

# Identification of an Outer Segment Targeting Signal in the COOH Terminus of Rhodopsin Using Transgenic *Xenopus laevis*

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**Abstract.** Mislocalization of the photopigment rhodopsin may be involved in the pathology of certain inherited retinal degenerative diseases. Here, we have elucidated rhodopsin's targeting signal which is responsible for its polarized distribution to the rod outer segment (ROS). Various green fluorescent protein (GFP)/rhodopsin COOH-terminal fusion proteins were expressed specifically in the major red rod photoreceptors of transgenic *Xenopus laevis* under the control of the *Xenopus* opsin promoter. The fusion proteins were targeted to membranes via lipid modifications (palmitoylation and myristoylation) as opposed to membrane spanning domains. Membrane association was found to be necessary but not sufficient for efficient ROS localization. A

GFP fusion protein containing only the cytoplasmic COOH-terminal 44 amino acids of *Xenopus* rhodopsin localized exclusively to ROS membranes. Chimeras between rhodopsin and  $\alpha$  adrenergic receptor COOH-terminal sequences further refined rhodopsin's ROS localization signal to its distal eight amino acids. Mutations/deletions of this region resulted in partial delocalization of the fusion proteins to rod inner segment (RIS) membranes. The targeting and transport of endogenous wild-type rhodopsin was unaffected by the presence of mislocalized GFP fusion proteins.

**Key words:** rhodopsin • photoreceptors • transgenic animals • membrane proteins • cell polarity

## Introduction

Asymmetric or polarized distribution of membrane components in neuronal and epithelial cells allows the various cell surface domains to accomplish different tasks. Although it is known that membrane and secretory proteins are sorted at the trans-Golgi network before being transported to their final cellular destination, the signals that target these proteins to their functional domains and the mechanisms by which they are vectorially transported have not been fully elucidated.

To model vectorial targeting of membrane proteins, many studies employ polarized epithelial cells, which have distinct apical and basolateral surfaces separated by tight junctions. Basolateral sorting signals have been identified in the cytoplasmic faces of proteins. These signals, which commonly contain di-tyrosine or dileucine motifs (Hunziker et al., 1991; Hunziker and Fumey, 1994), can be associated with clathrin-coated pit-mediated endocytosis. Other basolateral targeting signals are independent of endocytotic signals (Casanova et al., 1991; Beau et al., 1998). Most apical sorting signals have been identified in the luminal aspects of proteins and are commonly lipid or carbohydrate modifications that allow the proteins to associate with lipid rafts (Ikonen and Simons, 1998). Glycophosphatidylinosi-

tol modification (Brown et al., 1989; Lisanti et al., 1989), and N- and O-linked glycosylation (Scheiffele et al., 1995; Gut et al., 1998; Monlauzeur et al., 1998; Alfalah et al., 1999) have all been identified as apical targeting signals in MDCK cells. From this ongoing body of work, a complex set of rules for vectorial transport in polarized epithelia is emerging in which there appears to be a hierarchy of signals with basolateral signals being dominant over apical signals (Alonso et al., 1997; Perego et al., 1997).

From their work on the targeting of viral proteins in cultured neurons, Dotti and Simons (1990) proposed that the dendritic and axonal surfaces of neurons were analogous to the basolateral and apical surfaces of epithelial cells, respectively, and that the two cell types employed similar protein sorting mechanisms. Dendritic proteins were thus expected to be transported basolaterally and axonal proteins apically in epithelia. The results of many protein targeting experiments have both supported and contradicted this hypothesis (Fuller and Simons, 1986; Cameron et al., 1991; Pietrini et al., 1994; De Strooper et al., 1995; Simons et al., 1995; Perego et al., 1997; Ghavami et al., 1999). While obviously an invaluable experimental tool in studying cell polarity, the results derived from polarized epithelial cells cannot always be applied directly to neurons.

Our group has studied the vectorial transport of rhodopsin, a heptahelical, G protein-coupled receptor found in rod photoreceptors. Rods are highly compartmentalized

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neuronal cells that consist of an inner segment (cell body), an outer segment (a light capturing organelle that contains rhodopsin in membranous disks), a 9+0 connecting cilium which joins the two compartments, and a synaptic terminal. Rhodopsin is dually glycosylated at its NH<sub>2</sub> terminus (Fukuda et al., 1979) which faces the lumen and is dually palmitoylated near its cytoplasmic COOH terminus (O'Brien and Zatz, 1984; Ovchinnikov et al., 1988). Newly synthesized rhodopsin is transported from the Golgi apparatus to the base of the connecting cilium and then to the rod outer segment (ROS)<sup>1</sup> where it is incorporated into nascent disks. In frogs, a highly specialized structure called the periciliary ridge complex exists at the base of the cilium and is the initial docking site of rhodopsin-bearing post-Golgi membranes (Peters et al., 1983; Papermaster et al., 1985). The distal tips of the photoreceptors are shed daily, thus requiring the concomitant synthesis of new rhodopsin and formation of new disks at the base of the ROS (Young, 1967; Young and Droz, 1968).

Mutations of rhodopsin have been linked to inherited forms of retinal degeneration such as retinitis pigmentosa and macular degeneration (Dryja et al., 1990). Although these mutations are found throughout the protein, many of those associated with early onset aggressive disease are clustered in the COOH-terminal cytoplasmic tail (Sandberg et al., 1995). Transgenic mice, rats, and pigs harboring such mutants exhibit substantial delocalization of rhodopsin to the rod inner segment (RIS) plasma membrane (Sung et al., 1994; Li et al., 1998; Green et al., 2000) where it is normally found at very low levels (Nir and Papermaster, 1983). This observation has led to the theory that the cytoplasmic tail is involved in sorting rhodopsin into appropriate post-Golgi membranes or in the highly polarized vectorial transport of rhodopsin to the ROS. This theory is also supported by studies of post-Golgi vesicle formation in broken retinal cell preparations (Deretic et al., 1996, 1998), rhodopsin targeting experiments in MDCK cells (Chuang and Sung, 1998), and by identification of a molecular motor which interacts with the COOH terminus of rhodopsin (Tai et al., 1999; Sung and Tai, 2000).

We have used transgenic *Xenopus laevis* to identify rhodopsin's ROS localization signal in rod photoreceptors. This transgenic system coupled with the use of the opsin promoter (Batni et al., 1996) has allowed us to study rhodopsin targeting in its native cell type under physiological conditions. The cost and speed of generating transgenic tadpoles permitted the analysis of many more transgenes than would have been feasible in a transgenic mammalian system. Moreover, the relatively large size of amphibian photoreceptors facilitated the localization of the fusion proteins.

## Materials and Methods

### Molecular Biology

All expression vectors are based on pGFP-C1 obtained from CLONTECH Laboratories, Inc. The vector was modified to contain the *X. laevis* opsin promoter in place of the cytomegalovirus promoter as described

<sup>1</sup>Abbreviations used in this paper: AAR,  $\alpha$ 2A adrenergic receptor; dpf, days postfertilization; mGFP, myristoylated GFP; RIS, rod inner segment; ROS, rod outer segment; TR-WGA, Texas red-conjugated wheat germ agglutinin.

previously (Moritz et al., 1999). Subsequently, a plasmid was made which contains only the proximal 1.3-kb BglII–BamHI fragment of the opsin promoter. XOP5.5GFP-C1 was digested with NotI and BglII. The vector backbone was religated using a NotI–BclI linker to create XOP1.3GFP-C1. XOP1.3GFP-C1 was functionally equivalent to XOP5.5GFP-C1 and was used to make the GFP fusion constructs. Short *X. laevis* rhodopsin, porcine  $\alpha$  adrenergic receptor (AAR), and c-src sequences were synthesized either by annealing complimentary overlapping oligonucleotides or by PCR. Unique XhoI and EcoRI or BamHI restriction sites were introduced into the oligonucleotides for cloning into XOP1.3GFP-C1, in frame, behind the GFP cDNA. The mutants P353S, P353L, and C322/323S were created by PCR mutagenesis based on the protocol of Nelson and Long (1989). The NH<sub>2</sub>-terminal c-src sequence was incorporated into an oligonucleotide used for PCR amplification of the GFP cDNA. This 5' PCR oligonucleotide annealed to the region surrounding the start codon of the GFP cDNA in XOP1.3GFP-C1 and incorporated the 16 NH<sub>2</sub>-terminal amino acids of c-src. A 3' PCR oligonucleotide annealed downstream of the GFP cDNA. The PCR product was digested with AgeI–XhoI and ligated into the corresponding sites of XOP1.3GFP-C1, replacing the original GFP cDNA, to produce XOP1.3mGFP-C1. All PCR-amplified DNA fragments were analyzed by DNA sequencing to confirm that no PCR-induced errors were introduced. All expression constructs were linearized by digestion with NotI and purified using the GeneClean Kit (Bio101). Restriction endonucleases were obtained from GIBCO BRL and New England Biolabs, Inc.

### Transgenesis, GFP Screening, and Tadpole Rearing

Transgenic frogs were generated using a modified protocol (Moritz et al., 1999) based on that of Kroll and Amaya (1996) and Amaya and Kroll (1999). *X. laevis* sperm nuclei were incubated with 0.3 $\times$  high speed egg extract, 0.05 U restriction enzyme, and 100–200 ng linearized vector DNA. The reaction mixture was then diluted to 0.3 nuclei/nl and 10 nl was injected per egg. The resulting embryos were kept at 18°C in 0.1 $\times$  Marc's Modified Ringer, 6% Ficoll solution for 48 h and then switched to 0.1 $\times$  Gerhart's Ringer Solution (Wu and Gerhart, 1991). At 5–6 d postfertilization (dpf) roughly corresponding to stages 40–42 (Nieuwkoop and Faber, 1994), tadpoles were screened for GFP expression using a Leica MZ8 dissecting microscope equipped with epifluorescence optics and a GFP filter set. Animals were placed into glass Pasteur pipettes to immobilize them. Tadpoles expressing GFP were easily identified by the green fluorescence emitted from their eyes. At 14 dpf, transgenic tadpoles were placed in tanks with 0.1 $\times$  Gerhart's Ringer Solution and reared at 18°C on a 12/12 h light/dark cycle. Adult *X. laevis* were obtained from Nasco or Xenopus Express.

### Immuno-EM

Transgenic tadpoles were killed at 14 dpf (stage 47–48) and fixed in 4% paraformaldehyde, 0.1 M sodium phosphate buffer, pH 7.5. After overnight fixation, eyes were excised and embedded in LR White according to the manufacturer's instructions (London Resin Company). Thin sections were labeled either with a rabbit anti-GFP polyclonal antibody (CLONTECH Laboratories, Inc.) diluted 1:200 or a mouse anti-frog rhodopsin monoclonal antibody (11D5; Deretic and Papermaster, 1991) diluted 1:5,000 in 1% goat serum, 0.1 M Tris, pH 7.4, followed by incubation with an appropriate secondary antibody conjugated to 10 nm colloidal gold (Amersham Pharmacia Biotech) diluted 1:5 in 0.1 M Tris, pH 8.2, 1% goat serum. A minimum of two transgenic animals were examined by immuno-EM for each construct.

### Immunocytochemistry and Confocal Microscopy

Transgenic tadpoles were killed between stages 48–62. After immobilizing the tadpoles in 0.02% Tricaine, their eyes were excised and fixed in 4% paraformaldehyde, sodium phosphate buffer, pH 7.5, overnight. Fixed eyes were embedded in OCT tissue embedding medium (Tissue-Tek) and frozen in a dry ice/isopentane bath. Cryostat frozen sections (14  $\mu$ m) were blocked (10% BSA, 0.1% Triton X-100 in PBS) and labeled overnight with 0.1 mg/ml Texas red-conjugated wheat germ agglutinin (TR-WGA) (Molecular Probes) and 0.01 mg/ml Hoescht 33342 stain (Sigma-Aldrich) to label Golgi/post-Golgi membranes and nuclei, respectively. Labeling was done in the presence of 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 1% BSA, and 0.1% Triton X-100 in 1 $\times$  PBS. Labeled sections were analyzed by confocal microscopy using a scanning laser microscope (model 410; ZEISS). A minimum of three transgenic animals were examined by confocal microscopy for each construct.

**A**

rho 311 KQFRNCLITLCCGK<sup>\*\*</sup>NPFG<sup>del25</sup>DEDGSSAATSKTEASSVSS<sup>del5</sup>QVSPA<sup>@</sup> 354

AAR HDFRRAF<sup>\*</sup>KKILCRGDRKRIV

myr <sup>^</sup>MGSNKS<sup>^</sup>KPKDASQRRR

**B**

CONSTRUCT	DIAGRAM	MEMBRANE ASSOCIATION	LOCALIZATION
GFP		-	IS
GFP-CT44		+	OS
GFP-CT25		-	IS
GFP-CT44del25		+	IS, OS
GFP-CT44del5		+	IS, OS
GFP-CT44P353S/L		+	IS, OS
GFP-AAR		+/-	IS
GFP-AAR(CC)		+	IS, OS
GFP-AAR(CC)rho6		+	IS, OS
GFP-AAR(CC)rhoCT8		+	OS
mGFP		+	IS, OS
mGFP-CT9		+	IS, OS
mGFP-CT25		+	IS, OS
mGFP-CT44		+	OS
mGFP-CT44C322/323S		+	IS, OS

*Figure 1.* The GFP-fusion proteins. (A) Amino acid sequences of the cytoplasmic tails of *X. laevis* rhodopsin (rho), the porcine AAR, and the NH<sub>2</sub> terminus peptide from human c-src (myr). Numbers refer to the amino acid sequence of *X. laevis* rhodopsin. The sites of palmitoylation (\*), the site of myristoylation (^), and proline353 (@) are indicated. Arrows mark the sites of truncation. The solid line indicates the rho6 sequence. The dashed line indicates the rho8 sequence. (B) Diagrams of the fusion proteins and their cellular localization. Solid bars represent GFP. Open bars represent rhodopsin peptides. Vertically striped bars represent the c-src peptide. Diagonally striped bars represent adrenergic peptides. Straight vertical lines represent palmitoyl groups and zigzag lines represent myristoyl groups. +, membrane association; -, its absence. Inner segment (IS) and/or outer segment (OS) localization of the fusion protein is indicated.

## Results

### Generation of Transgenic *X. laevis* Tadpoles and Localization of the Transgenic Fusion Proteins

All fusion proteins were successfully expressed at easily observed levels in transgenic frog retinas and at low levels in the pineal gland. The subcellular localization of the fusion proteins in retinal photoreceptors was determined from confocal microscopy of frozen sections and immunocytochemistry of ultrathin sections. The results are summarized in Fig. 1. We distinguished between cytoplasmic and membrane associated and between ROS and RIS compartments (i.e., lateral plasma membrane, intracellular membranes, and synaptic terminal).

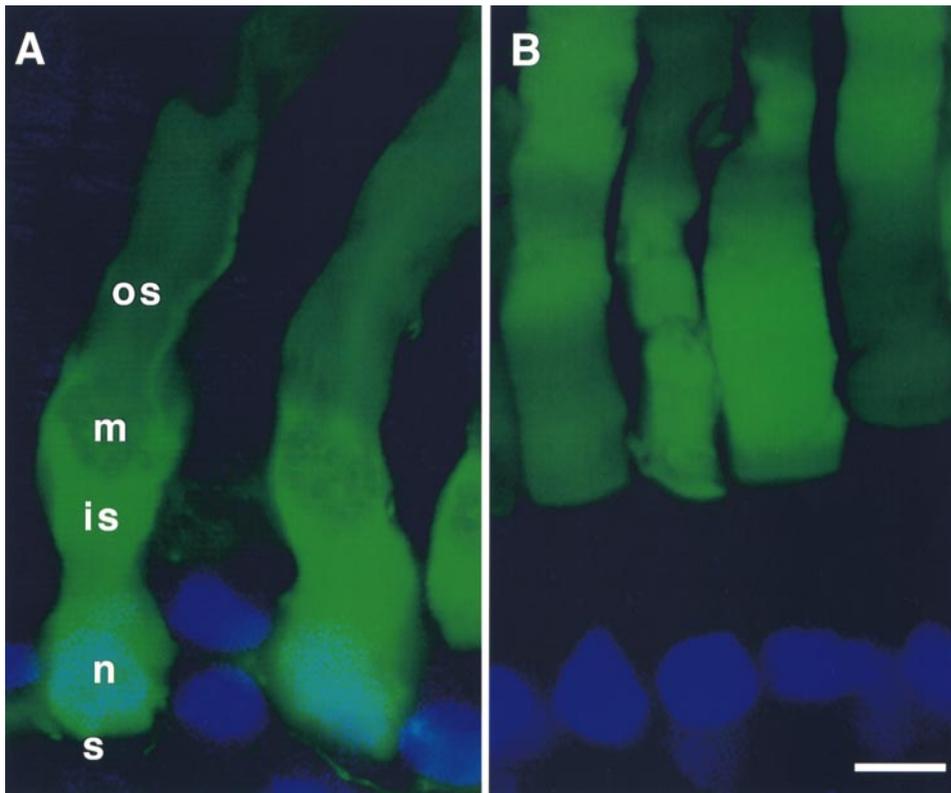
### The Cytoplasmic Tail of Rhodopsin Contains an ROS Localization Signal

The distribution of GFP expressed in transgenic *Xenopus* rod photoreceptors was determined for comparison with subsequent GFP fusion proteins. High levels of GFP distributed to the RIS cytoplasm and the nucleus, whereas low levels were evident in the ROS cytoplasm (Fig. 2 A). GFP was excluded from the mitochondria and ROS disks, as might be expected of a soluble protein. These observations are consistent with those described previously by Knox et al. (1998) and Moritz et al. (1999) and suggest a distribution based on diffusion rather than active transport to any cellular compartment.

To test whether the COOH-terminal 44-amino acid cytoplasmic tail of rhodopsin could confer rhodopsin-like sorting, residues 311–354 of *Xenopus* rhodopsin were fused to the COOH terminus of GFP (GFP-CT44). Unlike GFP alone, GFP-CT44 is membrane bound via its two naturally occurring palmitoylation sites (see below) and localized almost exclusively to the ROS. Little or no GFP-CT44 was detected in the RIS, either in the cytoplasm or the lateral plasma membrane (Fig. 2 B). These results indicate that the cytoplasmic tail of rhodopsin contains sufficient information to direct ROS localization.

### Truncating or Mutating the Cytoplasmic Tail Results in Partial Delocalization to the RIS

Mutations of the COOH terminus of rhodopsin cause particularly aggressive forms of retinitis pigmentosa with early onset and blindness (Sandberg et al., 1995). Specific examples include a proline 347 to leucine or serine (P347L, P347S) point mutation and a premature stop codon following glutamine 344 resulting in the deletion of the last five amino acids of rhodopsin (Q344ter). In transgenic mice and pigs, these mutations correlate with partial delocalization of the mutant rhodopsin to the RIS plasma membrane (Sung et al., 1994; Li et al., 1998; Green et al., 2000). To test whether analogous mutations in the *Xenopus* rhodopsin COOH terminus cause similar delocalization, transgenic tadpoles were generated that express the cytoplasmic tail of rhodopsin with a truncation of the ter-



**Figure 2.** The cytoplasmic tail of rhodopsin can redirect GFP to the ROS. Confocal micrographs of transgenic retinas expressing GFP and GFP-CT44. Nuclei were stained with Hoescht 33342. (A) GFP distributed predominantly to the cytoplasm of the inner segment (is) and nucleoplasm. GFP resided at low levels in the outer segment (os) and was not associated with membranes. (B) GFP-CT44 localized almost exclusively to the outer segments. GFP (green) and Hoescht 33342 (blue). n, nucleus; m, mitochondria; and s, synaptic terminal. Bar, 5  $\mu$ m.

terminal 25 amino acids (GFP-CT44del25) or the terminal 5 amino acids (GFP-CT44del5) fused to GFP. Both of these fusion proteins localized to the ROS but in contrast to GFP-CT44, significant levels of fusion protein were also detected in inner segment intracellular membranes, the plasma membrane, and the synaptic terminal (Fig. 3, A and B). Like GFP-CT44, no fusion protein was visible in the cytoplasm or the nucleus. Similar results were obtained when proline 353 (analogous to P347 in human rhodopsin) was mutated to either serine or leucine (GFP-CT44/P353S, GFP-CT44/P353L) (Fig. 3, C and D). These results indicate that the extreme COOH terminus of rhodopsin contains an ROS transport/localization signal and that the penultimate proline is part of that signal.

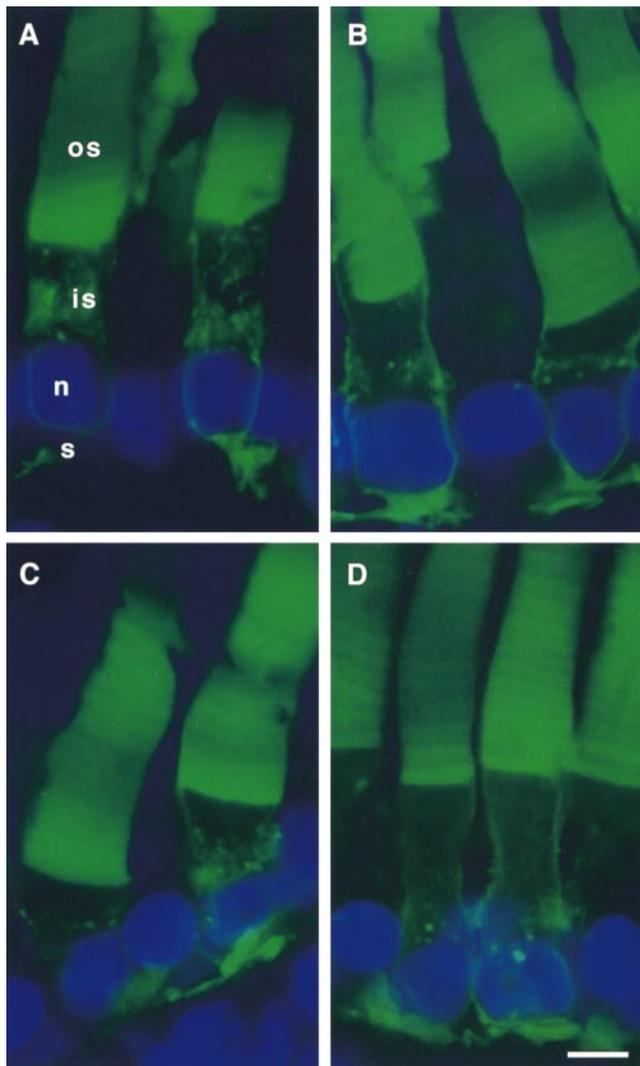
#### ***Membrane Association Is Necessary but Not Sufficient for ROS Localization***

Rhodopsin is an integral membrane protein with seven membrane spanning regions (Hargrave et al., 1983). The cytoplasmic tail of rhodopsin also contains two cysteines (cys322 and cys323) that are palmitoylated and provide another point of membrane attachment (Moench et al., 1994). The GFP fusion proteins in this study did not include rhodopsin's membrane spanning domains yet appeared to associate with membrane compartments, presumably due to palmitoylation of the cysteines. To confirm membrane association and determine the identity of the membranes with which the fusion proteins associate, we labeled retinal sections expressing GFP-CT44del25 with TR-WGA, a marker for Golgi/post-Golgi membranes (Virtanen et al., 1980). In rod cells, TR-WGA also labels ROS and plasma membranes. GFP fluorescence colocalized

with TR-WGA-labeled membranes (Fig. 4, A–C), signifying that the fusion proteins are present in Golgi/post-Golgi membranes as well as ROS membranes. Furthermore, absence of GFP-CT44del25 from the cytoplasm and nucleoplasm suggests that this molecule was no longer soluble but likely to be palmitoylated and membrane bound.

To test whether membrane association is required for outer segment transport, we fused the terminal 25 amino acids of rhodopsin to GFP (GFP-CT25). This region of rhodopsin is downstream of cys322/323 and therefore GFP-CT25 would not be palmitoylated or membrane bound. As seen in Fig. 4 D, GFP-CT25 localized predominantly to the RIS cytoplasm. The expression pattern is essentially identical to that of GFP (Fig. 2 A) and suggests that both membrane association and a targeting/retention sequence are required for efficient and exclusive transport to the ROS.

To further evaluate this issue, we fused the NH<sub>2</sub>-terminal 16-amino acid peptide of c-src upstream of GFP. This region of c-src is myristoylated at the glycine at residue 2 (Schultz et al., 1985) and contains six positively charged residues which are required, in conjunction with the myristoyl group, for efficient membrane attachment (Resh, 1994). This myristoylated form of GFP (mGFP) was found at high levels in the ROS but also was detected in the RIS plasma membrane and diffusely through inner segment membranes including Golgi and mitochondrial membranes (Fig. 4 E). mGFP was not appreciably soluble since the nucleoplasm exhibited little or no fluorescence. When the COOH-terminal 44 amino acids of rhodopsin were fused to mGFP (mGFP-CT44), the fusion protein again localized exclusively to the ROS (Fig. 4 F). Taken together, these results demonstrate that although membrane associ-



**Figure 3.** Truncations or mutations of the rhodopsin COOH-terminal domain cause the fusion proteins to partially delocalize to the RIS. Confocal micrographs of transgenic retinas expressing (A) GFP-CT44del25; (B) GFP-CT44del5; (C) GFP-CT44/P353S; and (D) GFP-CT44/P353L. Nuclei were stained with Hoescht 33342. Truncation of the distal 25 (A) or 5 amino acids (B) or mutation of the penultimate proline to either serine (C) or leucine (D) resulted in partial delocalization of the fusion proteins to the lateral plasma membrane, Golgi, and synaptic terminal. GFP (green) and Hoescht 33342 (blue). os, outer segment; is, inner segment; n, nucleus; and s, synaptic terminal. Bar, 5  $\mu$ m.

ation of proteins is necessary, it is not sufficient for restricted ROS localization.

### **The ROS Localization Signal Is Specific to Rhodopsin**

AAR is a heptahelical G protein-coupled receptor and, like many others of this family, contains a palmitoylation site in its cytoplasmic tail (Kennedy and Limbird, 1993). Although sharing overall structural similarities, AAR and rhodopsin reside in different cell types and perform different physiological roles. We were interested to see whether the cytoplasmic tail of another heptahelical receptor would promote ROS targeting or would direct its trans-

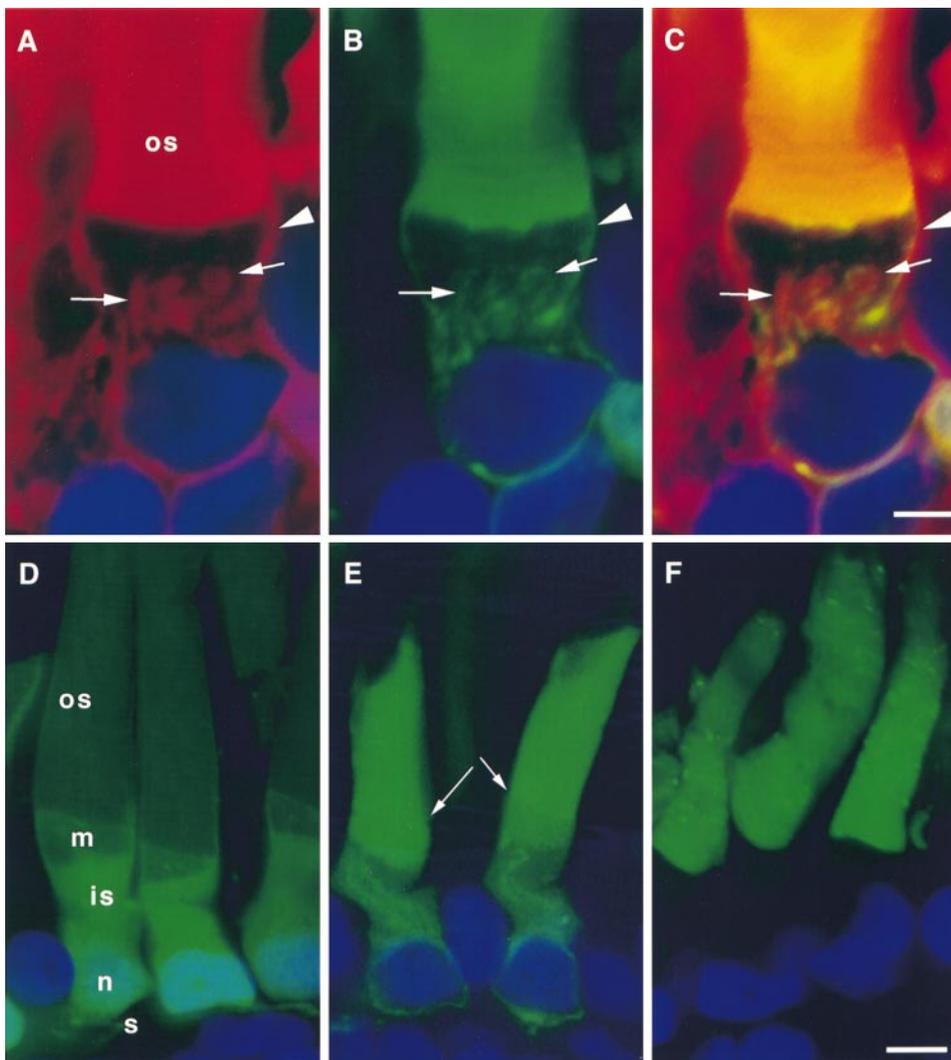
port to another domain of the cell. Therefore, the cytoplasmic tail of AAR was fused to GFP (GFP-AAR). GFP-AAR localized predominantly to the RIS plasma membrane and especially to the synaptic terminal. A low concentration of GFP-AAR was also seen in the cytoplasm of the RIS, the ROS, and the nucleus, perhaps due to incomplete palmitoylation (Fig. 5 A). Since membrane association appears to be required for efficient outer segment localization, we incorporated a second cysteine (GFP-AAR[CC]) to create a second palmitoylation site. As evidenced by the clearing of the nucleoplasm, GFP-AAR[CC] exhibited a higher degree of membrane association (Fig. 5 B). The distribution of the fusion protein shifted from predominantly synaptic to include also the base of the outer segment. Significant levels were still detectable in inner segment and synaptic membranes. This demonstrates that the ROS sorting signal is specific to the rhodopsin sequence and not a general feature of the cytoplasmic tails of heptahelical receptors.

### **The Terminal Eight Amino Acids of Rhodopsin Are Sufficient to Redirect Membrane-associated GFP to the ROS**

Although we established that the distal region of rhodopsin's cytoplasmic tail was involved in ROS localization, we wished to further define the localization signal. Therefore, we constructed an AAR/rhodopsin chimera (GFP-AAR[CC]rho8). The terminal eight residues of GFP-AAR[CC] were replaced with the terminal eight residues of *X. laevis* rhodopsin (residues 347–354). GFP-AAR(CC)rho8 localized exclusively to the ROS and was not detected in the RIS, either in the cytoplasm or any membrane domains (Fig. 5 C). As a control, the fusion protein GFP-AAR(CC)rho6 was made in which the terminal octapeptide of the AAR cytoplasmic tail was replaced with residues 324–330 of rhodopsin. GFP-AAR(CC)rho6 was found in significant levels in internal membranes, RIS plasma membrane, and the synaptic terminal (Fig. 5 D). These results indicate that removal of the last eight amino acids of AAR does not unmask a ROS localization signal but rather that a ROS localization signal resides within the terminal eight amino acids of *X. laevis* rhodopsin.

### **Trafficking of Endogenous Rhodopsin Is Not Disrupted in Transgenic Photoreceptors**

To determine whether delocalization of the mutated/truncated fusion proteins reflected a general disruption of the trafficking machinery, we analyzed the distribution of endogenous rhodopsin. We labeled rods expressing GFP-CT44del25 with an anti-rhodopsin antibody (11D5) that recognizes the extreme COOH terminus of frog rhodopsin (Deretic and Papermaster, 1991) and therefore does not detect GFP-CT44del25, which lacks the epitope. Immunofluorescence revealed that endogenous rhodopsin was not significantly delocalized to the inner segment plasma membrane or synapse (Fig. 6 B). A neighboring section from the same cell was labeled with an anti-GFP antibody. The fusion protein was present in the ROS, RIS plasma membrane, trans-Golgi, and synapse (Fig. 6 A). Therefore, the rhodopsin trafficking machinery of these cells was not compromised and delocalization of GFP-CT44del25 was



**Figure 4.** Membrane association is necessary but not sufficient for ROS localization. Fusion proteins are associated with Golgi and post-Golgi membranes. (A–C) Confocal micrographs of a cell expressing GFP-CT44del25 labeled with TR-WGA (red) and Hoescht 33342 (blue). (A) TR-WGA labeling; (B) GFP fluorescence (green); (C) the overlap of the two images. Regions of colocalization are represented by yellow/orange. Together, these demonstrate that the fusion protein colocalizes with membranes, specifically Golgi and post-Golgi membranes (arrows), plasma membrane (arrowheads), and ROS membranes. (D–F) Confocal micrographs of transgenic retinas expressing GFP-CT25 (D), mGFP (E), and mGFP-CT44 (F). Nuclei were stained with Hoescht 33342 dye. GFP-CT25 did not associate with membranes and was not efficiently transported to the ROS. mGFP was membrane associated and was found in both ROS and RIS membranes including mitochondrial membranes found immediately below the inner/outer segment junction (arrows). mGFP-CT44 distributed almost exclusively to the ROS. Together, these demonstrate that both membrane attachment and a targeting signal are required for restricted ROS localization. GFP (green) and Hoescht 33342 (blue). os, outer segment; is, inner segment; n, nucleus; m, mitochondria, and s, synaptic terminal. Bars: (A–C) 2.5  $\mu\text{m}$ ; (D–F) 5  $\mu\text{m}$ .

due to insufficient targeting information. The anti-GFP labeling also supported the confocal microscopy results (Fig. 4, A–C) which showed that although they are not integral membrane proteins, the GFP fusions are present in the Golgi/post-Golgi trafficking pathway.

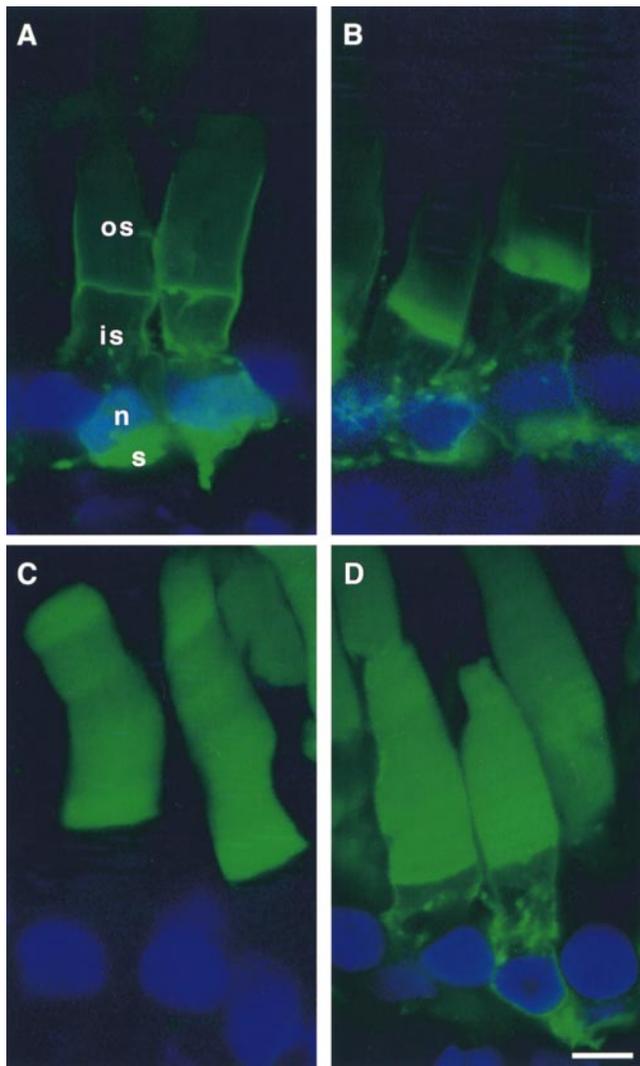
#### **Presentation of the Targeting Signal Is Important for ROS Localization**

The role of palmitoylation in the function of rhodopsin is still unclear. To test whether palmitoylation of the cysteines might serve a structural role in presenting rhodopsin's ROS localization signal to sorting/transport machinery, we used the myristoylated form of GFP. A modified version of mGFP-CT44 was made in which cys322 and cys323 were mutated to serines (mGFP-CT44C322/323S) and expressed in rods. mGFP-CT44C322/323S was efficiently localized to the ROS (significantly more than mGFP alone), but very low levels of the fusion protein were still detected in the RIS plasma membrane and inter-

nal membranes (Figs. 4 E and 7 C). A similar result was seen when the COOH-terminal nine amino acids of rhodopsin were fused to mGFP (mGFP-CT9) (Fig. 7 A). However, when the terminal 25 amino acids were fused to GFP (mGFP-CT25), the fusion protein was substantially delocalized (similar to mGFP), suggesting that some aspect of its conformation was interfering with access to or interaction with the COOH-terminal sequence. These results indicate not only that the presence of the targeting signal is required, but also that the way it is presented is important.

#### **Delocalization Is Not a Function of the Expression Level of the Transgenes**

Due to the nature of the transgenesis protocol, each transgenic tadpole has the potential to be different from every other one with regard to the transgene copy number and the site(s) of integration. Therefore, it is not surprising that we observed that expression levels varied among tadpoles expressing the same transgene. However, the total



**Figure 5.** Rhodopsin's outer segment targeting/retention signal resides within its distal most eight amino acids. Confocal micrographs of transgenic retinas expressing (A) GFP-AAR; (B) GFP-AAR(CC); (C) GFP-AAR(CC)rho8; and (D) GFP-AAR(CC)rho6. GFP-AAR distributed mainly to RIS membranes, especially the synaptic terminal, but was also partially soluble. GFP-AAR(CC) displayed greater membrane association than its single cysteine counterpart and localized to both ROS and RIS membranes. GFP-AAR(CC)rho8 localized exclusively to the ROS while GFP-AAR(CC)rho6 localized to both ROS and RIS membranes. The last eight amino acids of rhodopsin are therefore sufficient to direct ROS localization. GFP (green) and Hoescht 33342 (blue). os, outer segment; is, inner segment; n, nucleus; and s, synaptic terminal. Bar, 5  $\mu$ m.

range of expression levels among the various transgenes significantly overlapped. Furthermore, the presence or absence of RIS GFP fluorescence for animals expressing a particular transgene was consistent regardless of the overall expression level. Highly expressed transgenes reached  $\sim$ 50% of endogenous rhodopsin levels as determined by Western blots of solubilized transgenic retinas (data not shown). Many of the retinas expressed the transgene at significantly lower levels (Fig. 8, A and B). To rule out the possibility that RIS delocalization of certain transgenes re-

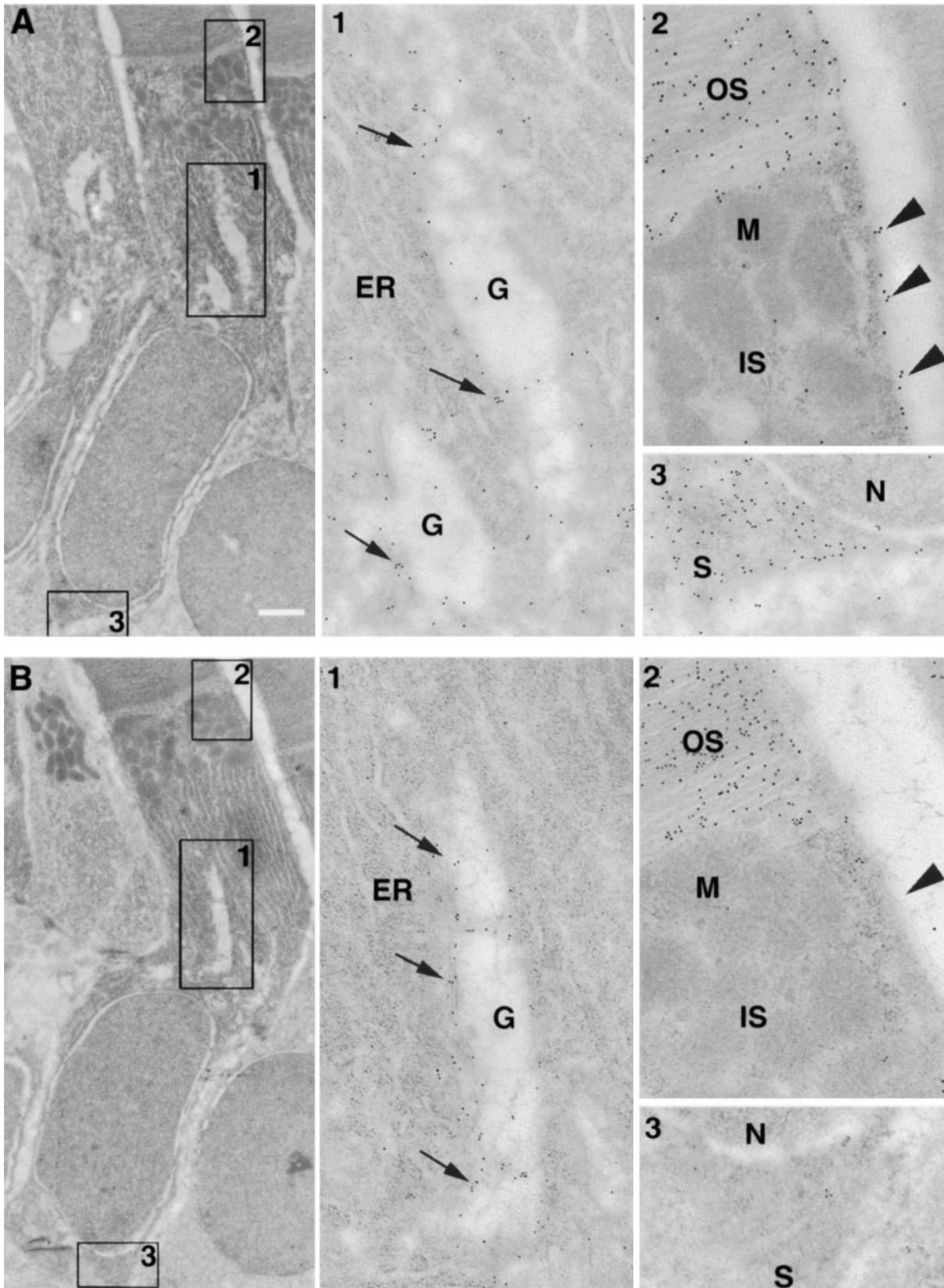
sulted from overexpression, a retina expressing high levels of transgene that did not delocalize (GFP-CT44) was compared to a retina expressing low levels of a transgene that did not delocalize (GFP-CT44P353L). Confocal micrographs were taken consecutively of frozen retinal sections from the two animals using the same settings (i.e., laser attenuation, brightness, and contrast). GFP intensity and thus protein concentration is directly comparable between the two micrographs. As seen in Fig. 8, although GFP-CT44 is being expressed at a much higher level (Fig. 8 B), there is very little GFP fluorescence in the RIS. In contrast, the concentration of GFP-CT44P353L (Fig. 8 A) is drastically lower, but RIS localization of the transgene is readily detected. Thus, delocalization is a result of lack of targeting information and not overexpression.

## Discussion

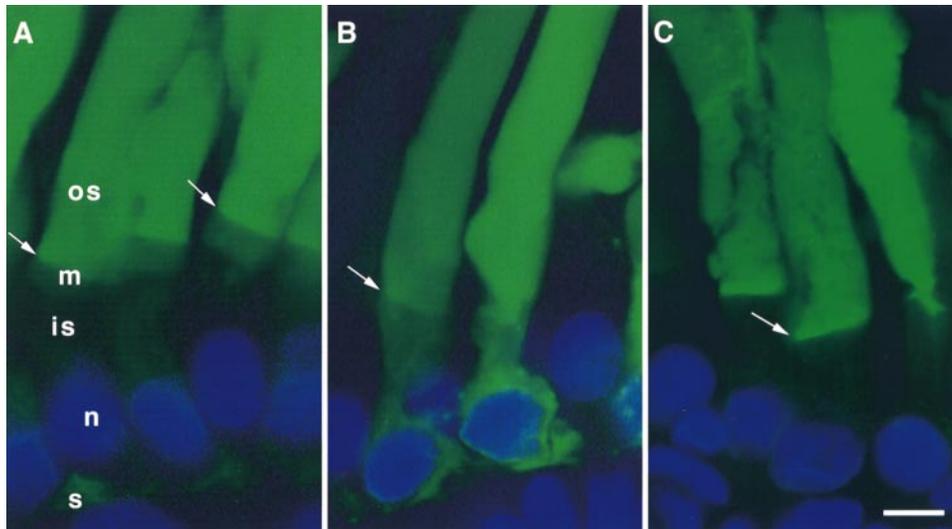
The localization signals of many epithelial and neuronal proteins are currently being sought as well as the cellular components that interact with these signals. In the retinal degenerative disease retinitis pigmentosa, it has been hypothesized that a group of naturally occurring mutations clustered at the COOH terminus of rhodopsin might potentiate the disease by disrupting targeting of rhodopsin. Many *in vitro* studies support this hypothesis but because of the lack of a suitable expression system, the essential premise of the theory, that a sequence within the COOH terminus is a ROS targeting signal, has remained untested *in vivo*. Studies have been done in transgenic mice, rats, and pigs but have not excluded contributions from the upstream domains of rhodopsin.

We have now identified a ROS localization signal within the cytoplasmic tail of rhodopsin. By both loss and gain of function, we have shown that the last eight amino acids of rhodopsin are necessary and sufficient for ROS localization when the peptide sequence is membrane bound. Truncation of the distal five amino acids or mutation of the penultimate proline of rhodopsin resulted in partial mislocalization of the fusion proteins to RIS membranes, whereas the fusion protein containing the entire COOH-terminal tail targeted exclusively to the ROS. Furthermore, the COOH-terminal octapeptide of rhodopsin contains sufficient information to direct a predominantly RIS-synapse-localized protein (GFP-AAR) to the ROS (GFP-AAR[CC]rho8). This is the first demonstration of a gain of ROS sorting function of a rhodopsin domain. Although the mechanisms governing polarized targeting of neuronal proteins are widely studied, only a few amino acid-based axonal or dendritic targeting motifs have been identified. There were no obvious sequence homologies among the last eight amino acids of rhodopsin and the targeting signals of these other proteins.

Rhodopsin localization in rods may involve several steps, not all of which are necessarily relevant in epithelial cells, and vice versa. In a study of rhodopsin localization in MDCK cells, rhodopsin exhibited polarized apical distribution. However, truncation of the COOH-terminal 32, but not 22, residues resulted in partial delocalization to the basolateral surface (Chuang and Sung, 1998). This result suggests that the apical targeting signal resides in the 10 amino acids between the two truncations, a region which



**Figure 6.** GFP-CT44del25, but not endogenous rhodopsin, accumulates abnormally in the Golgi, RIS plasma membrane, and synapse. EM micrographs of a rod cell expressing GFP-CT44del25. The cell was labeled either with anti-GFP antibody (A) or antirhodopsin antibody, 11D5 (B), followed by a gold-conjugated secondary antibody. The boxed regions are magnified to show detail. Arrows point to GFP (A 1) and rhodopsin (B 1) labeling present in the Golgi. Arrowheads indicate the presence of GFP-CT44del25 (A 2), but not rhodopsin (B 2), on the RIS plasma membrane. Likewise, GFP-CT44del25 (A 3) is abundant in the synaptic terminal; rhodopsin (B 3) is not. Therefore, delocalization of the fusion protein does not result in delocalization of endogenous rhodopsin. os, outer segment; is, inner segment; n, nucleus; m, mitochondria; G, Golgi; and S, synaptic terminal. Bar, 2.5  $\mu$ m.



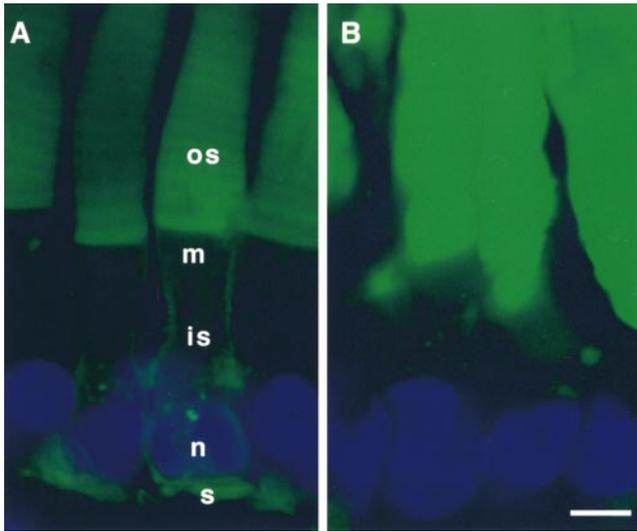
**Figure 7.** Presentation of the targeting signal affects the efficiency of ROS localization. Confocal micrographs of cells expressing (A) mGFP-CT9; (B) mGFP-CT25; and (C) mGFP-CT44C322/323S. Varying the lengths of the rhodopsin peptides fused to GFP affected the distribution patterns. mGFP-CT9 and mGFP-CT44C322/323, but not mGFP-CT25, were significantly more efficient than mGFP (see Fig. 4 E) in targeting to the ROS. Mitochondria were also labeled to varying degrees by the myristoylated fusion proteins. Arrows indicate the inner/outer segment junction. GFP (green) and Hoechst 33342 (blue). os, outer segment; is, inner segment; n, nucleus; m, mitochondria; and s, synaptic terminal. Bar, 5  $\mu$ m.

includes the two palmitoylated cysteines. In rods, however, the targeting of GFP-AAR(CC)rho8 to the ROS and the delocalized distribution of GFP-CT44del25 demonstrate that the distal COOH-terminal region is important for ROS localization and not the region immediately surrounding the palmitoylated cysteines. Unpalmitoylated rhodopsin localized to the apical surface of MDCK cells, indicating that palmitoylation was not required for apical targeting (Chuang and Sung, 1998). These results may be a consequence of a peptide-based apical targeting signal within rhodopsin's COOH-terminal 22 amino acids that functions in epithelial cells, but that in its absence, palmitoylation may be a second distinct signal for apical localization. We have shown, however, that palmitoylation cannot act by itself as a ROS localization signal in rod photoreceptors. The issues raised here highlight some of the problems with interpreting the targeting of neuronal proteins in polarized epithelial cells as a predictor of targeting in neuronal cells *in vivo*.

Two potential roles for rhodopsin's ROS localization signal are sorting of rhodopsin into the correct post-Golgi vesicular compartment and transporting rhodopsin-containing membranes from the TGN to the base of the connecting cilium. The abnormal accumulation, in the RIS plasma membrane, Golgi, and synapse, of the GFP fusion proteins lacking the intact ROS localization signal (Fig. 4, A–C, and Fig. 6, A and B) can most easily be explained by their inability to sort to the proper post-Golgi vesicles. Without the proper ROS signal, individual GFP fusion proteins might randomly associate with any or all vesicles exiting the Golgi including those destined for the ROS, lateral plasma membrane, and synapse. In broken retinal cell preparations, COOH-terminal rhodopsin peptides and antibodies to this region inhibit the production of post-Golgi vesicles, also suggesting that this signal is required for sorting (Deretic et al., 1996, 1998). The ability of wild-type, but not mutant, rhodopsin COOH terminus to interact directly with Tctex-1 (a cytoplasmic dynein light chain) was interpreted to indicate involvement of the signal in vectorial transport of rhodopsin-containing membranes (Tai et al., 1999). However, the rate of rhodopsin production pre-

cludes the possibility that Tctex-1 could transport individual rhodopsin molecules. Given that post-Golgi vesicles containing rhodopsin have a mean diameter of 300 nm (Deretic and Papermaster, 1991), the density of rhodopsin on disk membranes is 20,000  $\mu$ m<sup>2</sup> (Chen and Hubbel, 1973), and the density of molecules on a periciliary vesicle is  $\sim$ 40% that found in the disks (Besharse and Pfenninger, 1980), a post-Golgi vesicle would contain  $\sim$ 2,000 rhodopsin molecules. This estimate coupled with the low ratio of transgene product to endogenous rhodopsin ( $<1:2$ ) suggests that there should be abundant rhodopsin COOH termini present in each vesicle to supply the required targeting signal and to drive transport of both rhodopsin and fusion protein to the ROS. We do in fact see this bulk flow phenomenon to a certain extent (see discussion below). However, we see that even transgene products lacking an intact targeting signal expressed at very low levels consistently delocalized to RIS membranes. The probability of a vesicle containing only the mutant rhodopsin COOH-terminal fusions, and therefore entirely unable to interact with Tctex-1, due to random sorting is exceedingly small. Even in human retinitis pigmentosa disease states where 50% of the rhodopsin molecules are mutated (i.e., 1:1 ratio), the vast majority of vesicles should contain abundant targeting information. Therefore, a sorting event, involving the COOH terminus, must exist that separates individual rhodopsin molecules from proteins destined for other parts of the cell and Tctex-1, given its specificity, may even be a part of that event. Expression of our GFP fusion proteins did not disrupt general transport mechanisms since endogenous rhodopsin did not delocalize. Furthermore, Green et al. (2000) showed that peripherin and the cGMP-gated channel localization were, likewise, unaffected in transgenic rats by the presence of a rhodopsin COOH-terminal truncation mutant.

A paraciliary transport pathway has been proposed in which rhodopsin-bearing vesicles bud from the apex of the RIS and fuse with nascent disks of the ROS (Besharse and Wetzell, 1995). In transgenic mice, mutant rhodopsin (P347S) is released in vesicles into the interphotoreceptor space (Li et al., 1996). This might support a paraciliary



**Figure 8.** Overexpression of the fusion proteins is not the cause of delocalization to the RIS. Transgenic eyes expressing GFP-CT44 and GFP-CT44/P353L were excised, fixed, and sectioned in parallel. Frozen sections from each eye were consecutively imaged by confocal microscopy. All image acquisition settings were identical for both samples and postmicroscopy processing was done in parallel to the images. GFP-CT44 (A) was expressed at a much higher level than GFP-CT44/P353L (B) and yet was not found in significant levels in the RIS in contrast to the mutated fusion protein. GFP (green) and Hoescht 33342 (blue). os, outer segment; is, inner segment; n, nucleus; m, mitochondria; and s, synaptic terminal. Bar, 5  $\mu$ m.

pathway if the COOH terminus of rhodopsin were required for fusion of the extracellular rhodopsin-bearing vesicles with nascent disks. However, our study and other studies of transgenic animals expressing COOH-terminal rhodopsin mutants did not reveal the same vesicular accumulation (Sung et al., 1994; Li et al., 1998; Green et al., 2000). Alternatively, rhodopsin may be transported to the ROS via the cilium after docking at the periciliary ridge complex (Peters et al., 1983; Papermaster et al., 1985; Wolfram and Schmitt, 2000). Additional sorting steps may take place at these locations.

Finally, the distal amino acids of rhodopsin may form an ROS retention signal as well as, or rather than, a targeting signal. Because rhodopsin exhibits a high degree of rotational and lateral mobility in disk membranes (Brown, 1972; Poo and Cone, 1974), a mechanism exists to limit re-entry from the ROS to the RIS plasma membrane. However, a defect in retention alone cannot explain the presence of delocalized fusion proteins in the Golgi since loss of retention would result primarily in mislocalization to the RIS plasma membrane. It is possible, however, that rhodopsin in the lateral membrane might be endocytosed and recycled to the Golgi. Kinetic labeling studies would be required to determine if this is the case.

In our experimental system, we used posttranslational lipid modifications to confer membrane association properties to the fusion proteins. Membrane targeting via palmitoylation and/or myristoylation is well documented (Pellman et al., 1985; Gonzalo and Linder, 1998; McCabe and Berthiaume, 1999; Resh, 1999). Since the overwhelming majority of both membrane lipids and proteins synthe-

sized by photoreceptors are destined for the ROS, membrane proteins that lack a ROS localization signal may be passively cotransported with the rhodopsin-bearing vesicles (i.e., bulk flow). This would explain the high levels of GFP-CT44del5, GFP-CT44del25, GFP-CT44P353S, GFP-CT44P353L, GFP-AAR(CC)rho6, and mGFP in the ROS even though these molecules lack the ROS targeting signal. In contrast, GFP-AAR appeared to be almost excluded from the ROS. The COOH-terminal tail of AAR may contain a synaptic sorting/retention signal recognized by photoreceptors which is partially disrupted by the addition of a second cysteine in GFP-AAR(CC) or eliminated by removal of its last eight residues in GFP-AAR(CC)rho8. Expression of a COOH-terminal mutant rhodopsin in a rhodopsin knockout mouse could unambiguously address the issue of bulk flow in photoreceptors.

Although the role of COOH-terminal palmitoylation has been elucidated in other heptahelical G protein-coupled receptors (Moffett et al., 1996; Tanaka et al., 1998), the function of rhodopsin palmitoylation is unclear. Its role on rhodopsin phosphorylation, transducin activation, and rhodopsin regeneration remains controversial (Morrison et al., 1991; Karnik et al., 1993). Recently, Sachs et al. (2000) proposed that the palmitoyl moieties form a second retinal binding pocket. A potential role for the palmitoylation of rhodopsin, suggested by our results, may be to anchor the extreme COOH terminus in an orientation and proximity to the membrane that is optimal for interaction with its cognate sorting/transport components. It is important to note, however, that many cone opsins are not palmitoylated but achieve polarized outer segment localization in their respective cells (Ostrer et al., 1998).

Although we showed that the last eight amino acids of rhodopsin can direct ROS localization, both mGFP-CT9 and mGFP-CT25 delocalized, to different extents, to RIS membranes. It is possible that complete ROS localization of these two fusion proteins was inhibited through steric hindrance of the rhodopsin peptide by GFP with the cell's sorting/transport components. However, mGFP-CT44C322/323S also partially delocalized in contrast to the palmitoylated version of the same protein (mGFP-CT44) which targeted only to ROS, even though the lengths of these two rhodopsin peptides were identical. Although palmitoylation has been described as a reversible process, the relative absence of GFP-CT44 in the cytoplasm suggests that it was predominantly attached to membranes, and thus at least one of the cysteines was palmitoylated at any given time.

Colocalization of palmitoylated GFP fusion proteins with TR-WGA-labeled intracellular membranes indicates that they were present in Golgi/post-Golgi membranes and therefore may have used the same trafficking pathway as rhodopsin. It is intriguing that previous studies of rhodopsin missorting in transgenic animals have not documented accumulation in the Golgi. This may be due to the fact that detection of the intrinsic fluorescence of our fusion proteins may be more sensitive than indirect antibody labeling. Although we used only the COOH terminus of rhodopsin and not the entire protein, a rhodopsin-GFP fusion protein which lacked the final 14 amino acids of rhodopsin was also detected in the Golgi as well as the RIS plasma membrane and synapse (our unpublished results). The palmitoylation consensus sequence might act

as an ER recruiting signal since palmitoylation of rhodopsin occurs in the ER before entering the Golgi (St. Jules and O'Brien, 1986; St. Jules et al., 1990). Once attached to ER membranes, the palmitoylated fusion proteins can follow normal membrane protein transport pathways. In contrast, myristoylation is a cotranslational process (Wilcox et al., 1987). Once released from the ribosomes, the myristoylated fusion proteins distributed throughout all RIS membranes, including mitochondrial membranes and the plasma membrane, where they would not be accessible to the post-Golgi sorting/transport pathway. This provides an alternate explanation for why the myristoylated, but not palmitoylated, GFP fusion proteins (e.g., mGFP-CT9) were not fully localized to the ROS.

The mislocalization of mutant rhodopsins may be involved in the pathogenesis of rod photoreceptors in retinitis pigmentosa and macular degeneration. It is therefore necessary to determine how rhodopsin achieves its specific localization in order to understand how mutations disrupt the normal process. Using transgenic frog photoreceptors as an expression system has aided us in approaching and answering some of these issues. In the future it will also be important to determine how other ROS proteins achieve their localization.

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