dependent on expression of both hB-AR and rat  $G_s \alpha$  and required a yeast strain in which the endogenous G protein  $\alpha$  subunit was disrupted (5). The final  $\beta$ -galactosidase activity achieved in response to isoproterenol in transformed 8c1 cells was comparable to that induced by  $\alpha$  factor in nontransformed cells that express GPA1 (strain NNY19)—although basal β-galactosidase activity in NNY19 cells was considerably lower than in 8cl cells. Taken together, our results indicate that coexpression of hB-AR and rat  $G_s \alpha$  is sufficient to place key aspects of the mating signal transduction pathway in yeast under catecholamine control. However, the  $\beta$ -AR agonist did not stimulate mating in either 8c cells or NNY19 cells coexpressing h $\beta$ -AR and rat G<sub>s</sub> $\alpha$  (5)—in agreement with recent observations that yeast pheromone receptors, in addition to binding pheromones, participate in other recognition events required for mating (8).

The hβ-AR stimulates adenylyl cyclase in animal cells via the action of  $G_s\alpha$ . In contrast, mating factor receptors in yeast trigger their effector via the action of the G protein  $\beta\gamma$  subunits (9). Our results indicate that activation of h\beta-AR in yeast leads to dissociation of mammalian  $G_s \alpha$  from yeast G protein  $\beta\gamma$  subunits, and it is the  $\beta\gamma$  subunits that presumably elicit the response.

The ability to control the yeast pheromone response pathway by expression of a heterologous adrenergic receptor and its cognate G protein  $\alpha$  subunit may facilitate structural and functional characterization in yeast of mammalian G protein-coupled receptors. By scoring for growth arrest or  $\beta$ galactosidase induction, the functional properties of mutant receptors can now be rapidly tested. Similarly, as additional genes for putative G protein-coupled receptors are isolated (10, 11), numerous ligands can be screened to identify those with activity toward the unidentified receptors. Finally, our results underscore the conservation of function between analogous receptor and receptor-coupling components of signal transduction pathways in yeast and mammals.

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## Rhodopsin Mutants That Bind But Fail to Activate Transducin

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Rhodopsin is a member of a family of receptors that contain seven transmembrane helices and are coupled to G proteins. The nature of the interactions between rhodopsin mutants and the G protein, transducin (Gt), was investigated by flash photolysis in order to monitor directly Gt binding and dissociation. Three mutant opsins with alterations in their cytoplasmic loops bound 11-cis-retinal to yield pigments with native rhodopsin absorption spectra, but they failed to stimulate the guanosine triphosphatase activity of Gt. The opsin mutations included reversal of a charged pair conserved in all G protein-coupled receptors at the cytoplasmic border of the third transmembrane helix (mutant CD1), replacement of 13 amino acids in the second cytoplasmic loop (mutant CD2), and deletion of 13 amino acids from the third cytoplasmic loop (mutant EF1). Whereas mutant CD1 failed to bind G<sub>t</sub>, mutants CD2 and EF1 showed normal Gt binding but failed to release Gt in the presence of guanosine triphosphate. Therefore, it appears that at least the second and third cytoplasmic loops of rhodopsin are required for activation of bound G<sub>t</sub>.

HOTOACTIVATED RHODOPSIN, METArhodopsin II (MII), binds to G<sub>t</sub> and catalyzes guanosine diphosphate (GDP)-guanosine triphosphate (GTP) exchange (1). The GTP-bound form of the  $\alpha$ subunit of Gt then dissociates from MII and interacts with a guanosine 3',5'-monophosphate (cyclic GMP) phosphodiesterase, a process that ultimately results in the generation of a neural signal (2). Evidence suggests that the cytoplasmic surface of rhodopsin (Fig. 1A) participates in Gt binding and activation. The sites of rhodopsin that interact with G<sub>t</sub> have been proposed to include loop CD (3), loop EF (4, 5), or a combination of these loops, as well as a third loop formed by the COOH-terminal tail (Fig. 1A) (6). To

further study the specificity of interaction between rhodopsin and G<sub>t</sub>, we prepared rhodopsin mutants in which cytoplasmic loops CD or EF were altered by amino acid replacements or deletions (Fig. 1B). In mutant CD1, a charged pair (Glu<sup>134</sup>Arg<sup>135</sup>) was mutated to Arg<sup>134</sup>Glu<sup>135</sup> (7). A GluArg or AspArg charged pair is conserved at the cytoplasmic border of helix C in all known G proteincoupled receptors. In mutant CD2, a segment of 13 amino acids (residues 140 to 152) in loop CD was replaced by an unrelated sequence of equal length. A 14-amino acid segment (residues 137 to 150) was deleted from loop CD to form mutant CD3, and a 13-amino acid segment (residues 237 to 249) was deleted from loop EF to form mutant EF1. The mutant opsin genes were expressed in COS-1 cells, regenerated with 11-cis-retinal, and purified in detergent solution by an immunoaffinity procedure (4, 8).

Ultraviolet (UV)-visible spectroscopy and in vitro guanosine triphosphatase (GTPase) assays were performed on these opsin mutants (Table 1). Mutant CD3 did not bind

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11-cis-retinal and, thus, did not yield a chromophore. Mutants CD1, CD2, and EF1 formed pigments with absorption spectra indistinguishable from that of native rhodopsin [wavelength of maximal absorption  $(\lambda_{max}) = 500 \text{ nm}$  and showed the characteristic shift in  $\lambda_{max}$  to 380 nm ( $\lambda_{max}$  of MII) on illumination. However, these mutants did not activate Gt, as assayed by lightdependent inorganic phosphate (Pi) release. A mutation resulting in a loss of light-induced GTPase activity in this assay might be attributable to a defect in MII formation or to the formation of an MII-like species that either failed to bind Gt or that bound Gt but failed to induce the guanine nucleotide exchange necessary for the activation process.

To identify the specific defect that prevented light-induced activation of transducin, we studied the rhodopsin mutants by flash photolysis under conditions that allowed the distinct monitoring of MII formation, Gt binding, and Gt activation and release (9, 10) (Fig. 2). Mutant CD1 formed an MII species that failed to bind Gt. Mutants CD2 and EF1 formed an additional amount of MII species ("extra MII") in the presence of G<sub>t</sub> to the same degree as wild-type rhodopsin, indicating unaltered binding to Gt. However, neither CD2 nor EF1 allowed dissociation of the MII-G<sub>t</sub> complex in the presence of GTP, indicating that a defect existed in the G<sub>t</sub> activation pathway. Thus, Gt binding was uncoupled from Gt release in mutants CD2 and EF1.

Thus, the failure of mutant CD1 to bind  $G_t$  may indicate that a direct interaction exists between the Glu<sup>134</sup>Arg<sup>135</sup> charged pair and

Fig. 1. (A) Structural model of rhodopsin (20). The seven putative transmembrane helices (A through G) are represented by cylinders. Cytoplasmic loops linking the successive helices are designated AB, CD, and EF. A fourth putative loop is formed by a portion of the COOHterminal tail between helix G and a pair of palmitoylated cysteines (21). The cytoplasmic surface of rhodopsin interacts with soluble Gt. The charged pair Glu<sup>134</sup>Arg<sup>135</sup> has been proposed to form the cytoplasmic border of transmembrane helix C (7) 22). (B) Amino acid sequences of loops CD and EF in bovine opsin (23) and the corresponding mutants. In mutant CD1, the charged pair Glu<sup>134</sup>Arg<sup>135</sup> was reversed. Because mutant CD3, a deletion of 14 amino acids (residues 137 to 150), failed to bind 11-cis-retinal and yield a chromophore, mutant CD2 was prepared in which 13 amino acids (residues 140 to 152) were replaced by a sequence derived from the NH2terminal tail region (residues 2 to 14) of rhodopsin. Mutant EF1 contained a deletion of 13 amino acids (residues 237 to 249). Site-directed mutagenesis was performed on a synthetic opsin gene

Fig. 2. Flash-induced formation of MII and Gtdependent stabilization of MII. Flash photolysis experiments were performed according to a dualwavelength spectrophotometric method (6, 27). Results are for purified COS cell rhodopsin, CD1, CD2, and EF1. Three traces are shown for each pigment: a, pigment alone; b, pigment plus Gt; and c, pigment plus Gt plus GTP. COS cell rhodopsin produced a small amount of MII on illumination. In the presence of G<sub>t</sub>, the amount of MII increased because Gt bound MII to shift the MI-MII equilibrium toward MII. In the presence of Gt and GTP, activated Gt dissociated from MII so that the MI-MII equilibrium was unaffected and no additional MII was observed. Mutant CD1 formed a spectrally normal MII species that



did not bind to  $G_t$  to stabilize MII. Mutants CD2 and EF1 both formed MII species that were stabilized by  $G_t$  binding so that extra MII signals were observed. However, GTP did not cause the release of  $G_t$ and the extra MII signal persisted. Traces show the formation of MII in arbitrary absorbance units as a function of time. The small negative deflection in the trace at the flash is due to formation of early lightinduced products including MI (28).

Gt. However, this charged pair may help other cytoplasmic sites to form the Gt binding domain. Because mutations in loop CD or EF allowed a stable MII-Gt complex to form but prevented G<sub>t</sub> activation, it is likely that these two loops are part of a Gt activation site on the cytoplasmic surface of rhodopsin. Our results are consistent with peptide competition studies on native rhodopsin showing that any two of three cytoplasmic loops (loop CD, loop EF, or the loop consisting of the proximal portion of the COOH-terminal tail) were sufficient to bind  $G_t$  (6). This implies that at least one interaction site exists on each of these loops. An indirect effect of mutagenesis that causes a conformational perturbation or alteration of a posttranslational modification cannot be excluded. However, formation of MII is extremely sensitive to changes in the



(24) cloned into a modified version of the mammalian expression vector, pMT-2 (4). All mutants were prepared by restriction fragment replacement ("cassette") mutagenesis (25). Cloned synthetic DNA sequences were confirmed by the dideoxy chain termination method of DNA sequencing (26). Abbreviations for the amino acid residues are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

molecular environment of rhodopsin (10, 11) and should therefore act as an indicator of overall rhodopsin structure. Thus, a significant conformational perturbation is improbable because each mutant displayed a normal  $\lambda_{max}$  and formed a normal amount of MII with the same kinetics. We conclude that (i) binding of G<sub>t</sub> and stabilization of MII is not sufficient for G<sub>t</sub> activation and (ii) activation of bound G<sub>t</sub> requires at least two sites (loops CD and EF) on the cytoplasmic surface of rhodopsin.

The state of the nucleotide binding site of

Table 1. Characteristics of mutant rhodopsins. Opsin apoproteins were expressed in transiently transfected COS cells (8). Harvested cells were incubated with 11-cis-retinal (10 µM) to regenerate pigments. The pigments were solubilized in dodecyl maltoside, purified according to an immunoaffinity procedure (8, 18), and subjected to UV-visible spectroscopy and transducin activa-tion assays (7). The  $\lambda_{max}$  was determined in 0.1% dodecyl maltoside (pH 7.1) at 20°C. On illumination with light of greater than 495-nm wavelength, each of the pigments was converted to an MII species absorbing at 380 nm. However, none of the mutant rhodopsins stimulated the GTPase activity of Gt. GTPase activity refers to the lightdependent release of Pi in a system consisting of purified pigment, purified bovine Gt, and GTP  $(SE \le 10.7\%, n = 20)$  (7). The values used for extinction coefficients of rhodopsin and the mutant pigments were 42,700  $M^{-1}$  cm<sup>-1</sup> (19). The specific activity of rhodopsin was 61.6 ± 20.4 pmol of Pi released per picomole of pigment per minute (mean  $\pm$  SE, n = 6). The GTPase activity values are normalized to the activity of rhodopsin purified from COS cells in parallel with the mutant pigments. Mutant opsin CD3 did not bind 11-cis-retinal to yield a chromophore.

Rho- dopsin	$\begin{array}{c} \text{Pigment} \\ \lambda_{\max} \\ (\text{nm}) \end{array}$	GTPase activity (normalized)
Wild type	500	1.00
EFI	500	0.004
CD1	500	0.009
CD2	500	0.012

Gt, which binds to mutants CD2 and EF1, is not measured directly in these experiments. Hypothetically, the G<sub>t</sub> nucleotide binding site may be unoccupied or may contain GDP or GTP. We find it likely that the GDP form of Gt binds to photoactivated rhodopsin, and that the release of GDP then allows MII stabilization (12). The activation step that is blocked in mutants CD2 and EF1 may be the signal that induces formation of the GTP binding pocket in Gt. Thus, we speculate that mutants CD2 and EF1 bind to Gt that is nucleotide free.

Many receptors that couple to G proteins have been identified, and deciphering the mechanism of G protein activation is central to an understanding of G protein-mediated signal transduction. Mutants of the  $\beta_2$ -adrenergic receptor ( $\beta$ -AR), which is structurally related to rhodopsin (13), have been studied in whole-membrane preparations with steadystate agonist binding assays. A 34-amino acid deletion from the third cytoplasmic loop (EF) of  $\beta$ -AR produced a receptor with a single high-affinity agonist state, which did not activate adenylate cyclase (14, 15). The interpretation of this result was that the mutant receptor was uncoupled from the G protein, G<sub>s</sub>. A seven-amino acid deletion in the same loop (EF) of  $\beta$ -AR results in a moderate impairment of the adenylate cyclase response and a single high-affinity agonist state (16). It was concluded that this receptor domain in loop EF might participate in the transmission of an agonist-induced stimulatory signal to G<sub>s</sub>. Our direct demonstration of an inactive mutant rhodopsin-Gt complex supports the interpretation that these deletion mutants of  $\beta$ -AR bound G<sub>s</sub> to form a complex with impaired activity. Furthermore, the combined results of our and other studies (14-17) suggest that the  $G_s$ -induced high-affinity agonist state of  $\beta$ -AR is analogous to the G<sub>t</sub>-induced stabilization of MII. The correlation between rhodopsin and  $\beta$ -AR might be tested further by purifying mutant  $\beta$ -ARs and studying agonist binding affinity and the stimulation of G<sub>s</sub> in artificial vesicles. Thus, the study of rhodopsin-Gt interactions may provide general information regarding transmembrane signaling, and our direct spectroscopic assay of purified rhodopsin mutants should allow a more detailed analysis of discrete steps in the G protein activation pathway.

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- Solubilization of rhodopsin in steroid-containing micelles allows MII formation, Gt binding, and G activation and release to be monitored separately (1, 10). The light-induced intermediates, metarhodopsin I (MI) and metarhodopsin II (MII), exist in equilibrium, and binding of  $G_t$  to MII shifts the equilibrium toward MII. The amount of MII- $G_t$ complex and, therefore, the binding of  $G_t$  to MII can be quantitated by measuring the G<sub>r</sub>-dependent shift from MI ( $\lambda_{max} = 480$  nm) to MII ( $\lambda_{max} = 380$  nm). The resulting MII-G<sub>t</sub> complex remains stable for minutes in the absence of GTP and the kinetics of its formation and steady-state concentration can be determined. However, in the presence of GTP, MII stimulates the exchange of GDP for GTP on  $G_t$ . The GTP-bound form of the  $\alpha$ subunit of  $G_t$  then dissociates from MII and the equilibrium shifts back to MI [D. Emeis and K. P. Hofmann, FEBS Lett. 136, 201 (1981)]. 10. A. Schleicher, R. Franke, K. P. Hofmann, H. Fin-
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- 28 Transiently transfected COS-1 cells (~108) treated with 11-cis-retinal were solubilized in buffer A [7 mM deoxylysolecithin, 5 mM cortisone, 10 mM Hepes (pH 8), and 1 mM CaCl<sub>2</sub>] supplemented with 1 mM phenylmethylsulfonyl fluoride at room temperature for 1 hour. Insoluble material was separated by centrifugation at 100,000g for 30 min at 20°C, and pigment was purified by an immunoaf-finity procedure (8), with the exception that washing and peptide elution were performed in buffer A. UV-visible spectra of rhodopsin and mutants CD1, CD2, and EF1 were identical (visible  $\lambda_{max} = 500$ nm). In the flash photolysis experiments, the effective path length was 0.7 cm on 0.8 ml of sample that contained pigment (200 nM),  $G_t$  (40 nM), and GTP (20  $\mu$ M) in buffer A. Each light flash (520 ± 15 nm) bleached 5% of the pigment present. The formation of MII was measured as the difference between absorbances at 380 nm and 417 nm (the isosbestic point between MI and MII). Experiments were performed at 4°C, but for each sample, a separate experiment was performed with pigment alone at 20°C. At the higher temperature, the normal extent (60%) of conversion to an MII species was observed for each mutant tested. COS cell rhodopsin was identical to both washed disk membrane and purified solubilized bovine rhodopsin with respect to MII formation, extra MII formation in the presence of G<sub>t</sub>, and the absence of the extra MII signal in the presence of G<sub>t</sub> and GTP (R. R. Franke, B. König, T. P. Sakmar, H. G. Khorana, K.
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## **Technical Comments**

## Inhibition of HIV-1 Infectivity by **Phosphate-Methylated DNA: Retraction**

We recently reported (1) that phosphatemethylated 20-nucleotide DNA oligomers are able to inhibit human immunodeficiency virus type-1 (HIV-1) infectivity through hybridization to the viral RNA or integrated viral DNA. The oligomers we used to test the biological activity of phosphatemethylated DNA with regard to the inhibition of HIV-1 infectivity were synthesized according to the method of Moody et al. (2) in which methylation of natural oligomers is achieved by a three-step process.

After the publication of our report (1), we analyzed our original samples of phosphatemethylated DNA with reversed-phase C18 and strong anion exchange (SAX) highperformance liquid chromatography (HPLC). Analysis by reversed-phase C18 HPLC is largely based on a difference in polarity

between compounds, while the elution order in SAX HPLC is dictated for the most part by the negative charge of the compounds. Therefore, completely or partially phosphate-methylated DNA oligomers are expected to elute before their natural DNA counterparts in SAX HPLC, while for the reversed-phase C18 HPLC the order of elution is reversed. A reversed-phase C18 chromatogram and a SAX chromatogram of a sample of the phosphate-methylated antisense -) NEF sequence are shown in Fig. 1.

We had assumed that the first peak in the SAX profile represented the completely phosphate-methylated oligonucleotide sequence. However, treatment of part of the sample with tert-butylamine/water (1:1 v/v) for 16 hours at 50°C [which causes complete demethylation of methyl phosphotri-

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