Expression of Transducin in Retinal Rod Photoreceptor Outer Segments

A guanine nucleotide–binding (G) protein "translocation" mechanism has been proposed (*1*) to explain long-term adaptive physiology in the rod photoreceptor. Large, prolonged changes in immunoreactivity of the α subunit of transducin (α_T) were detected within the rod outer segment (ROS) at both light onset and light offset (*1*). Concurrent and opposite changes in α_T immunoreactivity within the rod inner segment suggested that the amount of transducin within the photoreceptor is altered daily by massive movement of transducin subunits from one cellular locale to another.

To independently check the putative transducin translocation event upon which the model is based, we quantified the amount of the α and β subunits (β_T) of transducin within the ROS at several times during the day and night. We also checked the influence of protein denaturation on transducin antibody binding in light and dark. Tissue sections were prepared and stained as described in (*1*). Before antibody staining, sections were either fixed by immersion in 4% formaldehyde in saline for 20 min or in cold (−20°C) acetone for 10 min. The primary antibody was a previously characterized antiserum specific for α_T (*2*), GI-2, at 1:100 dilution and the secondary antibody was goat antiserum to rabbit immunoglobulin G labeled with fluorescein isothiocyanate.

We prepared ROS (*3*) from retinas of 45-day-old Long-Evans or Sprague-Dawley rats raised on a 12-hour light:12-hour dark cycle. At each of six times evenly spaced throughout the light:dark cycle, ROS were isolated from 4 to 12 retinas. The recovery of ROS from the retinas was greater than 50% as determined from absorbance at 500 nm. Purity and intactness of the ROS were estimated from the absorbance ratio 280/500 nm (*4*), the appearance of the ROS with light microscopy, and SDS-polyacrylamide gel profiles. The α and β subunits of transducin were identified in these preparations by three criteria. Transducin subunits are the second largest polypeptide components of outer segment membranes and show a characteristic mobility on SDS-polyacrylamide gels with a low ratio of *N,N'*-methylenebisacrylamide to acrylamide (*5*). In addition, transducin is characteristically eluted from bleached ROS disk membranes at guanosine triphosphate (GTP) concentrations greater than 40 μ*M* under low salt concentrations (*5, 6*). A previously characterized antiserum to transducin (*7*) was used to confirm the localization of α and β subunits on immunoblots of ROS polypeptides.

Peak heights from densitometric profiles of Coomassie blue–stained SDS-polyacrylamide gels (Ultroscan 2202 laser densitomer, LKB Instruments) were used to quantify α_T and β_T in each ROS preparation. The transducin peak heights were normalized to the height of the rhodopsin peak in each gel lane. The resulting ratio of transducin to rhodopsin allows comparison of the relative transducin concentrations from different ROS samples with variable amounts of ROS polypeptides (*8*).

A "soluble" pool of transducin was also recovered to check the possibility that transducin was preferentially lost into the ROS supernatant during preparation of dark-adapted membranes. Transducin subunits were recovered from both dark- and light-adapted ROS supernatants with DE52 column chromatography (*3, 5, 6*).

When prepared as above, the ROS were free from major contamination by non–ROS proteins. An absorbance ratio (280/500 nm) of 2.6 indicated ROS of relatively high purity and, although inner segment and cell debris were visible in light micrographs, these contaminants were present in low amounts. Further, SDS-polyacrylamide gel profiles of rat ROS showed all major polypeptides present in purified bovine, and frog ROS (*9*) (rhodopsin, transducin, phosphodiesterase, and 48K) with no additional

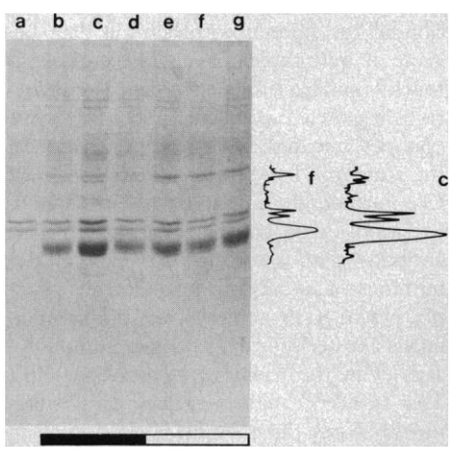

Fig. 1. Coomassie blue–stained gel of purified bovine transducin (a) and dark-adapted ROS isolated at 9:30 p.m., 1:30 a.m., and 5:30 a.m. (b, c, and d) and light-adapted ROS isolated at 9:30 a.m., 1:30 p.m., and 5:30 p.m. (e, f, and g). The light-dark cycle is indicated schematically below the gel. Densitometry scans of lanes f (light-adapted ROS) and c (dark-adapted ROS) are shown at right (*8*).

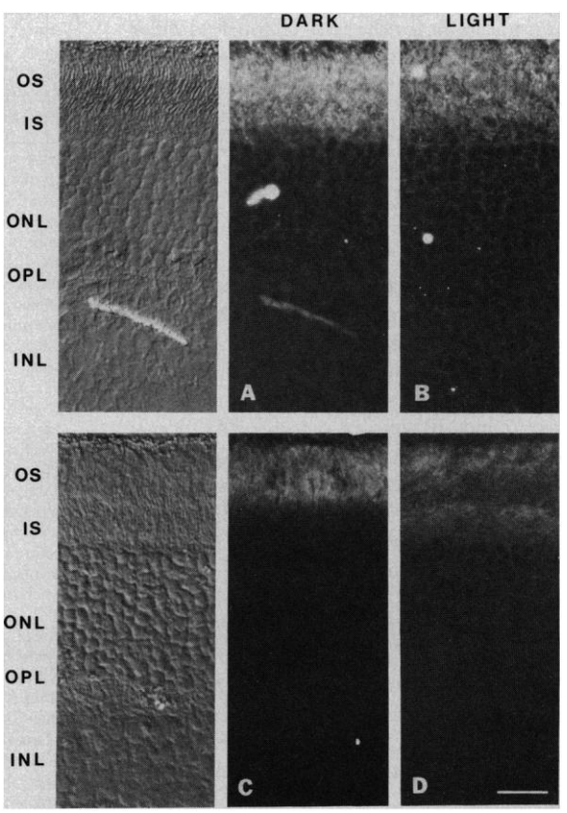

Fig. 2. Localization of α_T immunoreactivity in frozen sections of eyes from dark-adapted (A and C, 9:30 p.m.) and light-adapted (B and D, 5:30 p.m.) rats. Section were pretreated with cold acetone (A and B) or 4% formaldehyde in saline (C and D) and stained with a polyclonal antibody specific for α_T (*2*) and FITC-labeled secondary antibody. Nomarski micrographs of sections in (A) and (C) are on the left. Scale bar, 22 μm. Abbreviations: OS, outer segments; IS, inner segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer.

Table 1. The amounts of α_T, β_T, and rhodopsin (Rh) measured by laser densitomer scans of Coomassie blue–stained gels. Values are expressed as the mean ± SEM ($n = 6$).

	α_T/Rh	β_T/Rh	$\alpha_T/\alpha_T + \beta_T$
Light	0.43 ± 0.04	0.50 ± 0.05	0.46 ± 0.02
Dark	0.45 ± 0.04	0.56 ± 0.06	0.44 ± 0.02

major contaminants. The identification of rat transducin subunits was unambiguous (*10*) by (i) comparison with purified bovine transducin standard, (ii) total elution from ROS washed with 1 m*M* GTP, and (iii) cross-reactivity with the antiserum to transducin. In addition, loss of transducin due to proteolysis was unlikely as shown by the presence of only the 37,39-kD transducin α, β doublet on immunoblots.

Quantification of transducin:rhodopsin ratios for purified rat ROS demonstrated that the concentration of α and β transducin subunits did not vary during the daily light:dark cycle (Fig. 1 and Table 1). In addition, no soluble pool of transducin was preferentially lost during preparation of dark- or light-adapted ROS. In contrast, the major dark-soluble ROS protein, 48K (*11*), was reduced in concentration in ROS membranes prepared from dark-adapted retinas (Fig. 1, b through d).

Thus, massive depletion of transducin polypeptides from ROS did not occur at any time during the daily light-dark cycle. Explanations other than subunit translocation must account for the two- to fourfold differences (*1*) in light-dark transducin immunoreactivity within the ROS. One possibility is that transducin, like rhodopsin, undergoes light-induced (*12*) covalent modifications. Other possibilities include conformational changes in α_T or masking of antigenic sites on transducin by tight binding to rhodopsin in light-adapted ROS.

To test this we repeated the tissue-staining experiments of Brann and Cohen (*1*) on light and dark retinas under two different conditions: (i) with formaldehyde fixation to preserve the native conformation of transducin and (ii) with cold acetone fixation to denature the transducin in the tissue section. The two conditions produced very different results (Fig. 2). Formaldehyde fixation, as shown originally by Brann and Cohen, resulted in light-dark differences in α_T staining. In contrast, a more equal light-dark staining of ROS in acetone-fixed sections suggests the relevant transducin epitopes are unmasked by denaturation. Therefore, both biochemical and immunocytochemical results support the conclusion that transducin is present in both light- and dark-adapted ROS in comparable amounts, but that at least one antigenic site on the native α_T molecule is masked under light-adapted conditions.

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REFERENCES AND NOTES

1. M. R. Brann and L. V. Cohen, *Science* **235**, 585 (1987).
2. M. Pines, P. Gierschik, G. Milligan, W. Klee, A. Spiegel, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4095 (1985).
3. Rat retinas were dissected under room lights or under dim red light and placed in buffered sucrose (34% sucrose, 65 m*M* NaCl, 2 m*M* $MgCl_2$, 5 m*M* tris acetate, *p*H 7.4) at 4°C. The retina suspension was gently shaken for 1 min and large retina pieces were removed by centrifugation (5 min, 4500*g*). The ROS-containing supernatant was collected and diluted with two volumes of TBS (10 m*M* tris, 0.15*M* NaCl, *p*H 7.4), and centrifuged (10 min, 4500*g*). The supernatant was removed and assayed for soluble transducin. The ROS pellet was resuspended in 50 μl of TBS and sampled for light microscopy. The remainder was solubilized in SDS sample buffer for analysis by SDS–polyacrylamide gel electrophoresis (*5*).
4. The 500-nm and 280-nm absorbance of dark-adapted ROS was determined by solubilizing the ROS in 1% Ammonyx LO (Onyx Chemical Co.) and measuring peak heights before and after complete bleaching and used to estimate the purity and intactness of ROS preparations [H. G. Smith, Jr., G. W. Stubbs, B. J. Litman, *Exp. Eye Res.* **20**, 211 (1975)].
5. W. Baehr, M. J. Devlin, M. L. Applebury, *J. Biol. Chem.* **254**, 11669 (1979).
6. W. Godchaux III and W. F. Zimmerman, *ibid.* **254**, 7874 (1979); H. Kühn, *Nature* **283**, 587 (1980).
7. D. J. Roof, M. L. Applebury, P. C. Sternweis, *J. Biol. Chem.* **260**, 16242 (1985).
8. Quantification of rhodopsin and α_T and β_T was performed on gel lanes loaded with similar amounts of protein (10 to 20 μg). However, the ratios of rhodopsin to transducin were also checked for selected samples over a threefold range of protein concentration (7 to 20 μg) and were independent of the amount of total protein loaded. Transducin subunits were normalized to rhodopsin values rather than to total protein because rhodopsin, an integral membrane protein, does not change concentration in the ROS during the light-dark cycle. The small amount of rhodopsin dimer in both dark- and light-adapted samples was not included in the quantitation.
9. H. Kühn, *Nature* **283**, 587 (1980); *Curr. Top. Membr. Transp.* **15**, 171 (1981); for review, P. A. Hargrave, in *The Retina: A Model for Cell Biology Studies*, R. Adler and D. Farber, Eds. (Academic Press, Orlando, FL, 1986), part I, pp. 207–237.
10. Two major sources of error in quantifying ROS transducin can be excluded: (i) Contribution of transducin from retinal cells other than rod photoreceptors. Within the retina, transducin is found only in photoreceptors. [G. B. Grunwald, P. Gierschik, M. Nirenberg, A Spiegel, *Science* **231**, 856 (1986); C. L. Lerea, D. E. Somers, J. B. Hurley, I. B. Klock, A. H. Bunt-Milam, *ibid.* **234**, 77 (1986)]. (ii) Contributions from "contaminating" G proteins with α subunits identical in mobility to transducin (that is, G_0). The ratio of α_0:α_T in the ROS is less than 0.1% [S. M. Mumby, R. A. Kahn, D. R. Manning, A. G. Gilman, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 265 (1986)].
11. 48K (S-antigen, arrestin) was identified in these preparations by its characteristic solubility in the dark and from immunoblots of dark- and light-adapted preparations with a previously characterized antiserum to 48K [N. Mangini, D. Roof, W. Baehr, M. Applebury, D. Pepperberg, *Invest. Ophthalmol. Vis. Sci.* **25**, 112 (1984)].
12. G. W. Balkema and U. C. Draeger, *Nature* **316**, 630 (1985).
13. We thank R. Swanson and B. Knox for helpful discussions on the quantification of ROS proteins, A. Speigel for antiserum to transducin, and M. Adamian for help in the preparation of the figures. Supported by NIH grants EY06514 (DJR) and EY05790 (CAH) and a grant from the Retinitis Pigmentosa Foundation.

27 August 1987; revised 11 April 1988; accepted 20 May 1988

Response: Roof and Heth report a disagreement between subcellular fractionation and immunocytochemical data concerning the amount of the alpha subunit of transducin (α_T) within rod outer segments (ROS). As we previously reported (*1*), they observe that, under certain fixation conditions, the α_T immunoreactivity of ROS is less during the day then at night. On the other hand, when they isolated ROS and measure α_T content by scanning SDS-polyacrylamide gels, α_T levels appear constant. On the basis of this disagreement, the authors interpret the data as showing that the amounts of α_T do not change and that light induces a masking of antigenic sites on α_T. I believe this conclusion is unlikely to be correct. First, the observed changes in α_T immunoreactivity are difficult to explain in terms of antigen masking. Second, the presented measurements of α_T levels in isolated ROS are confounded by technical limitations. These limitations are reinforced by the recent publication of a report by Philp *et al.* (*2*) which demonstrates that the α_T levels in isolated ROS change as predicted by immunocytochemical data.

Epitope masking is an unlikely explanation of the immunocytochemical data because at least three spatially separated epitopes would have to be involved. The antisera to α_T used in our study recognize two epitopes, one in the NH_2-terminal region of the protein and one within a central segment (*3*). Further, the immunocytochemical observations were confirmed with a second antibody that is directed to the COOH-terminus of α_T. Even more difficult to explain with such a model are the simultaneous and reciprocal changes in immunoreactivity in the rod inner segments (RIS). That is, even if light were able to simultaneously block three antigenic sites on α_T in the ROS, what is the mechanism of reciprocal changes in immunoreactivity in the RIS? In support of their epitope-masking hypothesis, Roof and Heth present a comparison of immunocytochemical data in which two fixation procedures were used. When tissue was fixed with acetone, a less marked change in immunoreactivity was observed than