A G Protein–Coupled Receptor Phosphatase Required for Rhodopsin Function

Javier Vinós, Kees Jalink, Robert W. Hardy, Steven G. Britt,* Charles S. Zuker†

Heterotrimeric guanine nucleotide-binding protein (G protein)–coupled receptors are phosphorylated by kinases that mediate agonist-dependent receptor deactivation. Although many receptor kinases have been isolated, the corresponding phosphatases, necessary for restoring the ground state of the receptor, have not been identified. *Drosophila* RDGC (retinal degeneration C) is a phosphatase required for rhodopsin dephosphorylation in vivo. Loss of RDGC caused severe defects in the termination of the light response as well as extensive light-dependent retinal degeneration. These phenotypes resulted from the hyperphosphorylation of rhodopsin because expression of a truncated rhodopsin lacking the phosphorylation sites restored normal photoreceptor function. These results suggest the existence of a family of receptor phosphatases involved in the regulation of G protein–coupled signaling cascades.

G protein–coupled receptors (GPCRs) mediate responses to a wide range of extracellular stimuli. These receptors contain seven putative transmembrane domains and clustered serine and threonine residues in the cytoplasmic COOH-terminus (*1*). The β-adrenergic receptor (*2*) and the light receptor rhodopsin (*3*) are GPCRs that share common desensitization and inactivation mechanisms. Ligand- or light-dependent activation of these receptors results in the activation of a GPCR kinase (GRK) that phosphorylates several residues in the COOH-terminus of the receptor (*4*). Phosphorylation results in a slight decrease in receptor activity, but also causes the receptor to become a high-affinity substrate for arrestin (*5–9*). Arrestin binding terminates the active state of the receptor by preventing its coupling to G protein (*5, 6, 10*). Although receptor dephosphorylation is essential for completing the signaling cycle (*11, 12*), the molecular identification of a GPCR phosphatase has proven difficult (*12*). We have used *Drosophila* phototransduction, a phospholipase C–mediated, calcium-regulated G protein–coupled pathway for a genetic dissection of GPCR function and regulation (*13*).

Several mutations that lead to light-dependent retinal degeneration have been isolated in *Drosophila* (*14–16*). Most of these define genes important for photoreceptor cell signaling, suggesting that photoreceptor cell integrity depends on the normal functioning of this pathway (*17*). The *rdg*C (retinal degeneration C) gene encodes an unusual type of serine-threonine phosphatase, consisting of an NH_2-terminal domain that has high sequence similarity to the catalytic domain of protein phosphatases 1, 2A, and 2B and a COOH-terminal domain containing multiple EF-hand calcium-binding motifs (*18*). On the basis of its primary structure, RDGC has been proposed to be a calcium-regulated phosphatase (*18*) and has been shown to be required for efficient dephosphorylation of rhodopsin in vitro (*19*).

To determine whether RDGC functions in vivo as a rhodopsin phosphatase, we analyzed *rdg*C mutants biochemically, physiologically, and genetically. We examined the light-dependent phosphorylation of rhodopsin in wild-type and *rdg*C mutant photoreceptor neurons. Unlike vertebrate opsin, most invertebrate photopigments are not bleached after light activation but can be photoconverted between the rhodopsin (R) form and a thermally stable, active metarhodopsin (M) form (*20*). The major rhodopsin in the fly retina, rhodopsin 1 (Rh1), has R and M forms that absorb light maximally at 480 nm (blue) and 580 nm (orange), respectively (*21*). Thus, blue or orange light can be used to shift between the active and inactive states of rhodopsin.

As expected, in white-eyed control flies, Rh1 was phosphorylated in a blue light–dependent manner, whereas subsequent exposure to orange light promoted its dephosphorylation (Fig. 1, A, B, and C). In contrast, Rh1 was hyperphosphorylated in *rdg*C mutants, and it remained in this state even after exposure to orange light (Fig. 1B). This result is consistent with the loss of a major rhodopsin phosphatase activity in *rdg*C mutant photoreceptor cells. No other proteins displayed altered light-dependent phosphorylation profiles in *rdg*C flies (*22*).

Because most GPCRs are phosphorylated by GRKs at a series of serine and threonine residues on the COOH-terminal tail of the receptor (*23*), we predicted that the lack of a GPCR phosphatase would lead to hyperphosphorylation of the COOH-terminal region of rhodopsin. Thus, we generated transgenic flies that express a truncated rhodopsin molecule, *Rh1Δ356*. This mutation eliminates the last 18 amino acid residues that include the serines and threonines in the Rh1 cytoplasmic tail. This truncated rhodopsin was expressed in a *ninaE* mutant background such that the only rhodopsin present in these photoreceptors was the one directed by the transgene (Fig. 1B, bottom panel). The truncated receptor

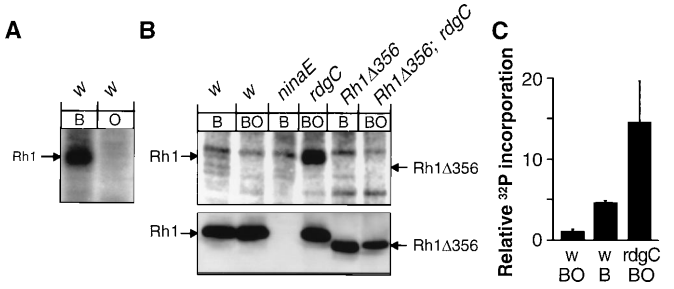

Fig. 1. In vivo phosphorylation of retinal proteins. (**A**) Autoradiogram of SDS–polyacrylamide gel electrophoresis (PAGE) of $^{32}PO_4$ in vivo labeled retinal proteins from flies that were exposed to blue light (B) or orange light (O) for 15 min (*37*). Blue light promoted phosphorylation of Rh1, whereas orange light promoted dephosphorylation (*w*, white-eyed control flies). (**B**) The experiment in (A) was repeated with a 20-s pulse of light to quantitatively examine the *rdgC* phenotype. Upper panel: Autoradiogram of SDS-PAGE of $^{32}PO_4$ in vivo labeled retinal proteins. B denotes flies exposed to 20 s of blue light; BO denotes flies exposed to 20 s of blue light followed by 20 s of orange light (*37*). *ninaE* represents a null mutation in the structural gene for Rh1, *rdgC* is a mutation in the RDGC phosphatase gene, and *Rh1Δ356* is the truncation of the last 18 residues of the COOH-terminal tail of rhodopsin. The results are representative of three independent experiments. Lower panel: The same gel blotted and probed with antibodies to Rh1. The truncation of rhodopsin results in a faster migrating polypeptide. (**C**) Histogram of the relative extent of ^{32}P incorporation into Rh1. Samples were normalized to control flies exposed to blue and orange light sequentially (BO) and corrected for the amount of rhodopsin loaded. Data are means ± SEM of triplicate determinations.

Howard Hughes Medical Institute and Departments of Biology and Neuroscience, University of California at San Diego, La Jolla, CA 92093, USA.

*Present address: Institute of Biotechnology and Department of Molecular Medicine, University of Texas Health Science Center, San Antonio, TX 78245, USA.
†To whom correspondence should be addressed. E-mail: czuker@ucsd.edu

was expressed in near normal amounts, and the cells displayed normal light responses (*24*). As predicted, rhodopsin was not hyperphosphorylated in the *Rh1Δ356; rdg*C flies (Fig. 1B).

The finding that Rh1 was hyperphosphorylated in *rdg*C mutants suggested that improper rhodopsin function may underlie the retinal degeneration phenotype. Some forms of light-dependent retinal degeneration in *Drosophila* are the result of uncontrolled signaling and thus are suppressed by second-site mutations that prevent signaling. For instance, light-dependent retinal degeneration induced by a mutation in arrestin (*Arr2*) is suppressed by a null mutation in the effector phospholipase C (encoded by *norp*A) (*16*). Like arrestin mutants, the *rdg*C degeneration is completely light-dependent (Fig. 2, A and B) because mutant flies that are grown in the dark have morphologically normal photoreceptor cells (*15*). However, this degeneration is not prevented by *norp*A, whereas it is suppressed by loss-of-function mutations in rhodopsin (*25*) or mutations that reduce rhodopsin amounts (*26*). Thus, *rdg*C acts at or downstream of rhodopsin, but upstream of *norp*A.

Two lines of evidence demonstrate that hyperphosphorylated rhodopsin is the cause of the light-dependent retinal degeneration. We genetically mapped the site of action of RDGC by generating double mutants between *rdg*C and other components of the phototransduction cascade. In *Drosophila*, photoconversion of rhodopsin activates a Gαq, encoded by the *Dgq* gene, which in turn stimulates a phospholipase C. A mutation in *Dgq* (*27*) does not protect *rdg*C flies from retinal degeneration, because *Dgq; rdg*C double mutants still degenerate (Fig. 2, C and D). This indicates that degeneration does not require the activation of components immediately downstream of rhodopsin. We also examined the effect of the *Rh1Δ356* transgene on light-induced, *rdg*C-dependent retinal degeneration. *Rh1Δ356* by itself does not affect retinal morphology (Fig. 2E). However, if the COOH-terminal hyperphosphorylation of Rh1 is directly responsible for retinal degeneration in *rdg*C, then elimination of the phosphorylation target sites in *Rh1Δ356; rdg*C double mutants should prevent the degeneration. Indeed, *Rh1Δ356* acts as a strong suppressor of *rdg*C degeneration (Fig. 2F).

In wild-type photoreceptor cells, termination of the light response results from the concerted action of regulatory events at multiple steps (*3*, *11*, *17*, *28*). Because *rdg*C mutants accumulate phosphorylated rhodopsin, we expected alterations in the kinetics of photoreceptor cell inactivation. Examination of photoreceptor light responses by electroretinograms (ERGs) and intracellular recordings showed that *rdg*C mutants exhibited a notable decrease in the rate of deactivation relative to control flies (Fig. 3, A and B). The deactivation rate of *rdg*C mutant cells was less than 10% the rate of control cells (time to 85% deactivation t_{85} = 1.78 s versus 0.17 s; Fig. 3C). If the deactivation defect of *rdg*C is attributable to the hyperphosphorylation of the serine and threonine residues in the COOH-terminal tail of rhodopsin, then the

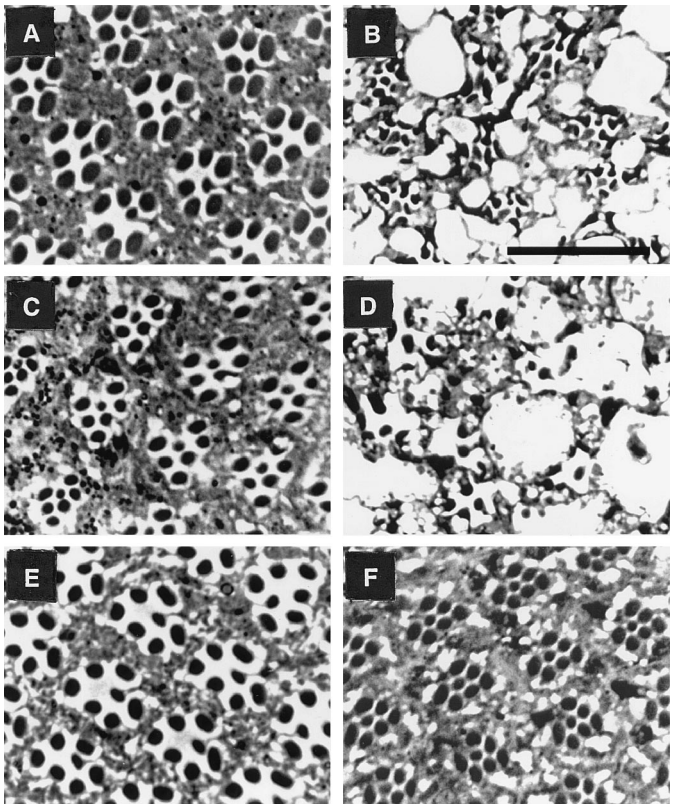

Fig. 2. *Rh1Δ356* suppresses *rdgC* light-dependent retinal degeneration. Shown are cross sections (1 μm thick) through adult retinas after 6 days of light exposure (*38*). (**A**) Control flies showing normal retinal morphology, with ommatidial clusters organized as a patterned array. (**B**) Retinas from *rdgC* flies show marked degeneration. (**C**) *Dgq* mutants that lack the α subunit of the G protein that couples rhodopsin to PLC do not show light-dependent degeneration. (**D**) *Dgq* is unable to suppress the degeneration of the *Dgq; rdgC* double mutants. (**E**) *Rh1Δ356* transgenic flies display normal retinal morphology. (**F**) *Rh1Δ356* drastically suppresses *rdgC* degeneration in *Rh1Δ356; rdgC* double mutants. If kept in the dark, all genotypes display normal retinal morphology (*39*). Scale bar, 40 μm.

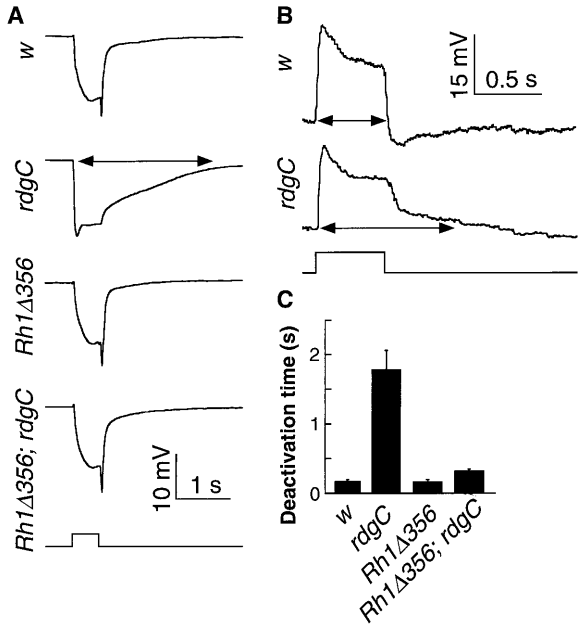

Fig. 3. *rdgC* mutants have defective deactivation kinetics. (**A**) ERGs of *w*, *rdgC*, *Rh1Δ356*, and *Rh1Δ356; rdgC* mutant flies (*40*). Photoreceptors were given a 0.5-s pulse of orange light. *rdgC* mutants display strong defects in the deactivation kinetics (arrow), and this phenotype is suppressed by the *Rh1Δ356* truncation. Traces from 15 independent measurements were averaged. (**B**) Intracellular recordings of light-activated responses from control and *rdgC* photoreceptors. This recording configuration demonstrates the photoreceptor specificity of the phenotype; note the strong deactivation phenotype (arrow). For control flies, t_{50} = 34 ± 2 ms; for *rdgC* flies, t_{50} = 136 ± 22 ms (n = 26). Data are means ± SEM. (**C**) Histogram of the deactivation time for the different ERG phenotypes. For control flies, t_{85} = 0.17 ± 0.03 s; for *rdgC* flies, t_{85} = 1.8 ± 0.3 s; for *Rh1Δ356* flies, t_{85} = 0.16 ± 0.03 s; for *Rh1Δ356; rdgC* flies, t_{85} = 0.32 ± 0.03 s (n = 15). Data are means ± SD.

Rh1Δ356 truncation should restore normal physiology to the mutant cells. Recordings from control flies expressing *Rh1Δ356* showed that these photoreceptors displayed normal kinetics of activation and deactivation. As predicted, *Rh1Δ356* suppressed the deactivation defect of *rdg*C mutants (Fig. 3A). Thus, continued phosphorylation of Rh1 is responsible for the defects in deactivation kinetics.

Arrestin binding is required for termination of the active state of GPCRs (*5*, *6*, *29*, *30*). In vertebrate photoreceptors, the formation of the rhodopsin-arrestin complex is largely determined by the phosphorylation state of rhodopsin (*5*, *6*, *8*–*10*, *31*). Because *rdg*C mutants accumulate hyperphosphorylated rhodopsin, the deactivation defect may result from a defect in the rhodopsin-arrestin interaction. A manifestation of this interaction in vivo is the prolonged depolarizing afterpotential (PDA) (*16*, *32*), a sustained photoresponse that occurs when a substantial amount of rhodopsin has been photoconverted from R to the active M state (Fig. 4A). A PDA results when metarhodopsin is produced in excess of free arrestin. Wild-type photoreceptors require ~20% conversion of R to M to trigger a PDA. This amount of rhodopsin isomerization approximately equals the total number of arrestin molecules in the photoreceptor cell (*16*). Mutants expressing small amounts of arrestin enter a PDA with very little light (because only a small amount of M would readily saturate arrestin availability), whereas mutants that reduce amounts of Rh1 to less than those of arrestin prevent entry into a PDA (there could never be an excess of M over arrestin). In dark-raised *rdg*C mutants, the amounts of arrestin and rhodopsin are the same as in the wild type (*22*). Like arrestin mutant photoreceptors, *rdg*C photoreceptors entered a PDA with approximately one-eighth as much light as did photoreceptors in control flies (Fig. 4B) (*16*). In contrast, *Rh1Δ356; rdg*C double mutants displayed a normal PDA. Thus, RDGC is required for normal rhodopsin function, and the deactivation defect may be attributable to impaired arrestin function that results from rhodopsin hyperphosphorylation.

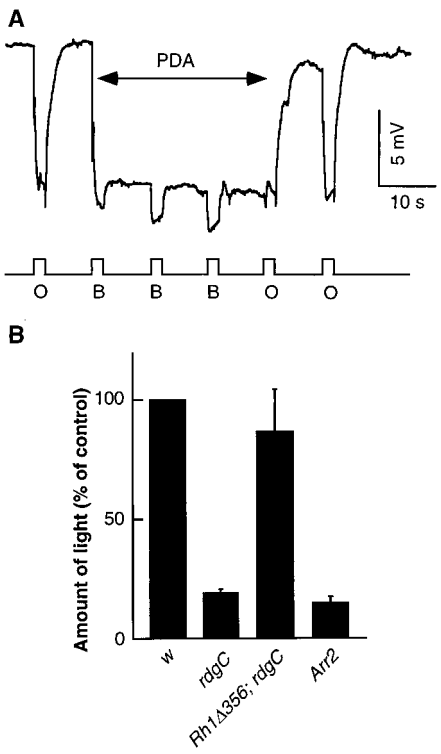

Fig. 4. Defects in the PDA response of *rdgC* flies. (**A**) ERG recording of a white-eyed control fly showing a prototypical PDA response. O, orange light (M → R conversion); B, blue light (R → M conversion). (**B**) Histogram of relative amount of blue light required to enter a PDA for the different genotypes. Values are normalized to control flies and represent means ± SEM of triplicate determinations. *Arr2*, a known mutant with a PDA defect (*16*), is shown for comparative purposes.

Extensive research in other systems had shown that receptor phosphorylation is required for arrestin binding; however, *Rh1Δ356* transgenic flies displayed normal deactivation physiology. This contrasts to vertebrate rhodopsin, in which a truncation of the COOH-terminal tail abolished arrestin binding (*9*) and led to severe defects in deactivation (*29*). *Drosophila* rhodopsin could be a special case, but this is not likely given the conservation of GPCR function in different systems. Instead, the COOH-terminus of rhodopsin may function as an autoinhibitory domain for arrestin binding. In this case, the lack of the COOH-terminus in *Rh1Δ356* would eliminate the need for receptor phosphorylation and would explain the rescue of *rdg*C phenotypes by the *Rh1Δ356* truncation.

Also, photoreceptor cell degeneration resulted directly from an excess of phosphorylated rhodopsin. Because rhodopsin dephosphorylation is essential for photoreceptor cell integrity, rhodopsin phosphatase mutations in vertebrates may also lead to retinal dysfunction and may account for some of the human retinal degenerative disorders. Indeed, these results could also help to explain the retinal degeneration seen in human retinitis pigmentosa patients and in transgenic mice that express a $Lys^{296} \rightarrow Glu$ missense mutation in rhodopsin (which eliminates the chromophore binding site and leads to light-independent receptor activation in vitro) (*33*, *34*). However, the $Lys^{296} \rightarrow Glu$ mutation does not lead to degeneration by constitutively activating phototransduction; instead the mutant opsin was found to be constitutively phosphorylated and inactivated by deactivation mechanisms (*34*, *35*).

Although there are hundreds of GPCRs, there are only a few G proteins, receptor kinases, and arrestins. RDGC is also expressed in the mushroom bodies of the fly brain (*18*), which suggests that this phosphatase participates in different pathways. The recent finding of RDGC homologs in the nervous systems of humans and mice (*36*) indicates that the conservation of GPCR function probably extends to receptor phosphatases.

REFERENCES AND NOTES

1. T. M. Fong, *Cell Signal.* **8**, 217 (1996); J. M. Baldwin, *Curr. Opin. Cell Biol.* **6**, 180 (1994).
2. L. Hein and B. K. Kobilka, *Neuropharmacology* **34**, 357 (1995); R. J. Lefkowitz *et al.*, *Adv. Second Messenger Phosphoprotein Res.* **28**, 1 (1993).
3. J. Nathans, *Biochemistry* **31**, 4923 (1992); P. A. Hargrave and J. H. McDowell, *Int. Rev. Cytol.* **137B**, 49 (1992); L. Lagnado and D. Baylor, *Neuron* **8**, 995 (1992).
4. H. Künh and W. J. Dreyer, *FEBS Lett.* **20**, 1 (1972); D. Bownds, J. Dawes, J. Miller, M. Stahlman, *Nature New Biol.* **237**, 125 (1972); X. Zhao, K. Palczewski, H. Ohguro, *Biophys. Chem.* **56**, 183 (1995); H. Ohguro, H. J. Van, A. H. Milam, K. Palczewski, *J. Biol. Chem.* **270**, 14259 (1995).
5. U. Wilden, S. W. Hall, H. Kuhn, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 1174 (1986).
6. K. Palczewski, *Protein Sci.* **3**, 1355 (1994).
7. A. Sitaramayya and P. A. Liebman, *J. Biol. Chem.* **258**, 12106 (1983).
8. K. Palczewski, G. Rispoli, P. Detwiler, *Neuron* **8**, 117 (1992).
9. V. V. Gurevich and J. L. Benovic, *J. Biol. Chem.* **268**, 11628 (1993).
10. N. Bennett and A. Sitaramayya, *Biochemistry* **27**, 1710 (1988).
11. J. B. Hurley, *Curr. Opin. Neurobiol.* **4**, 481 (1994); G. Langlois, C. K. Chen, K. Palczewski, J. B. Hurley, T. M. Vuong, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 4677 (1996).
12. S. D. Yang *et al.*, *J. Biol. Chem.* **263**, 8856 (1988); C. Fowles, M. Akhtar, P. Cohen, *Biochemistry* **28**, 9385 (1989); K. Palczewski, P. A. Hargrave, J. H. McDowell, T. S. Ingebritsen, *ibid.*, p. 415; K. Palczewski, J. H. McDowell, S. Jakes, T. S. Ingebritsen, P. A. Hargrave, *J. Biol. Chem.* **264**, 15770 (1989); S. D. Yang, J. L. Benovic, Y. L. Fong, M. G. Caron, R. J. Lefkowitz, *Biochem. Biophys. Res. Commun.* **178**, 1306 (1991); A. J. King, N. Andjelkovic, B. A. Hemmings, M. Akhtar, *Eur. J. Biochem.* **225**, 383 (1994); M. A. Kutuzov and N. Bennett, *ibid.* **238**, 613 (1996).
13. C. S. Zuker, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 571 (1996).
14. W. A. Harris and W. S. Stark, *J. Gen. Physiol.* **69**, 261 (1977); L. Wu, B. Niemeyer, N. Colley, M. Socolich, C. S. Zuker, *Nature* **373**, 216 (1995).
15. F. Steele and J. E. O'Tousa, *Neuron* **4**, 883 (1990).
16. P. J. Dolph *et al.*, *Science* **260**, 1910 (1993).
17. R. Ranganathan, D. M. Malicki, C. S. Zuker, *Annu. Rev. Neurosci.* **18**, 283 (1995).
18. F. R. Steele, T. Washburn, R. Rieger, J. E. O'Tousa, *Cell* **69**, 669 (1992).
19. T. Byk, Y. M. Bar, Y. N. Doza, B. Minke, Z. Selinger, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 1907 (1993).
20. B. Minke, in *The Molecular Mechanisms of Phototransduction,* H. Steive, Ed. (Springer-Verlag, New York, 1986), pp. 241–286.
21. J. E. O'Tousa *et al.*, *Cell* **40**, 839 (1985); C. S. Zuker, A. F. Cowman, G. M. Rubin, *ibid.*, p. 851.
22. J. Vinós and C. Zuker, unpublished data.
23. R. J. Lefkowitz, *Cell* **74**, 409 (1993); R. T. Premont, J. Inglese, R. J. Lefkowitz, *FASEB J.* **9**, 175 (1995).
24. No significant differences were found between *Rh1Δ356* flies and control flies (R. Ranganathan, K. Jalink, C. Zuker, unpublished data).

25. P. Kurada and J. E. O'Tousa, *Neuron* **14**, 571 (1995).
26. Such mutations include dominant mutations of rhodopsin (*25*) and mutations on the receptor-specific cyclophilin ninaA that reduce rhodopsin amounts to ≤10% those of wild-type flies (J. Vinós and C. Zuker, unpublished data).
27. K. Scott, A. Becker, Y. Sun, R. Hardy, C. Zuker, *Neuron* **15**, 919 (1995).
28. Z. Selinger, Y. Doza, B. Minke, *Biochim. Biophys. Acta* **1179**, 283 (1993).
29. J. Chen, C. L. Makino, N. S. Peachey, D. A. Baylor, M. I. Simon, *Science* **267**, 374 (1995).
30. J. L. Benovic *et al*., *Proc. Natl. Acad. Sci. U.S.A.* **84**, 8879 (1987); R. J. Lefkowitz *et al*., *Cold Spring Harbor Symp. Quant. Biol.* **57**, 127 (1992).
31. W. C. Smith *et al*., *J. Biol. Chem.* **269**, 15407 (1994).
32. W. L. Pak, in *Neurogenetics: Genetic Approaches to the Nervous System,* X. O. Breakfeld, Ed. (Elsevier, New York, 1979), pp. 67–99.
33. T. J. Keen *et al*., *Genomics* **11**, 199 (1991).
34. T. Li, W. K. Franson, J. W. Gordon, E. L. Berson, T. P. Dryja, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 3551 (1995).
35. J. Rim and D. D. Oprian, *Biochemistry* **34**, 11938 (1995).
36. E. Montini *et al*., *Hum. Mol. Genet.* **6**, 1137 (1997).
37. Flies used in the phosphorylation, degeneration, and electrophysiology experiments were 4 days old and were raised and kept in the dark. The following stocks were used: w^1, w^{1118}, w^{1118};;*ninaE*^{234}, w^{1118};; *rdgC*^{306} *cu red*, w^{1118};; P[*ninaE*$^{\Delta 356}$ *ry*$^+$] *ry ninaE*^{117} *e*^{s} (*Rh1Δ356*), w^{1118}; P[*ninaE*$^{\Delta 356}$ *ry*$^+$]; *rdgC*^{306} *cu red ry ninaE*^{117} *e*^{s} (*Rh1Δ356*; *rdgC*), w^{1118}; *Dgq*^{1} *cn*, w^{1118}; *Dgq*^{1} *cn*; *rdgC*^{306} *cu red*, and w^{1118};; *Arr2*^{3}. In phosphorylation experiments, the amount of label ingested was monitored by adding a green food dye to the radioactive food. For each sample, 10 flies were subjected to 4 hours of starvation in the dark and were then transferred to a vial containing 50 μl of 1% sucrose, 0.5% agarose, and 50 μCi of carrier-free $^{32}PO_4$. Flies were kept in a dark, humid chamber overnight and were then exposed to either 20 s or 15 min of blue or orange light. Light exposure times were selected to maximize differences between phenotypes. Flies were then frozen in liquid nitrogen and dried in cold acetone for 3 hours at –80°C followed by 16 hours at –20°C. Retinas were hand-dissected and homogenized in electrophoresis sample buffer containing the phosphatase inhibitors okadaic acid and microcystin. Proteins were separated by denaturing electrophoresis, blotted to nitrocellulose, analyzed on a phosphorimager, and then probed with specific antibodies.
38. For microscopy, flies (*37*) were exposed to white light (30-W white lamp, attenuated 1:100, at 15 cm) for 6 days. Fly heads were cut, fixed, and embedded in resin as described [D. P. Smith *et al*., *Science* **254**, 1478 (1991)]. Sections (1 μm thick) were stained with methylene blue and borax before analysis.
39. J. Vinós, R. Hardy, C. Zuker, unpublished data.
40. ERGs of 4-day-old adult, dark-raised flies were recorded as described (*32*). Stimulating light was delivered with a 450-W xenon lamp (Osram) and filtered with either a 480-nm bandpass filter (Oriel, 53850) for blue light or a 570-nm longpass filter (Oriel, 51310) for orange light. Recordings (Fig. 3A) were performed with orange light attenuated 1:1585. Similar responses were elicited with blue light. For intracellular recordings, a coronal section was made through the compound eye. Dorsal hemispheres of the head were mounted upside down and immediately immersed in Schneider's medium (Gibco). Photoreceptor cells were impaled with 90- to 150-MΩ electrodes filled with 2 M KCl. Maximal differences in deactivation time were found when stimuli 0.5 s or longer were used. Under whole-cell recording conditions, no significant differences in deactivation time were found.
41. We thank P. Dolph for suggestions and fly stocks, and A. Newton and members of the Zuker laboratory for critical reading of the manuscript. K.J. is an American Cancer Society junior fellow, R.W.H. is an associate of the Howard Hughes Medical Institute, and C.S.Z. is an investigator of the Howard Hughes Medical Institute. Supported in part by a grant from the National Eye Institute.

31 March 1997; accepted 18 June 1997

Simplification of DNA Topology Below Equilibrium Values by Type II Topoisomerases

Valentin V. Rybenkov, Christian Ullsperger, Alexander V. Vologodskii, Nicholas R. Cozzarelli*

Type II DNA topoisomerases catalyze the interconversion of DNA topoisomers by transporting one DNA segment through another. The steady-state fraction of knotted or catenated DNA molecules produced by prokaryotic and eukaryotic type II topoisomerases was found to be as much as 80 times lower than at thermodynamic equilibrium. These enzymes also yielded a tighter distribution of linking number topoisomers than at equilibrium. Thus, topoisomerases do not merely catalyze passage of randomly juxtaposed DNA segments but control a global property of DNA, its topology. The results imply that type II topoisomerases use the energy of adenosine triphosphate hydrolysis to preferentially remove the topological links that provide barriers to DNA segregation.

Randomly cyclized DNA molecules can be found in three topological forms (*1*): supercoils, knots, and catenanes (Fig. 1). If cyclization is sufficiently slow, the distribution of topological isoforms of circular DNA will be at thermodynamic equilibrium. This equilibrium distribution depends solely on the conformations adopted by DNA during its thermal fluctuations in solution (*2, 3*). The study of topological equilibrium has provided valuable information about DNA superhelical energy (*4, 5*), DNA effective diameter (*6*), and the conformations of supercoiled DNA (*3*).

V. V. Rybenkov, C. Ullsperger, N. R. Cozzarelli, Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720, USA.
A. V. Vologodskii, Department of Chemistry, New York University, New York, NY 10003, USA.

*To whom correspondence should be addressed. E-mail: cozzlab@mendel.berkeley.edu

A widely held belief is that, with the obvious exception of DNA gyrase and reverse gyrase, which introduce supercoiling in circular DNA (*7*), topological equilibrium is also achieved in the reactions catalyzed by DNA topoisomerases. Accordingly, topoisomerases were described as enzymes that convert a real DNA chain into a phantom chain that freely passes through itself to generate the equilibrium set of topological isomers. In support of this view, a mouse type I topoisomerase (topo I) generated the same Gaussian distribution of linking number (*Lk*) topoisomers as did ligation of a nicked circular DNA (*5*). Type II topoisomerases (topo II) can also relax supercoiled DNA. They can unlink knotted (*8, 9*) or catenated DNA (*10, 11*) and promote catenation if the DNA concentration is high enough (*11*). In all these reactions, the systems approach equilibrium. However, the question of whether the enzymes create an equilibrium distribution of relaxed, catenated, or knotted molecules has not been carefully examined.

Our previous studies of equilibrium DNA conformations (*3, 6*) provided the tools to determine whether the product distributions of the reactions catalyzed by prokaryotic and eukaryotic type II topoisomerases matched those at equilibrium. First, we measured the equilibrium fraction of catenanes between two nicked circular DNA molecules (*3, 6*). Bacteriophage P4 DNA (2 μg/ml) was cyclized by means of its long cohesive ends in the presence of excess (50 μg/ml) nicked plasmid pAB4 DNA, and the fraction of heterodimeric catenanes among all cyclized molecules was measured. More than 90% of the catenanes were singly linked dimers. The probability of catenation was proportional to the concentration of pAB4 DNA. Previous experimental data strongly support the conclusion that cyclization of DNA by its cohesive ends results in an equilibrium distribution of topological species, and this was confirmed by Monte Carlo simulations (*2, 3*).

To measure the fraction of catenanes in a topoisomerase-catalyzed reaction, we used the same mixture of pAB4 and P4 DNA molecules as a substrate but with twice the equilibrium fraction of catenanes (*12*). The fraction of catenanes as a function of the amount of *Escherichia coli* topoisomerase IV was measured by agarose gel electrophoresis (Fig. 2A). This fraction decreased to 0.004 when the molar ratio of enzyme to DNA, *s*, was 0.1 to 1 (Fig. 2B). Over this range of *s*, we found the same fraction of catenanes in the reverse reac-

A G Protein-Coupled Receptor Phosphatase Required for Rhodopsin Function

Javier Vinós, Kees Jalink, Robert W. Hardy, Steven G. Britt, and Charles S. Zuker

Science, 277 (5326), • DOI: 10.1126/science.277.5326.687