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An improved rhodopsin/EGFP fusion protein for use in the generation of transgenic *Xenopus laevis*

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Abstract Previous studies by Papermaster and coworkers introduced the use of rhodopsin–green fluorescent protein (rho–GFP) fusion proteins in the construction of transgenic *Xenopus laevis* with retinal rod photoreceptor cell-specific transgene expression [Moritz et al., J. Biol. Chem. 276 (2001) 28242–28251]. These pioneering studies have helped to develop the *Xenopus* system not only for use in the investigation of rhodopsin biosynthesis and targeting, but for studies of the phototransduction cascade as well. However, the rho–GFP fusion protein used in the earlier work had only 50% of the specific activity of wild-type rhodopsin for activation of transducin and only 10% of the activity of wild-type in rhodopsin kinase assays. While not a problem for the biosynthesis studies, this does present a problem for investigation of the phototransduction cascade. We report here an improved rhodopsin/EGFP fusion protein in which placement of the EGFP domain at the C-terminus of rhodopsin results in wild-type activity for activation of transducin, wild-type ability to serve as a substrate for rhodopsin kinase, and wild-type localization of the protein to the rod photoreceptor cell outer segment in transgenic *X. laevis*.

Key words: G protein-coupled receptor; Rod photoreceptor cell; Phototransduction; Protein folding; Protein trafficking

1. Introduction

The South African clawed frog, *Xenopus laevis*, has been used extensively in developmental biology as a model organism for more than half a century [2], but techniques for the generation of transgenic *Xenopus* have been introduced by Kroll and Amaya only relatively recently [3]. Subsequently, Knox and colleagues characterized the *Xenopus* rhodopsin gene [4] and used the promoter to generate transgenic *X. laevis* in which transgene expression was specific for retinal rod photoreceptor cells [5]. Papermaster and colleagues then exploited this approach beautifully to elucidate mechanisms of rhodopsin transport and trafficking within the rod photoreceptor [1,6–8]. Because of the efforts of Knox and Papermaster and others, *X. laevis* is rapidly becoming a model system of choice for understanding the molecular biology of vertebrate phototransduction.

To facilitate their studies of rhodopsin biosynthesis and transport in the transgenic frog model, Moritz et al. [1] generated a rhodopsin–green fluorescent protein (rho–GFP) fusion protein in which GFP was inserted in the C-terminus of bovine rhodopsin following Thr335 (the insertion also resulted in a duplication of amino acids Ser334 and Thr335 following the GFP sequence). The rho–GFP fusion protein was of great usefulness in identifying transgenic animals and in monitoring biosynthesis and trafficking patterns of rhodopsin. However, the specific activity of this protein under in vitro conditions was only half that of wild-type rhodopsin in transducin assays and only one-tenth that of wild-type in rhodopsin kinase (RK) assays. While this is not a problem for investigations of protein biosynthesis or gene regulation, it does present some concern for studies of the phototransduction cascade. Here we report a rhodopsin/enhanced GFP (rho/EGFP) fusion protein with significantly improved folding characteristics and in vitro activity for use in generating transgenic *X. laevis*.

2. Materials and methods

2.1. Materials

The design and chemical synthesis of the gene for rhodopsin used in these studies has been published [9]. pEGFP-C2 and pEGFP-N1, sources of the gene for EGFP, were from Clontech. *n*-Dodecyl-β-D-maltoside (DDM) was from Calbiochem. The anti-rhodopsin monoclonal antibody 1D4, which specifically recognizes the C-terminal eight amino acids of rhodopsin (Glu-Thr-Ser-Gln-Val-Ala-Pro-Ala) [10,11], was purified from hybridoma growth medium and coupled to Sepharose 4B [12] for use in immunoaffinity purification of the fusion proteins, as previously described for rhodopsin [13]. Peptide I, with sequence Asp-Glu-Ala-Ser-Thr-Thr-Val-Ser-Lys-Thr-Glu-Thr-Ser-Gln-Val-Ala-Pro-Ala corresponding to the C-terminal 18 amino acids of rhodopsin, was from American Peptide. 11-*cis*-Retinal was synthesized according to published procedures [14].

2.2. Genes for fusion proteins rho/EGFP and rho/EGFPi

We prepared genes for two fusion proteins in this work using standard methods. Both genes were cloned between the *Eco*RI and *Not*I cloning sites of the mammalian cell expression vector pMT3 [15]. Rho/EGFP (Fig. 1A) is composed of N-terminal rhodopsin followed

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Abbreviations: EGFP, enhanced green fluorescent protein; GFP, green fluorescent protein; DDM, *n*-dodecyl-β-D-maltoside; rho/EGFP, a rhodopsin/EGFP fusion protein consisting of amino-terminal bovine rhodopsin, followed by EGFP, followed by the 1D4-epitope at the carboxy-terminus; rho/EGFP*i*, a rhodopsin/EGFP fusion protein with EGFP inserted between Ala333 and Ser334 in bovine rhodopsin; rho–GFP, rhodopsin–GFP fusion protein described by Moritz et al. [1]; PBS, phosphate-buffered saline; RK, rhodopsin kinase

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by the entire coding sequence of EGFP followed by the C-terminal eight codons of rhodopsin (epitope for the 1D4 antibody). Thus, there are two copies of the C-terminal eight amino acids of rhodopsin in this construct. This was necessary to ensure proper targeting of the protein in rod photoreceptor cells [7] and to ensure that the fusion protein would be recognized by the 1D4 antibody which requires a C-terminal epitope [16].

Rho/EGFP*i* (Fig. 1B) is composed of full-length rhodopsin with the coding sequence for EGFP inserted between amino acids Ala333 and Ser334 in the C-terminal tail. Rho/EGFP*i* was designed to mimic the Moritz et al. construct [1], and while not identical it does display a similar phenotype with respect to in vitro assays for transducin activation and phosphorylation by RK, as is shown below.

2.3. Expression in COS cells and purification of the proteins

Rho/EGFP and rho/EGFP*i* were expressed transiently in COS cells transfected using DEAE-dextran as described previously for rhodopsin [13]. Fluorescence microscopy employed an Olympus IX50/IX70 microscope equipped with a U-MWB (exciter filter BP450–480 nm) cube to follow expression of the fusion proteins in intact cells (photographs presented in Fig. 1C,D were taken 40 h post transfection).

Cells were harvested 72 h after initial exposure to DNA and treated with 20 μM 11-*cis*-retinal in 10 mM sodium phosphate buffer, pH 7.0, containing 150 mM NaCl (PBS) for 1 h to reconstitute the pigments (all procedures beginning with and following initial treatment of the sample with retinal were performed in the dark under illumination from a 15 W incandescent bulb filtered through a Kodak Safelight #2 filter). Cell membranes were solubilized in PBS containing 1% (w/v) DDM and 0.1 mg/ml phenylmethylsulfonyl fluoride, and the post-nuclear supernatant fraction applied to a 1D4-Sepharose 4B matrix for purification of the fusion proteins by immunoaffinity chromatography, as has been described previously for rhodopsin [13]. Bound protein was eluted with 50 μM peptide I in PBS containing 0.1% (w/v) DDM.

2.4. Absorption spectroscopy

UV/visible absorption spectra were obtained on a Hitachi model U-3210 spectrophotometer modified by the manufacturer for dark room use. All spectra were recorded on samples of 1.0 cm path length and analyzed using Kaleidagraph (Version 3.08d). Fusion protein spectra were analyzed by simulation using a linear combination of constituent spectra recorded from purified samples of wild-type rhodopsin and EGFP after normalization on the basis of extinction coefficient (ε at 500 nm = 42 700 cm^{-1} M^{-1} for rhodopsin [17]; ε at 488 nm = 55 000 cm^{-1} M^{-1} for EGFP [Living Colors user manual, Clontech]). The concentration of the protein for use in activity assays was then determined from the rhodopsin component. An implicit assumption in this analysis is that the spectral properties (absorption) of rhodopsin and EGFP are unaffected by being brought together in the fusion protein. This assumption was judged to be reasonable on the basis of the close fit of simulated to experimental spectra.

2.5. Miscellaneous procedures for in vitro characterization of the fusion proteins

Western blot analysis [14] was performed using 2.8 pmol protein per lane with 1D4 as the primary antibody and horseradish peroxidase-conjugated anti-mouse IgG (Santa Cruz Biotechnology) as the secondary. Assays for the activation of transducin were performed with 5 nM purified pigment by following the binding of [^{35}S]guanosine 5′-*O*-(3-thiotriphosphate) as previously described [18]. Assays for phosphorylation by RK were performed as described [19] using RK from a post-nuclear supernatant fraction of transiently transfected COS cells [20]. Reactions were carried out in mixtures containing 125 nM rhodopsin or fusion protein, 6 μl RK extract, 75 mM [1,3-bis[tris(hydroxymethyl)methylamino]propane] buffer, pH 6.7, 10 mM $Mg(OAc)_2$, 5 mM dithiothreitol, 0.01% DDM, and 100 μM [γ-^{32}P]ATP (2000 cpm/pmol). Reactions were performed either in the dark or under light from a 300 W tungsten bulb filtered through a 490 nm cut-on filter. Samples were incubated for 30 min at 30°C and then assayed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and autoradiography.

2.6. Transgenic X. laevis

Transgenic experiments were performed as previously described [21]. Briefly, transgenic *X. laevis* were generated by the method of Kroll and Amaya using restriction enzyme-mediated integration of DNA into sperm chromosomes prior to transplantation of the sperm nuclei into unfertilized oocytes [3]. Cell-specific expression in the primary rod photoreceptor cells of the *Xenopus* retina was achieved with a vector (pXOP(−508/+41)Rho/EGFP) that is similar to those previously described [1,5,6] in which a fragment (nucleotides −508 to +41) from the *X. laevis* opsin promoter, XOP(−508/+41) [5,22], is used to drive expression of rho/EGFP. The XOP promoter was isolated by using polymerase chain reaction amplification of *X. laevis* genomic DNA with the primers ATAAACTGCAGCCCCTAGGCCA and CCAAAGGATCCCTAGAAGCCTG for the sense and antisense strands, respectively, followed by digestion with *Pst*I and *Bam*HI to obtain the −508/+41 fragment [4].

2.7. Histochemistry and confocal microscopy

Histochemistry was performed essentially as previously described [23]. Briefly, transgenic tadpoles (1 month post injection, ∼stage 51) were anesthetized in 0.25% (w/v) tricane solution, fixed with 4% (w/v) paraformaldehyde in 0.1 M sodium phosphate buffer (PB), pH 7.2, at 4°C overnight, incubated first with 5% and then 15% (w/v) sucrose in PB at room temperature, and finally embedded in TBS (Triangle Biomedical Sciences) and frozen on dry ice. Cryosections (16 μm thickness) were prepared with a Jung CM3000 cryostat (Leica) and then melt-mounted onto glass slides. Samples were allowed to thaw in 0.1 M sodium phosphate buffer, pH 7.2, containing 150 mM NaCl (PBS*) at 37°C. The tissue was then incubated in 5% (v/v) goat serum (Sigma) in PBS* containing 0.3% (v/v) Triton X-100 (PBS*-Tx) for 0.5 h. 1D4 antibody (1 μg/ml diluted in PBS*-Tx) was added and incubation continued for 3–4 h at room temperature, the sample washed four times with PBS*-Tx, and incubated with a 1:100 dilution of tetramethylrhodamine isothiocyanate-conjugated goat anti-mouse IgG (Sigma) for 1 h. The samples were covered with 50% (v/v) glycerol in PB and coverslips and then used to obtain images from a Leica TCS SP2 confocal scanning microscope (Leica Microsystems). Fluorescence images were obtained using excitation wavelengths of 488 nm for EGFP and 543 nm for rhodamine. The Nomarski DIC images were obtained from a Zeiss Axioplan-2 optical (Carl Zeiss) microscope equipped with Nomarski optic and GFP filter (FITC LP #41012, Chroma Technology).

3. Results and discussion

We prepared two different rho/EGFP fusion proteins (Fig. 1): one in which EGFP was inserted between amino acid residues Ala333 and Ser334 in the C-terminal tail of rhodopsin (rho/EGFP*i*), and a second in which the coding sequence for EGFP was placed after the last amino acid of full-length rhodopsin and was itself followed by a repeat of the C-terminal eight amino acids of rhodopsin (rho/EGFP). Rho/EGFP*i* was designed to mimic the previously reported rho–GFP fusion protein used to generate transgenic *X. laevis* [1]. Rho/EGFP was designed with hope of improving the functional characteristics of the fusion protein by moving the EGFP domain further from potentially important amino acid residues near the cytoplasmic surface of rhodopsin. As is shown in Fig. 1C,D, both proteins were expressed on the membrane surface of COS cells as judged by the characteristic pattern of diffuse green fluorescence covering the entire cell outline.

UV/visible absorption spectra for reconstituted and purified rho/EGFP and rho/EGFP*i* are shown in Fig. 2A,B. Both proteins display an absorption maximum at about 488 nm with long-wavelength shoulder above 530 nm, indicating the presence of chromophores from rhodopsin and EGFP. Both spectra are reasonably well simulated by a linear combination of spectra for the isolated wild-type rhodopsin and EGFP. Rho/EGFP is fit well by a combination of 1 rhodopsin plus 0.9 EGFP, indicating that both domains are folded properly in a majority of the molecules. In contrast, the spectrum of rho/EGFP*i* is fit with a combination of 1 rhodopsin and 0.5

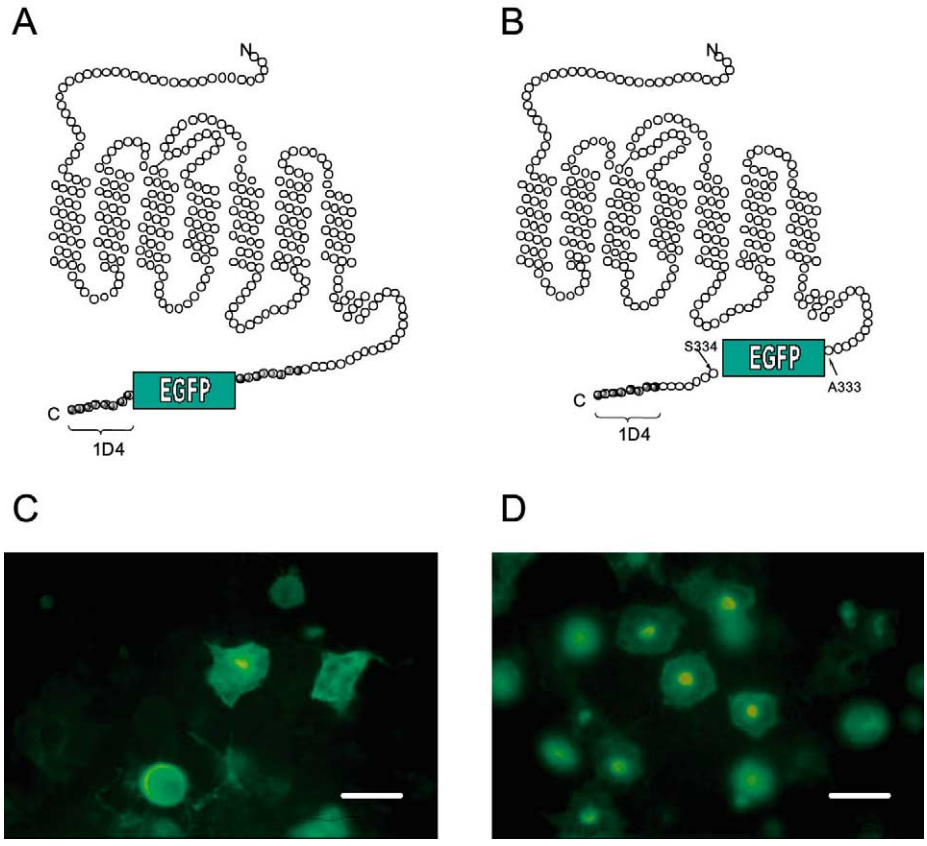


Fig. 1. Schematic representation of rhodopsin/EGFP fusion proteins rho/EGFP (A) and rho/EGFP*i* (B), and fluorescence images of COS cells expressing rho/EGFP (C) or rho/EGFP*i* (D). Shaded circles in A and B indicate location of the 1D4 epitope. Although there are two 1D4 epitopes in rho/EGFP (A), only the one at the C-terminus of the protein is expected to be recognized by the 1D4 antibody (see Section 2). Scale bar in C and D = 40 μm.

EGFP. This ratio suggests that the EGFP domain is unable to adopt a native conformation in at least 50% of the molecules. In addition, rho/EGFP expresses to higher levels in COS cells (80% that of wild-type rhodopsin) than does rho/EGFP*i* (38% that of wild-type rhodopsin), as judged by the yield of rhodopsin pigment (not shown).

Western blot analysis of rho/EGFP and rho/EGFP*i* is displayed in Fig. 3A. The fusion proteins separate into two bands, as has been described before for rho–GFP [1], with relative intensity a variable of different preparations. The electrophoretic mobilities are less than that of wild-type rhodopsin, as is expected from their larger molecular weight. We do

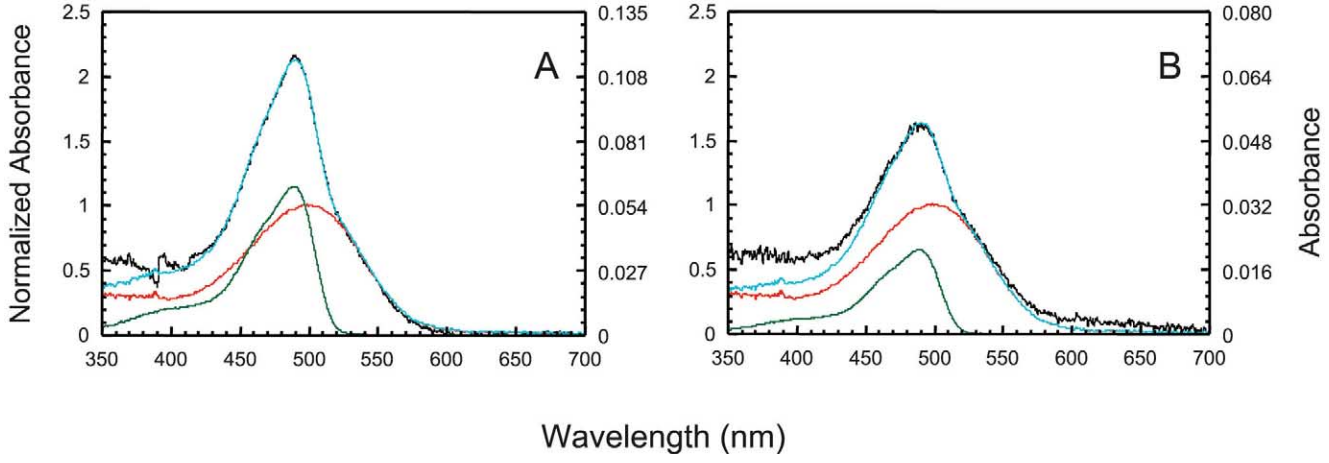
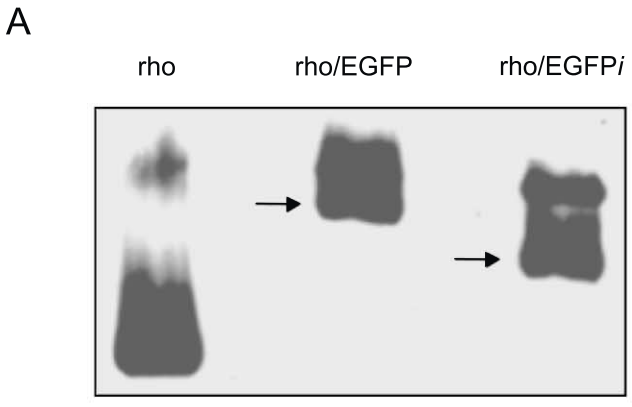
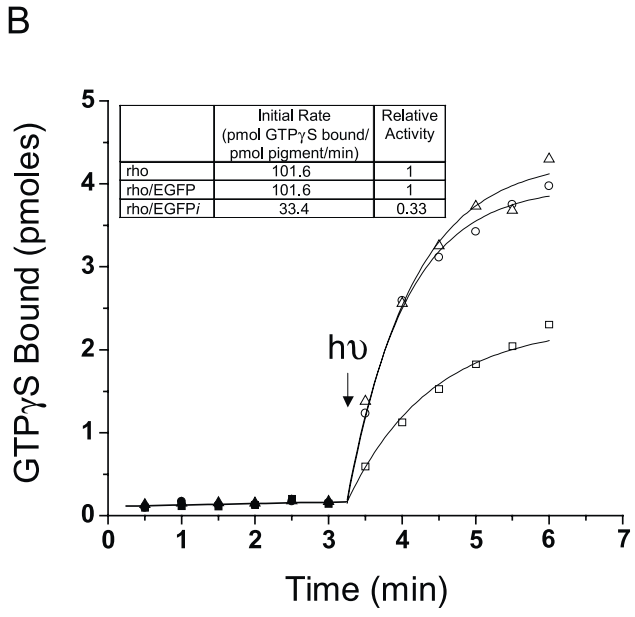


Fig. 2. Absorption spectra of purified rho/EGFP (A) and rho/EGFP*i* (B). In each case: black, experimental spectrum for the purified fusion protein; cyan, simulated spectrum generated by a linear combination of spectra for wild-type rhodopsin (red) and EGFP (green). A: Simulated spectrum generated from 1 rhodopsin and 0.9 EGFP. B: Simulated spectrum generated from 1 rhodopsin and 0.5 EGFP. Simulated and experimental spectra deviate somewhat at shorter wavelengths (less than 430 nm) due to increasing contribution of light scattering in the fusion protein samples that is not present in the wild-type rhodopsin and EGFP template spectra. This is more of a problem for the rho/EGFP*i* sample because of lower absorbance resulting from decreased expression level and ability to fold properly.





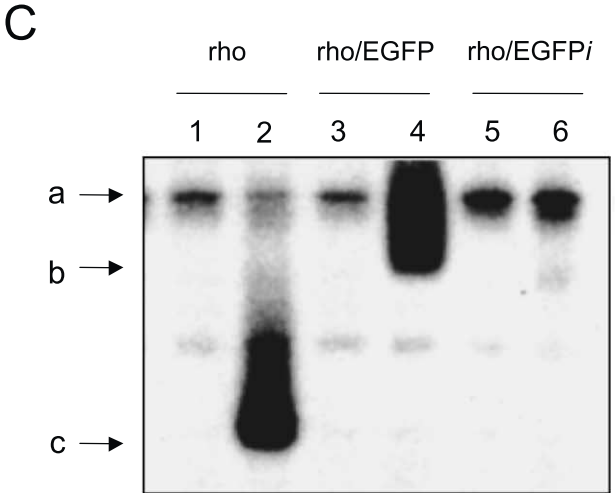


Fig. 3. In vitro characterization of the fusion proteins. A: Western blot analysis of the proteins purified from transfected COS cells. The stacking gel was removed, and only the running gel was transferred to nitrocellulose. Each lane contains 2.8 pmol pigment. Arrows indicate location of more rapidly migrating band. B: Transducin activity assays. Conditions were as described in Section 2. Circles, wild-type rhodopsin; triangles, rho/EGFP; squares, rho/EGFP*i*; solid symbols, reaction in the dark; open symbols, reaction after exposure to light (hυ). C: RK assays. Conditions were as described in Section 2. Substrates for RK were wild-type rhodopsin (rho), rho/EGFP, or rho/EGFP*i*, as indicated in the figure. Lanes 1, 3, and 5, reaction in the dark; lanes 2, 4, 6, reaction after exposure to light for 30 min. Arrows: a, autophosphorylated RK; b, phosphorylated rho/EGFP; c, phosphorylated wild-type rhodopsin. ←

not have a good explanation at this time for why the mobilities of fast bands of the two fusion proteins appear to be so different. The fact that the overall intensities of rhodopsin, rho/EGFP, and rho/EGFP*i* are similar suggests that the preparations do not contain substantial amounts of unfolded opsin (since the amount of protein in each lane was based on the amount of 11-*cis*-retinal chromophore).

Rho/EGFP and rho/EGFP*i* both activate transducin in a light-dependent manner, as is shown in Fig. 3B. However, rho/EGFP*i* has a much reduced specific activity in comparison to both wild-type rhodopsin and rho/EGFP, as is also the case for rho–GFP [1]. The specific activities of wild-type rhodopsin

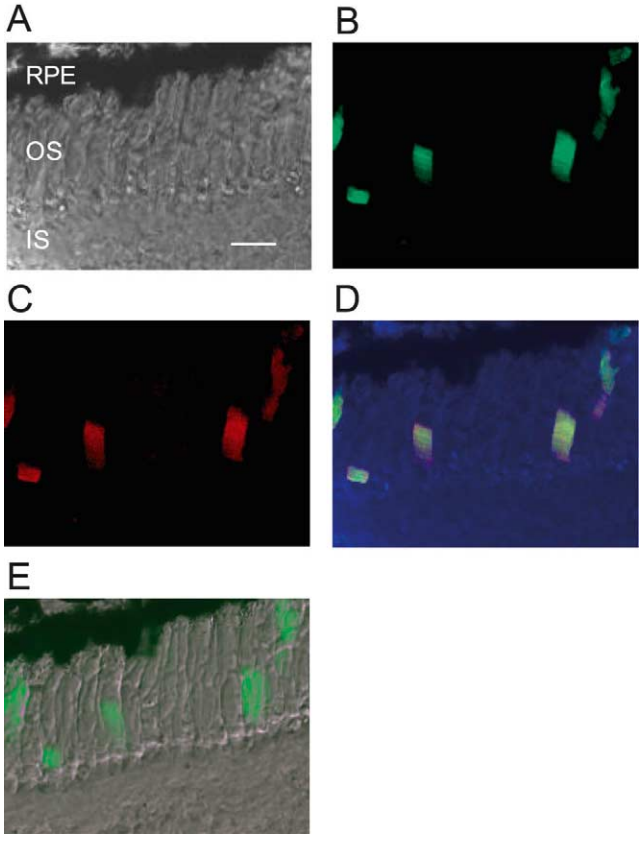


Fig. 4. Confocal and Nomarski images of a retina from a 1-month-old transgenic tadpole expressing rho/EGFP. Confocal images were obtained with transmitted light (A), or fluorescence following excitation at 488 nm (B, intrinsic fluorescence from the EGFP-tagged rhodopsin) or 543 nm (C, immunocytochemical fluorescence with 1D4 antibody). D: Superposition with blue background of images in A–C. E: Superposition of transmitted and fluorescence (GFP filter) light Nomarski DIC images taken from the same tissue as in A–D. Retina layers as indicated in A are: retinal pigmented epithelium (RPE), and photoreceptor cell outer (OS) and inner segments (IS). Scale bar, 15 μm.

and rho/EGFP (both 101.6 pmol/min/pmol) are three times that of rho/EGFP*i* (33.4 pmol/min/pmol). An even greater difference is observed in RK assays. As is shown in Fig. 3C, RK phosphorylates wild-type rhodopsin and rho/EGFP in light-dependent reactions with roughly the same amount of phosphate incorporated in the two proteins after a 30-min incubation period. In stark contrast, rho/EGFP*i* shows little if any incorporation of phosphate under the same conditions, as has been noted earlier by Moritz et al. for rho–GFP [1].

It is clear that the design of rho/EGFP represents an improvement over rho/EGFP*i* and rho–GFP [1] in terms of in vitro activity assays, but what about cellular location of the protein in rod photoreceptor cells? As is shown in Fig. 4, rho/EGFP does not disrupt proper targeting of rhodopsin to the outer segment of rod photoreceptor cells in the *Xenopus* retina. Rho/EGFP displays the same mosaic expression pattern and outer segment location as observed for rho–GFP by Moritz et al. [1]. Thus, movement of the EGFP domain to the C-terminus of rhodopsin (and followed by a repeat of the 1D4 epitope as indicated in Fig. 1) does not appear to adversely affect expression or trafficking of the protein in the rod photoreceptor cells of transgenic animals.

In summary, the placement of the EGFP domain in rho/EGFP results in a significant improvement in the ability of the fusion protein to activate transducin and serve as a substrate for RK, and maintains the signals responsible for proper targeting of the protein to outer segments of rod photoreceptor cells. As a consequence, rho/EGFP is a more appropriate choice for studies in which phototransduction is the focus of transgenesis experiments.

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