
Xenopus oocytes were isolated as previously described (29). Stage V and VI oocytes were injected with 50–100 nl of RNA solution and cultured for 3–6 days in MBS solution (88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 1.5 mM NaHEPES, pH 7.6, 0.3 mM Ca(NO₃)₂, 0.41 mM CaCl₂, 0.41 mM MgSO₄, 5 mM sodium pyruvate, and 10 μg/ml gentamycin).

Native Pigment
Limulus lateral eyes were dissected from 150 adult animals over a period of 3 days. Dissections of the retina from the cornea were done in ambient room light, and animals were not dark-adapted beforehand. Retinas were placed in saline solution (275 mM NaCl, 3.7 mM KCl, 8.0 mM CaCl₂, 7.0 mM MgCl₂, and 23 mM MgSO₄), covered in foil, and allowed to dark-adapt for 3 h. Retinas were divided into batches of 40 eyes, spun briefly to remove saline, flushed with N₂, quick frozen on dry ice and stored at −70 °C. All procedures were carried out in the dark. Limulus eyes (40) were allowed to thaw on ice, suspended in 67 mM NaPO₄, pH 6.5, containing 5 μg/ml protease inhibitors (benzamidine, pepstatin, leupeptin), and homogenized in a glass tissue grinder. This homogenate was layered on a 45% sucrose cushion and spun in an SW-27 rotor at 25,000 rpm at 4 °C for 25 min. The membrane band was collected, and the pellet was rehomogenized. The membrane fractions were pooled and diluted 10-fold with phosphate buffer to lower the sucrose concentration, and membranes were collected by centrifugation in a T35 rotor at 30,000 rpm for 25 min. The pellet was solubilized with 0.3 mL of a solution containing 2% digitonin or 1% DM in phosphate buffer and incubated in ice for 1 h. The solubilized solution was centrifuged in a JA-20 rotor at 19,000 rpm for 30 min. The top portion of the supernatant was removed and used for spectral analysis. Bleached membranes were prepared by suspending in 10 ml of 0.1 mM NH₄OH in phosphate buffer. The membranes were put on ice and exposed to the light projector (Eastman Kodak Co. projector equipped with a 300-watt tungsten bulb and a high pass 515-nm cut-off filter) for 3 h with occasional mixing. Membranes were centrifuged in a 70T.10 rotor at 50,000 rpm for 15 min. To remove the retinal oxime, the membranes were washed twice in phosphate buffer containing 2% BSA and resuspended in phosphate buffer.

Regeneration of Native Opin
Bleached membranes (from 40 eyes) were reconstituted with retinal using two methods. In method 1, membranes were incubated with 5 μM 11-cis-retinal at 4 °C. The incubated membranes were then centrifuged, washed twice with 2% BSA in phosphate buffer, and each solubilized with 0.6 μl of 2% digitonin in phosphate buffer for 1 h. The insoluble material was removed by centrifugation, and the top portion of the supernatant was taken for spectral analysis. In method 2, a Limulus eye extract, containing soluble proteins including putative photoreceptorase activity, was prepared (30), Limulus retina (~48) were homogenized in 5 ml of PI buffer (0.1 x NaPO₄, pH 6.8). The homogenate was spun at 8,000 x g at 4 °C for 40 min. The supernatant was saved, and the pellet was re-extracted in another 5 ml of buffer PI and respun. The two supernatants were pooled (10 ml total) and used as

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Limulus Rhodopsin

The abbreviations used are: BSA, bovine serum albumin; ERG, electroretinogram; DM, dodecyl maltoside.
a soluble Limulus eye extract. For reconstitution, membranes were prepared from Limulus retina as usual. The resuspended membranes were divided into two. One aliquot was bleached with NH₂OH and subsequently washed with 2% BSA as described above. The other aliquot was not bleached. Membranes were resuspended in either 2.5 ml of buffer P1 or 2.5 ml of eye extract, and a retinal solution containing a 2:1 molar ratio of all-trans- to 11-cis-retinal was added to a final 5 μm concentration. Aliquots were mixed at 4 °C overnight and washed twice with 2% BSA in phosphate buffer, and finally solubilized in 0.7 ml of 2% digitonin in phosphate buffer for spectral analysis.

**Western Blots and Immunofluorescence**

Protein samples were digested with N-glycanase as described by Kaushal et al. (31). Solubilized protein samples were run on 10% SDS-PAGE gels and transferred to nitrocellulose membranes. LimRh1-1D4 was detected using 1D4 IgG and anti-mouse horseradish peroxidase secondary antibody. Horseradish peroxidase was visualized using DAB/ NiC₃O₄. Blots were scanned, and figures were prepared using Photoshop (Adobe). COS1 cells were grown on gelatin-coated cover slips and transfected as usual. After 48 h, cells were fixed with cold (−20 °C) methanol followed by acetone. Opsin was detected using 1D4 antibody and a fluorescein-conjugated secondary antibody. UV-visible absorption spectra of purified visual pigments and solubilized membranes were recorded in the indicated buffers at 20 °C with a Beckman DU 640 single beam spectrophotometer. Bleaching illumination was provided with light from a 300-watt projector (Kodak) at a distance of 50–60 cm. Unfiltered light from the projector was tilted white light and filtered by a high pass glass cut-off filter (>355 nm; Edmund Scientific, Inc.) was used as indicated. Total radiant energy of the light stimulus was measured using an optical power meter (3M Optical). COS1 cells were transfected with 11-cis-retinal and the protein was detected using 1D4 IgG and anti-mouse horseradish peroxidase secondary antibody. Immunofluorescence—Opsin expression plasmids were transfected into COS1 cells by the calcium phosphate precipitation method, and isolated proteins were separated on 12% acrylamide gels and exposed to x-ray film. The confocal image was collected using a Zeiss LSM-310 microscope (Thornwood, NY).

**Analysis of Lim Rh1-1D4 Expression in Drosophila**

**Microscopy**—The immunohistochemistry using the 1D4 antibody and confocal imaging were performed as previously described (24). The confocal image was collected using a Zeiss LSM-310 microscope (Thornwood, NY).

**Electrophysiology**—Electoretinograms (ERGs) were recorded from immobilized white-eyed flies using glass microelectrodes filled with normal saline (0.9% NaCl, w/v) as previously described (23, 24). Spectral sensitivity was measured using a modification of the voltage clamp method of Franceschini (32, 33), which we have described in detail elsewhere (23). Briefly, the amplitude of the ERG response to a flickering (10 Hz) monochromatic stimulus was maintained at a criterion level while the wavelength of stimulating light was varied during a scan. Throughout the scan, the criterion response was maintained by the following log normal function:

\[
A = A_0 \times \exp(-ax^2/a_2^2)
\]

where \( x = 10(\log(x/\Lambda_{max})) \), \( A = 1, a_0 = 380, a_1 = 6.09, \) and \( a_2 = 30/18 \). The curve-fitting routine was implemented in KaleidaGraph™ (version 3.08d, Synergy Software, Reading, PA) using the Levenberg-Marquardt (nonlinear least-squares) algorithm. The computer solved for the \( \Lambda_{max} \) and amplitude of the rhodopsin absorption spectrum and calculated the S.D. value for each variable and the correlation coefficient (Pearson’s r). The effect of β-band absorption (\( \Lambda_{max} = 340 \) nm) was ignored, because this is likely to be minimal in the measured region (450–690 nm).

**Analysis of Lim Rh1-1D4 Expression in Oocytes**

**Immunoprecipitation**—Oocytes were injected with ~50 ng of LimRh1-1D4 cRNA and cultured in labeling medium (MBS containing 10% heat-inactivated newborn calf serum and 10 μCi/ml [35S] TRANS label (ICN, Inc.) for 4 days. The medium was replaced each day with fresh labeling medium. Oocytes were washed in modified Barth’s saline and homogenized in 150 mM NaCl, 10 mM NaPO₄, pH 7.0, and 0.5% dodecyl maltoside (10 μ/oocyte). Insoluble material was removed by centrifugation at 14,000 rpm for 15 min at 4 °C. Opsin was immunoprecipitated using 1D4-Sepharose and eluted from the resin with competing peptide. **RESULTS**

**Native Pigment**—As a preliminary study for heterologous expression of a Limulus opsin cDNA, we characterized the photobleaching and regeneration properties of detergent extracts from lateral eye membranes (Fig. 1). In diatomin, there was a broad absorbance between 400 and 500 nm that arose from screening pigments, thus preventing direct observation of the visual pigment. However, exposure of the extract to light caused a loss of absorbance at ~540 nm and an increase at ~370 nm (Fig. 1B). The difference spectrum fit the profile expected for an 11-cis-retinol-based rhodopsin. The visual pigment was quite stable in detergent extracts when kept in the dark, with only a minor loss of absorbance after 1 h (Fig. 1B).

The same results were obtained with membranes that were solubilized in dodecyl maltoside (data not shown). Rhodopsin could be isolated from dark-adapted and light-adapted animals as long as the retina was incubated in the dark prior to extraction (Fig. 1C). This shows that the M form of the visual pigment efficiently thermally converts back to R in the dark.

The chromophore could be completely removed from the visual pigment by exposing to light in the presence of hydroxylamine, resulting in no light-sensitive spectra. We attempted to regenerate the visual pigment by two methods. In the first, membranes were incubated with 11-cis-retinal in the dark before solubilization with digitonin. There were no light-sensitive (Fig. 1C) detectable responses. In the second method, membranes were incubated with an extract of soluble proteins from Limulus retina and either 11-cis- or all-trans-retinal. Again, no detectable visual pigment was regenerated. The reason that chromophore did not bind apoprotein is not clear. One possibility is that the chromophore must bind to the opsin co-translationally, prior to conformational changes that form the final pigment. Alternatively, there might be accessory proteins not extracted or stable under our conditions (e.g., integral or peripheral membrane proteins) required for insertion of chromophore into the apoprotein.

**Expression in COS1 Cells**—COS1 cells were transfected with an epitope-tagged Limulus opsin expression plasmid. Western analysis (Fig. 2A) of the purified protein showed that the expressed Limulus opsin was found in a number of different sized bands: a major broad band at 39 kDa, aggregated species of the size of dimers and trimers, and a proteolytic fragment at 21 kDa. Treatment of Limulus opsin with N-glycanase caused a mobility increase of monomer and higher aggregates, indicative of the removal of N-linked carbohydrate. Transfected COS1 cells were incubated with 11-cis-retinal, and the protein was solubilized in 1% dodecyl maltoside and then purified using immunoaffinity chromatography. The recovered protein did not have any associated retinal as measured by absorbance spectroscopy (Fig. 2B) or by dark-light difference. In order to determine whether the purification protocol caused the protein to release retinal, 11-cis-retinal was added directly to the purified protein sample. No light-sensitive pigment was formed. Transfected COS1 cell membranes were solubilized (conditions
in which the native Limulus rhodopsin was stable (Fig. 1) and analyzed by dark-light difference spectroscopy. Again, no detectable visual pigment was formed (Fig. 2D), in contrast to the situation with bovine rhodopsin (Fig. 2C). In order to determine whether 11-cis-retinal was required co-translationally, COS1 cells were transfected and then grown in the dark with 11-cis-retinal for 60 h. After isolation of membranes and solubilization, no detectable visual pigment was found in dark-light difference spectra. These results indicate that Limulus opsin is not correctly processed in COS1 cells. As such, we expect that it would not be transported to the plasma membrane. Immunohistochemistry on transfected COS1 cells (Fig. 2E, right panel) confirmed that Limulus opsin was localized predominantly in the endoplasmic reticulum and Golgi, and very little was found in the plasma membrane. This finding is in contrast to the normal membrane localization of bovine opsin expressed in these cells (Fig. 2E, left panel). From these results, we conclude that mammalian COS1 cells lack the ability to properly process and target the Limulus opsin.

Expression in Sf9 Cells—Sf9 cells, derived from the invertebrate fall armyworm, have been used to express a number of proteins in functional form, including vertebrate rhodopsin. We prepared a baculovirus vector encoding the epitope-tagged Limulus opsin and infected Sf9 cells. After 2 days, a dose-dependent expression of opsin was detected by Western blot (Fig. 3A, lanes 1 and 2). The expressed Limulus opsin was found in a number of bands, whose pattern was very similar to that observed in transfected COS1 cells: monomer, aggregates, and a proteolytic fragment. In Sf9 cells, the monomer band was actually a doublet of glycosylated and unglycosylated forms of the protein. Sf9-expressed Limulus opsin was not stable in detergent extracts and underwent rapid proteolysis at 4 °C. Inclusion of an extensive protease inhibitor mixture (see “Experimental Procedures”) slowed degradation significantly (data not shown). Next, Sf9 cells were infected with viral supernatants (four individual cultures inoculated under identical conditions) and harvested at 1, 2, 3, and 4 days postinfection. A Western blot of the solubilized samples revealed that under these conditions only the cells harvested on day 2 gave a substantial fraction of Limulus opsin monomer (Fig. 3, A (lanes 3–6) and B). Samples from the other harvest times were completely degraded. Sf9 cells were inoculated with viral supernatant encoding either bovine or Limulus opsin cDNA, either 11-cis- or all-trans-retinal was added to the culture medium, and the cultures were incubated in the dark for 2 days. No effect on the profile of expressed protein from infected Sf9 cells in Western blots was detected (Fig. 3, lanes 7–10). Cells grown in the presence of 11-cis-retinal were harvested, and membranes were prepared. Additional 11-cis-retinal was added, the membranes were solubilized with 1% dodecyl maltoside, and Limulus opsin was purified by immunoaffinity chromatography. Neither the absorbance spectra (Fig. 3C) nor dark-light difference spectra (Fig. 3D) had any detectable visual pigment. These results suggest, as in COS1 cells, that one or more components required for processing of Limulus opsin are missing in invertebrate Sf9 cells and that a component specifically found in invertebrate photoreceptors is required for correct formation of Limulus visual pigment.

Expression in the R1–R6 Photoreceptor Cells of Drosophila—To demonstrate that the Limulus lateral eye opsin cDNA encodes a functional visual pigment, we expressed the native and epitope-tagged forms of the Limulus opsin gene in the R1–R6 photoreceptor cells of Drosophila under the control of the ninaE opsin gene promoter. The R1–R6 photoreceptor cells are a suitable environment for the expression of novel opsins and have been used extensively for studying the spectral and physiological properties of novel or modified opsin genes in vivo, including those from other invertebrates such as the honeybee (23, 29, 36–38).

Transgenic flies expressing the epitope tagged Limulus opsin construct [P[Rh1+LimMT]] show proper localization of the tagged protein to the rhabdomeres of the R1–R6 cells of the compound eye (Fig. 4D). This demonstrates that the transgene is expressed in the proper cell types and that the protein is stable and appropriately targeted within the photoreceptor cells. To assess the functional activity of the Limulus opsin, we examined the ERGs of transgenic flies. As mentioned previously, ninaE flies were used as a host strain because they lack the visual pigment normally expressed in the R1–R6 photoreceptor cells. To avoid any interfering signal from the R7 and R8 cells, which are unaffected by the ninaE mutation and produce a small depolarization in the ERG of ninaE flies, we made use
Expression of an epitope-tagged Limulus opsin cDNA in COS1 cells. A. COS cells were transfected with bovine (lanes 2 and 3) or Limulus (lanes 4 and 5) opsin cDNAs, and protein was purified by immunoaffinity chromatography and analyzed by Western blots using 1D4 antibody. Limulus opsin (lane 4) was expressed to levels similar to those obtained with bovine opsin (lane 2) but exhibited significantly more protein in aggregated form. In addition, there was a proteolytic product at ~21 kDa, comprising the C-terminal fragment. Treatment of the Limulus (lane 5) or bovine (lane 3) samples with N-glycanase caused the major bands to increase mobility, reflecting the removal of N-linked carbohydrate moieties. Bovine rhodopsin from retina (lane 1) is shown for reference. Major bands observed were monomer (m), dimer (d), trimer (t), and proteolytic degradation product (p). B. Transfected COS cells were incubated with 11-cis-retinal, and protein was solubilized in 1% dodecyl maltoside and purified as before. The absorbance spectrum of Limulus opsin exhibited no chromophore bound in contrast to bovine opsin (inset), which had the typical λmax of 500 nm. C and D, membranes from COS cells transfected with bovine (C) or Limulus (D) opsin cDNA were isolated, incubated with 11-cis-retinal, and solubilized with 2% digitonin. Samples were illuminated with λ = 515 nm light, and dark-light difference spectra were calculated. No pigment formation was seen in the Limulus opsin sample. E, immunohistochemistry. Transfected COS cells were grown for 48 h, fixed, and stained with 1D4 antibody and fluorescent secondary antibody. In cells transfected with bovine opsin (left), cross-reactivity with antibody is observed predominantly in plasma membrane with some staining of organelles. In cells transfected with Limulus opsin (right), only intracellular staining is found, indicating that Limulus opsin is not correctly transported in COS cells.

of a special host strain, norpA. In this strain, the phospholipase C required in all photoreceptor cells of the compound eye is removed genetically by the norpA mutation (39). The norpA cDNA is expressed under the control of the ninaE (Rh1) promoter so that only the R1–R6 photoreceptor cells are competent for transducing the light response (40). Fig. 4A shows that norpA mutant flies that express norpA in the R1–R6 cells and also carry a single wild type copy of ninaE (denoted ninaE+/+) have a robust response to light at both 350 and 520 nm (Fig. 4B, top traces). Removing the wild type copy of ninaE to generate the host strain (that lacks the opsin in the R1–R6 cells and lacks the PLC in the R7 and R8 cells) yields no response to either 350- or 520-nm pulses of light (Fig. 4A, second row of traces). Introducing the epitope tagged or native forms of the Limulus lateral eye opsin cDNA into this background (Fig. 4A, third and fourth rows of traces, respectively) restored the electrophysiological response to light. This shows that both the native and epitope-tagged forms of the pigment are biologically active and functional when expressed in the Drosophila R1–6 photoreceptor cells, providing definitive proof that the Limulus cDNA encodes a functional visual pigment. The fact that these flies must be stimulated with very intense light in order to evoke a response (100-fold higher than those expressing Rh1) suggests that although the Limulus opsin is biologically active when expressed in fly photoreceptor cells, it may not be expressed as highly or couple as efficiently to the phototransduction cascade as the native Drosophila opsin. Interestingly, although there is a detectable response in the UV (350 nm) for both types of flies, the response at 520 nm is much greater. This indicates that the Limulus opsin does not effectively couple to the sensitizing pigment in the R1–R6 cells, which is responsible for their sensitivity to UV light (41, 42).

To test whether the Limulus opsin variants utilize the same signal transduction pathway as the native Drosophila rhodopsins, we examined two genetic mutants that lack the phospholipase C (norpA, as described above) or lack the G-protein Goα that mediate the light response in Drosophila photoreceptors and are required from normal phototransduction (39, 43). As shown in Fig. 4B, flies expressing the native Drosophila rhodopsin require Goα for phototransduction, because mutating this gene abolishes the light response (Fig. 4B, top trace). Similarly, expressing either of the Limulus opsin constructs in Goα mutant flies fails to produce a light response in the ERG (Fig. 4B, second and third traces from the top). Drosophila norpA mu-

Limulus Rhodopsin

E
Expression of an epitope-tagged Limulus opsin cDNA in Sf9 cells. A, viral inoculum. Sf9 cells were infected with two different volumes of viral supernatant containing baculovirus encoding the epitope-tagged Limulus opsin cDNA (lanes 1 and 2). Cells were harvested 2 days later, solubilized in 1% dodecyl maltoside, and analyzed by Western blot. Three major bands were observed: monomer (m), dimer (d), and proteolytic degradation product (p). Time of infection was as follows. Sf9 cells were infected with viral supernatant and harvested at 1, 2, 3, and 4 days postinfection (lanes 3–6). Only day 2 cells contained a significant proportion of monomer. Effect of retinal was as follows. Sf9 cells were infected with viral supernatants containing baculovirus encoding bovine opsin (lanes 7 and 8) or Limulus opsin (lanes 9 and 10) and grown in the dark in medium containing 2 μM all-trans-retinal (lanes 7 and 9) or 11-cis-retinal (lanes 8 and 10). There was no effect of retinal on expression levels or bands recovered. Bovine rhodopsin from retina (lane 11) is shown for reference. B, quantification of the distribution of various Limulus opsin forms from Sf9 cells at different times postinfection was performed using densitometry on lanes 3–6 in A. The m band contains two very closely spaced bands, one that is sensitive to N-glycanase (+CHO) and the other not (−CHO). The amount of protein in each portion of the m band was quantified separately. C and D, absorbance properties of rhodopsin purified from Sf9 cells infected with Limulus encoding baculovirus. The cells were grown in the presence of 11-cis-retinal, which was present in all solutions except the final one. The absorbance spectrum (C) was dominated by light scattering generated by aggregated protein, and no light-sensitive pigment was detectable in a difference spectrum (D).

Expression in Xenopus Oocytes—Transgenic experiments are not feasible in Limulus, so we utilized the Xenopus oocyte expression system, which has been used to express bovine rhodopsin as well as light-sensitive conductance from squid retinal mRNA. When Xenopus oocytes were injected with Limulus mRNA, light-sensitive chloride currents were generated after incubation with 11-cis-retinal (45). In these cells, the rhodopsin is able to couple to an endogenous, G-protein-coupled signal transduction pathway that regulates the membrane potential of the oocytes through a calcium-dependent chloride channel (46). In order to determine the kinetics of retinal binding to the apoprotein, we investigated the time course of regeneration of the light response. Oocytes were injected with mRNA from Limulus lateral eyes and incubated 4–7 days in culture medium without retinal. Oocytes were voltage-clamped and stimulated with light flashes. No responses were seen in the absence of 11-cis-retinal or in the presence of all-trans-retinal. However, the light-sensitive currents appeared 5 min after the addition of 11-cis-retinal (Fig. 5). These results indicate that 11-cis-retinal can bind to Limulus opsin and that a portion of it is properly folded. Although longer incubation times gave slightly larger currents, most visual pigment formation is rapid. Retinal binding appears to occur posttranslationally.

Next, we examined whether oocytes alone contained the components necessary for correct biosynthesis of Limulus opsin. Oocytes from a single donor were injected with either
cRNA encoding the epitope-tagged Limulus opsin or mRNA from Limulus lateral eye retina. The oocytes were tested for light-sensitive conductance changes following incubation with 11-cis-retinal (Fig. 6, A and B). None of the cRNA oocytes exhibited light-dependent currents, although they were synthesizing levels of polypeptide (Fig. 6C) comparable with that obtained with oocytes injected with cRNA encoding bovine opsin (29, 46). On the other hand, 11 of 13 oocytes injected with retinal mRNA produced light-sensitive currents. These results support the idea that Limulus retinal mRNA encodes one or
more proteins that are necessary for the proper folding of opsin and essential for retinal binding.

DISCUSSION

Development of an expression system for Limulus rhodopsin is motivated by the observation of a circadian rhythm in noise in lateral-eye photoreceptors (47). At night, an oscillator in the brain transmits efferent impulses to the photoreceptors reducing the rate of their generation of discrete “dark” electrical events, so-called quantum bumps. Both theoretical and experimental studies indicate that the circadian input reduces photoreceptor noise by reducing the small population of relatively unstable rhodopsin molecules in which Schiff base linkages between the chromophore and opsin are unprotonated (4, 5, 48). Deprotonation would destabilize rhodopsin by lowering the energy barrier for isomerization and thus allow activation of transduction mechanisms in the absence of light. We investigated expression systems for Limulus rhodopsin in order to test this molecular mechanism for photoreceptor noise.

The results presented here show that Limulus opsin can be expressed in COS1, Sf9 cells, Drosophila R1–6 photoreceptors, and Xenopus oocytes. In the heterologous cell culture systems, the polypeptide is made efficiently and is glycosylated. However, a functional visual pigment was formed only in Drosophila photoreceptors and Xenopus oocytes that co-express additional retinal proteins. We performed experiments on purified preparations and directly on solubilized membranes with equivalent results. Experiments using native Limulus membranes showed that rhodopsin was stable in either digitonin or DM in the dark, indicating that the failure to detect bound retinal was not a result of instability under our conditions. In the dark, invertebrate visual pigments appear to have similar stability in DM as vertebrate pigments, as shown in our previous study that characterized purified Drosophila Rh1 (49). However, once Limulus rhodopsin was bleached, it was not possible to regenerate the pigment.

In both COS1 and Sf9 cells, there is substantial degradation of the protein, suggesting that at least a portion is misfolded. Immunofluorescence of the transfected COS cells showed the mislocalization of the protein during biosynthesis. In our expression constructs, the C terminus of Limulus opsin was altered to that of bovine opsin. This sequence has been shown to be important in the proper localization of vertebrate rhodopsin...
in rods (50); however, an opsin mutant lacking the carboxyl terminus does bind retinal and exhibit light-dependent activation of the phototransduction cascade (51). Thus, it does not appear that this change in the Limulus opsin sequence would be expected to have a deleterious effect on pigment generation. We have tested this directly in transgenic Drosophila and found that constructs with either carboxyl terminus were equivalent in pigment formation and in light-induced ERG response (Fig. 4A). In our previous study of Drosophila Rh1 (49), we introduced a 1D4-epitope tagged Rh1 gene into transgenic Drosophila and found that this protein was comparable with the wild type protein in function and fully capable of generating a PDA. The use of the 1D4 epitope to purify other G-protein-coupled receptors in a functional state has also been described (e.g. see Refs. 52–55). Thus, the modification of the carboxyl terminus of Limulus rhodopsin does not account for the inability of Limulus opsin to bind retinal in COS1 or Sf9 cells.

Bovine rhodopsin mutants that have folding defects exhibit similar behavior in COS1 cells. Since both vertebrate COS1 and invertebrate Sf9 cells give similar results, there does not seem to be a general invertebrate-specific protein required for Limulus opsin biosynthesis. Rather, it appears most likely that there are one or more proteins specifically expressed in invertebrate photoreceptors that are required for forming visual pigment. Although no protein cofactor has been reported for rhodopsin, the C-terminal fragment of RAN BP2 (RBD4) was shown to act as a chaperone for human red opsin in COS cells (60) and invertebrate Sf9 cells. The deficiency may be similar to one observed here for Limulus opsin and could reflect a general property of these opsins, namely a requirement for specific chaperones and/or retinal shuttle proteins to form a functional pigment. Further work on Limulus folding and regeneration may help address these broader questions.

Acknowledgments—We acknowledge the assistance of W. Wang with the Sf9 expression experiments; H. Tran and L. Ferrara for performing the Xenopus oocyte experiments; and D. Starace, A. Surya, and M. Max for advice and helpful discussions.

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