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Expression and localization of an exogenous G protein-coupled receptor fused with the rhodopsin C-terminal sequence in the retinal rod cells of knockin mice

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Abstract

Vertebrate rod cell outer segments are highly differentiated compartments consisting of closely packed disk membranes, in which the photoreceptor rhodopsin is embedded at high density. To explore the unusually efficient mechanism of rhodopsin biosynthesis, folding and transport, we challenged it with the ectopic expression in rod cells of human endothelin receptor subtype B (hET_BR) fused with the C-terminal 10 residues of rhodopsin, under the control of the mouse opsin promoter/enhancer, by gene targeted replacement (knockin), because the C-terminal eight residues are essential to target rhodopsin to the outer segment. The hET_BR, a type-I G protein-coupled receptor, was successfully expressed and folded in a functional structure in the rod cells of knockin mice. However, while the mRNA level of hET_BR was one tenth of that of rhodopsin, the hET_BR protein level was approximately one-thousandth of the rhodopsin level in heterozygous mice, suggesting an intrinsically distinct efficiency in the production of functional receptor protein. In addition, a substantial fraction of the hET_BR was successfully transported to the outer segment, suggesting that the addition of the C-terminal sequence of rhodopsin enabled hET_BR to be translocated to the outer segment.

Keywords: rhodopsin biosynthesis; rhodopsin transport; photoreceptor cells; knockin mice; G protein coupled receptors

The retinal rod cell in vertebrates is a highly polarized cell, composed of outer and inner segments (OS and IS), a cell body, and a synaptic region. Phototransduction takes place in the OS, which contains a stack of 1000–2000 independent disk membranes (Filipek et al., 2003), in which photoreceptor rhodopsin molecules constitute more than 90% of the integral membrane proteins (Pugh and Lamb, 1993; Calvert et al., 2001).

Photoreceptor cells do not divide after maturation, but components of the disc membranes are continuously renewed with a high membrane turnover rate (Papermaster et al., 1986; Papermaster, 2002). To maintain this organization, an efficient biosynthesis and vectorial transport system must exist in the cells. Opsin molecules are synthesized in the proximal IS and are transported to the OS through the connecting cilium. As many mutations in the rhodopsin gene causing folding and transport defects, often lead to inherited retinal degeneration, retinitis pigmentosa (Sung et al., 1991, 1994; van Soest et al., 1999), the efficient folding of rhodopsin following its biosynthesis is an important process. Opsin protein folding may be assisted by various chaperone proteins specific to opsin, as well as nonspecific chaperones in rod cells (Baker et al., 1994;

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Chapple et al., 2001; Chapple and Cheetham, 2003). In the transport process, small GTP-binding proteins, such as rab6 and rab8, associate with rhodopsin-laden vesicles budding from the trans-Golgi network and regulate the fusion of the vesicles in the vicinity of the connecting cilium, together with ezrin/moesin, rac1, and other proteins (Deretic and Papermaster, 1993; Deretic, 1998; Moritz et al., 2001; Deretic et al., 2004). In addition, the C-terminal opsin sequence is required for delivering opsin to the post-Golgi membranes, and for interacting with the dynein light chain in the cytoplasmic dynein complex, which is responsible for the translocation of rhodopsin-bearing vesicles along microtubles (Deretic et al., 1998; Tai et al., 1999; Tam et al., 2000). However, it is not fully understood how rhodopsin is synthesized and translocated to the OS in an efficient manner.

A cytosolic green fluorescent protein (GFP), fused with the C-terminal eight residues of rhodopsin, becomes membrane-associated by the attachment of a lipid modification signal and is translocated specifically to the rod outer segment (ROS), as shown in *Xenopus laevis* photoreceptor cells (Tam et al., 2000). We were interested to see if membrane proteins, particularly other G protein-coupled receptors carrying the C-terminal rhodopsin sequence, whose secondary structure resembles that of rhodopsin, were able to mimic the biosynthesis and translocation of rhodopsin in the retinal rod cells. Therefore, we wanted to determine if the proteins could be synthesized efficiently, folded properly in the IS, and translocated to the OS specifically. In the present work, we tested the ectopic expression of another G protein-coupled receptor (GPCR) protein, human endothelin receptor subtype B (hET_BR), in rod cells under the control of the mouse opsin (mOPS) promoter/enhancer, and carried out comparative analyses with rhodopsin in mice.

The hET_BR is a type I GPCR, with relatively high sequence similarity to rhodopsin. It binds a 21 amino acid-long peptide, endothelin, and mediates various signals to regulate vasoconstriction, development, mitogenesis, cell proliferation, nociception, and other functions, mainly via coupling with the G_i and G_q proteins. Because hET_BR was expressed at a high level ($\sim$100 pmol/mg membrane protein) in insect Sf9 cells, we thought that it could be expressed as well as rhodopsin in the retinal rod cells (Doi et al., 1997). The hET_BR gene was fused with the sequence encoding the C-terminal 10 amino acids of rhodopsin at the C-terminus (hET_BR-CT), which was expressed at levels comparable to those of the wild-type hET_BR in Sf9 cells (Doi et al., 1999) and in COS-1 cells (Yamaguchi et al., unpublished work). This fusion was introduced into the opsin gene in the mouse genome by targeted replacement (referred to as 'knockin'). Here, we report the expression of the correctly-folded hET_BR-CT in the knockin retina, and describe its abundance and subcellular localization as compared with those of rhodopsin.

1. Materials and methods

All animal experiments were performed according to the guidelines of 'the Association for Research in Vision and Opthalmology', and were approved by Animal Care and Use Committee of Kyoto University.

1.1. Materials

The hET_BR cDNA was generously supplied by Dr T. Masaki (Kyoto University). Endothelin-1 (ET-1) and biotinyl-ET-1 were purchased from Peptide Institute (Osaka, Japan). [^{125}I]ET-1 (81·4 TBq/mmol) was purchased from Perkin–Elmer Life Sciences. Insect Sf9 cell membranes separately expressing hET_BR and $6hNET_BR$ (Doi et al., 1999) were kindly provided by Drs S. Nishimura and T. Yamaguchi (Kyoto University), respectively. The hybridoma producing the anti-bovine rhodopsin monoclonal antibody 1D4 was provided by Dr R. Molday (University of British Columbia). The anti-transducin α subunit antibody (rabbit) was purchased from DuPont NEN (AS/7) and SIGMA.

1.2. Gene targeted replacement

The targeting construct was made from the 129/SvJ genomic clone by replacing the sequence of exon 1 of the mOPS gene (from +97 to +431) with the hET_BR-CT gene and the neomycin-resistance gene (PGKneo) (Fig. 1A). The start codon of the hET_BR-CT gene was placed in the same position as the original start codon of the mOPS gene. The hET_BR-CT gene, composed of the hET_BR cDNA fused with the sequence corresponding to the C-terminal 10 amino acids (KTETSQVAPA) of rhodopsin (referred to as CT) immediately before the stop codon (Fig. 1B), was followed by 0·9 kb of the 3′ untranslated region of the mOPS gene containing three polyadenylation (poly(A)) signals (Fig. 2A). Mouse CJ7 embryonic stem cells were electroporated with the linearized targeting vector and were selected with G418 (200 μg/ml). Of 138 clones, 4 were identified as homologous recombinants by Southern blot analysis. One of them was microinjected into C57BL/6J blastocysts and the chimeric mice were produced. Heterozygotes were further crossed to produce homozygotes. All mice purchased were from Japan SLC, Inc. (Shizuoka, Japan). All following experiments were performed on the hET_BR-CT knockin mice and the wild-type littermates derived from chimeric mice, F1 or F2 C57BL/6J-129/SvJ mixed background litters. All mice were provided food and water ad lib, and were maintained in a constant 12 hr light and dark cycle.

1.3. Histology

Eyecups were fixed with 4% paraformaldehyde in 0·1 M phosphate buffer (pH 7·4) for 4 hr at 4°C,