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STRUCTURE-FUNCTION STUDIES OF BOVINE RHODOPSIN: INTERACTIONS WITH TRANSDUCIN[1]

Roland R. Franke, Thomas P. Sakmar, Robert M. Graham[2], and H. Gobind Khorana

Departments of Biology and Chemistry,
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139

ABSTRACT To elucidate the nature of the activation of transducin by rhodopsin, we have prepared a series of rhodopsin mutants by *in vitro* site-specific mutagenesis. The mutations involved amino acid substitutions or deletions in the cytoplasmic loops linking putative transmembrane helices and in the cytoplasmic carboxyl-terminal tail of rhodopsin. Three mutants in which charged amino acid residues in the cytoplasmic loop linking transmembrane helices E and F were replaced by neutral residues have been characterized. The mutant genes were expressed in monkey kidney (COS-1) cells and regenerated *in vivo* with exogenous 11-*cis* -retinal. The mutants were purified by immunoaffinity adsorption. Each of the mutants displayed a wild-type visible absorption spectrum. Mutant EF1 (Glu^{239}--Gln) and mutant EF3 (Glu^{247}--Gln, Lys^{248}--Leu, and Glu^{249}--Gln) were able to stimulate the GTPase activity of transducin. Mutant EF2 (Lys^{248}--Leu) was inactive with respect to transducin

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[2]Cellular and Molecular Research Laboratories, Cardiac Unit, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02114.

activation. These results are consistent with the involvement of cytoplasmic loop EF in transducin activation.

INTRODUCTION

In mammalian visual transduction, photoreceptor cells transduce a light stimulus into membrane hyperpolarization. The visual pigment rhodopsin is the major component of retinal rod outer segment (ROS) disc membranes. Photoisomerization of the rhodopsin chromophore, 11-*cis* -retinal, induces a conformational change in the protein which triggers an interaction with transducin (T), the rod outer segment G protein (guanine nucleotide-binding protein). The α-subunit (Tα) exchanges GDP for GTP and dissociates from rhodopsin and the $\beta\gamma$-subunits (T$\beta\gamma$). The interaction of rhodopsin with transducin is regulated, at least in part, by a soluble kinase which phosphorylates sites mainly in the carboxyl-terminal tail of rhodopsin.

In many hormone receptor systems, an analogous relationship exists between receptor and G protein. Furthermore, striking structural and functional similarities between the visual system and hormone-mediated signaling systems have been identified so that it has become clear that rhodopsin and transducin are members of a "superfamily" of related receptors and G proteins. The study of structure-function relationships involved in the interaction between membrane receptor and G protein is vital to the understanding of signal transduction. We have chosen to study the visual system as a model for transmembrane signal transduction. We employed techniques of recombinant DNA to prepare rhodopsin mutants to attempt to identify structural elements responsible for its interaction with transducin.

To facilitate site-specific mutagenesis by restriction fragment replacement, a synthetic gene for bovine rhodopsin was synthesized (1). The high level expression of this gene in mammalian cells (COS-1) was previously reported (2). The opsin expressed in COS-1 cells was shown to bind

11-*cis* -retinal which was added to the cells *in vivo* . The COS-1 cell rhodopsin was purified by immunoaffinity adsorption. The purified rhodopsin had a visible absorption spectrum indistinguishable from rhodopsin purified from retinas. The COS-1 cell rhodopsin stimulated the GTPase activity of transducin with the same specific activity as ROS rhodopsin (2).

According to currently accepted models of rhodopsin secondary structure and topography (3), the amino terminal tail is located on the intradiscal surface of the disc membrane, seven

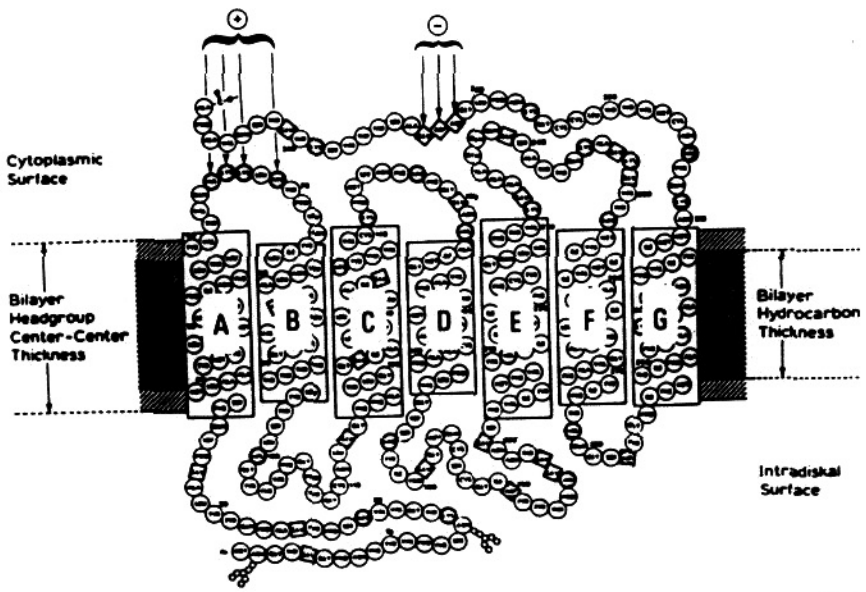

FIGURE 1. A model of rhodopsin structure with respect to the disc membrane according to Dratz and Hargrave (3). The seven putative transmembrane helical segments are labeled A-G. A series of rhodopsin mutants was prepared to identify domains on the cytoplasmic surface responsible for GTPase activation. Table 1 lists loop EF mutants. Table 2 lists loop AB and carboxyl tail (CT) mutants. As indicated, loop AB has a cluster of positively charged amino acids whereas the carboxyl tail contains three consecutive acidic residues.

helices (A-G) span the membrane bilayer, and the carboxyl terminal tail is on the cytoplasmic surface as shown in figure 1. Three classes of mutant rhodopsin genes are described in which the cytoplasmic domain had been altered: 1). loop EF mutants, 2). loop AB mutants, and 3). cytoplasmic tail (CT) mutants. To date three of the mutants have been characterized as to their ability to activate transducin: mutant EF1, Glu^{239}--Gln; mutant EF2, Lys^{248}--Leu; and mutant EF3, Glu^{247}--Gln, Lys^{248}--Leu, and Glu^{249}--Gln. Mutants EF1 and EF3 activated transducin similarly to wild-type rhodopsin, whereas mutant EF2 failed to activate transducin (4).

RESULTS AND DISCUSSION

Expression of a Synthetic Rhodopsin Gene in COS-1 Cells.

Our goal has been to develop a system in which techniques of molecular biology can be applied to structure-function studies of the proteins of the visual cascade. We aim to reconstitute purified proteins containing specific mutations and perform a number of *in vitro* functional assays. Toward this goal, a synthetic gene for bovine rhodopsin was synthesized (1). The gene was expressed in COS-1 cells. After addition of 11-*cis* -retinal *in vivo*, rhodopsin was purified by an immunoaffinity procedure in which detergent solubilized cells were incubated with an anti-rhodopsin monoclonal antibody. Bound protein was then eluted with a synthetic peptide corresponding to the epitope of the antibody. The COS-1 cell rhodopsin was compared to ROS rhodopsin. Electrophoretic mobility of the COS-1 cell rhodopsin was slightly less than that of ROS rhodopsin due to different extents of glycosylation. The visible absorption spectra and specific activities of GTPase activation were nearly identical (2).

Characterization of Rhodopsin Mutants.

Using this system, we have prepared a number of mutants designed to answer the question, what

are the structural elements of rhodopsin responsible for the light-dependent activation of transducin. Existing results from proteolysis experiments of rhodopsin (5) and from mutagenesis of the structurally related β-adrenergic receptor (6) supported a role for cytoplasmic loop EF in transducin activation. Furthermore, a cluster of charged amino acid residues near the beginning of helix F seemed to be a common feature of the family of receptors that activate G proteins. The first three mutants prepared, mutants EF1-3 in Table 1, were designed to neutralize some of these charges.

TABLE 1

RHODOPSIN CYTOPLASMIC LOOP EF MUTANTS

	230 240 250	
WILD-TYPE	- V K E A A A Q Q Q E S A T T Q K A E K E V T R -	WILD-TYPE
MUTANT		MUTANT
1	Q	1
2	L	2
3	Q L Q	3
4	Q L	4
5	L Q	5
6	Q — Q	6
7	L	7
8	A — V V	8
9	L — L	9
10	← DEL →	10
11	← DEL →	11
12	← DEL →	12

Mutant genes were prepared by replacing a restriction fragment in the synthetic rhodopsin gene by a synthetic duplex containing the desired codon alteration(s). Mutations were confirmed by dideoxy DNA sequencing. The cloning of the synthetic gene into the expression vector pMT-2 (7) has been described (4). The rhodopsin mutants were expressed in COS-1 cells and purified.

The mutants bound 11-*cis* -retinal to give wild-type absorption spectra. Whereas mutants EF1 and EF3 were able to activate transducin normally, mutant EF2 (Lys^{248}--Leu) was inactive as shown in figure 2 (4). The same Lys^{248}--Leu substitution, when combined with Glu^{247}--Gln and Glu^{249}--Gln substitutions as in mutant EF 3, produced a mutant capable of activating rhodopsin. These results are

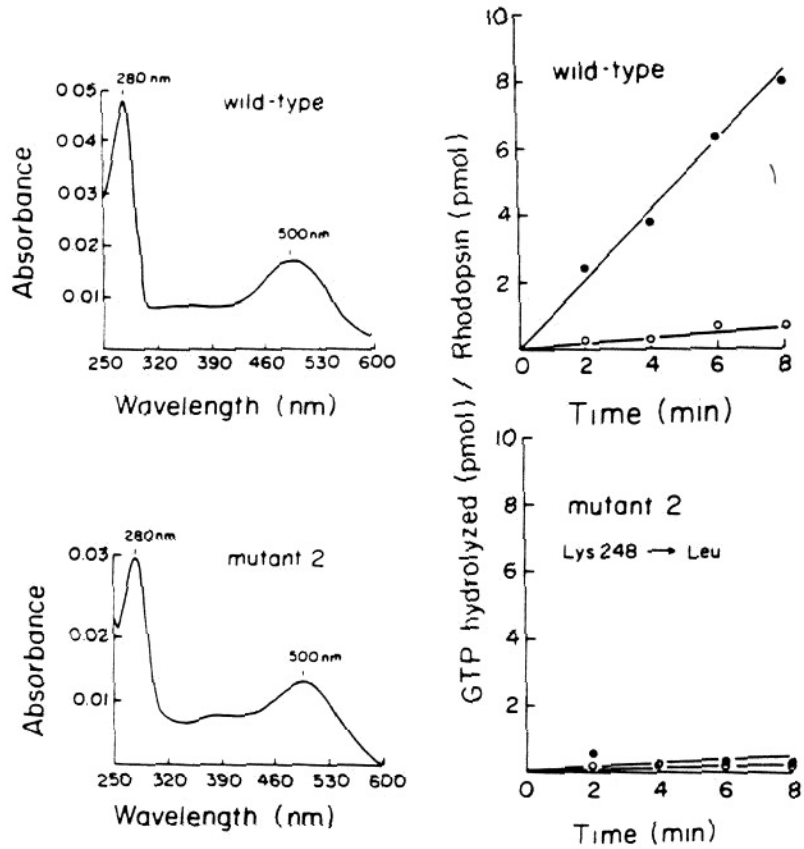

FIGURE 2. Rhodopsin mutant EF2 displayed a visible absorption spectrum identical to wild-type, but failed to activate the GTPase activity of transducin. Wild-type and mutant rhodopsins were purified from COS-1 cells. Activity assays were performed in the dark (○) and in room light (●).

TABLE 2

RHODOPSIN CYTOPLASMIC LOOP AB AND CARBOXYL TAIL MUTANTS

WILD-TYPE	$-\overset{60}{Y}\ V\ T\ V\ Q\ H\ K\ K\ L\ R\ \overset{70}{T}\ P\ L-$	WILD-TYPE
MUTANT		MUTANT
AB 1	——— Y G G — Q ———	AB 1
AB 2	——— D E E — E ———	AB 2
WILD-TYPE	$-N\ P\ \overset{330}{L}\ G\ D\ D\ E\ A\ S\ T\ T-$	WILD-TYPE
MUTANT		MUTANT
CT 1	- ——— N N N ———	CT 1
CT 2	——— K K K ———	CT 2
MUTANT		MUTANT
AB 2/CT 2	$-\overset{60}{Y}\ V\ T\ V\ Q\ D\ E\ E\ L\ E\ \overset{70}{T}\ P\ L-$ $-N\ P\ \overset{330}{L}\ G\ K\ K\ K\ A\ S\ T\ T-$	AB 2/CT 2

consistent with the involvement of loop EF in transducin interaction. Definitive conclusions regarding the exact nature of this involvement cannot be proposed from these results alone. The interaction between transducin and rhodopsin loop EF may be direct, or indirect through another cytoplasmic loop or the carboxyl-terminal tail.

A series of additional loop EF mutants have been prepared as shown in Table 1. These mutants were designed to clarify the role of the net charge in the portion of the loop ear helix F implied by the results of the first 3 mutations. The possible role of loop EF phosphorylation will also be addressed as will the question of whether or not loop EF deletions affect retinal binding or transducin activation. Mutants in loop AB and the carboxyl-terminal tail have also been prepared as shown in Table 2. These mutants have been designed to investigate the possibility of an interaction between the cluster of basic amino acid residues in loop AB and a cluster of acidic

residues in the carboxyl tail. Such an interaction may alter accessibility of loop EF to transducin binding or phosphorylation.

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