Preparation and Characterization of Monoclonal Antibodies to Several Frog Rod Outer Segment Proteins

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ABSTRACT Monoclonal antibodies to proteins important in phototransduction in the frog rod outer segment have been obtained. These include 6 different antibodies to rhodopsin, 50 to a guanine nucleotide binding protein (G-protein; 40,000 daltons), and 2 to cytoplasmic proteins. The antigens used were Percoll-purified rod outer segments, a rod outer segment soluble protein fraction, or a soluble plus peripheral membrane protein fraction. Antibodies were assayed by solid phase assay using a fluorogenic detection system. Proteins to which antibodies bound were assayed on Western blots, and the sensitivities of three different detection systems were compared. Most antibodies bound to only one rod outer segment protein band on Western blots. Immunofluorescence microscopy demonstrated binding of both anti-rhodopsin and anti-G-protein to isolated frog rod outer segments. Antibodies were purified from either culture supernatants or ascites fluid on protein A affinity columns. Two purified anti-G-protein antibodies have binding affinities to 125I-labeled G-protein of <10^-6 M^-1. Of 11 antibodies to frog or bovine G-protein tested in solid phase and Western blot assays, all bind to the a rather than the b or g subunits. Procedures developed here are being used in preparing other antibodies that affect reactions in the phototransduction pathway.

INTRODUCTION

In the vertebrate photoreceptor, light initiates a series of biochemical steps that ultimately effect a hyperpolarization of the cell. While several light-dependent reactions in this process have been identified, a complete characterization would include descriptions of the functions of all components, their interrelationships, and their locations in the rod outer segment (ROS). Several proteins involved in the light response have been identified so far. These include rhodopsin, a GTP-binding protein (G-protein), and a cyclic GMP phosphodiesterase, all involved in initial events (for a review, see Pober and Bitensky, 1979; Hubbell and Bownds, 1979; Kuhn, 1981; Stryer et al., 1981). A kinase responsible for light-dependent...
phosphorylation of rhodopsin has been described in frog and bovine ROS (Shichi and Somers, 1978; Kuhn, 1978; Lee et al., 1981). Two low molecular weight (12,000 and 13,000 mol wt) proteins that are reversibly dephosphorylated in the light (components I and II; Polans et al., 1979; Hermolin et al., 1982) have also been described. Other proteins whose exact role is not yet known include a 220,000-dalton glycoprotein found in disk membranes (Papermaster, 1978b; Clark and Molday, 1979) and a 50,000-dalton protein that binds to the ROS disk membrane in a light-dependent manner (Kuhn, 1980, 1981). Also, a number of cytoplasmic soluble proteins of unknown function have been identified (H. E. Hamm and M. D. Bownds, manuscript submitted for publication).

As one approach to identifying more proteins involved in phototransduction, we have begun developing a variety of monoclonal antibodies to ROS proteins, to be used for immunocytochemical localization, for identifying proteins separated on polyacrylamide gels, for altering the function of proteins, and for purification. To date, antibody studies on photoreceptors have focused on proteins already characterized, primarily rhodopsin, but similar techniques are applicable to uncharacterized proteins as well. For example, immunocytochemical techniques have been used to localize antigenic proteins in photoreceptors, including rhodopsin (Dewey et al., 1969; Jan and Revel, 1974; Papermaster et al., 1978a; MacKenzie and Molday, 1982; Wong et al., 1982), the 220,000-dalton protein (Papermaster et al., 1978b; MacKenzie and Molday, 1982), and enkephalin (Mancillas et al., 1981), as well as other unknown proteins specific to photoreceptors (Barnstable, 1980). Using immunocytochemical or Western blotting techniques to detect antibody binding, homologous proteins have been recognized in different cell types within species or between species (rhodopsin homology in bovine and frog ROS [Molday and MacKenzie, 1983] and in squid and octopus photoreceptors [Kremer et al., 1982]). An antibody has been obtained that distinguishes between native and detergent-solubilized, photo-bleached rhodopsin (Molday and MacKenzie, 1983). A monoclonal antibody that recognizes a topographic gradient along the avian retina has also been obtained (Trisler et al., 1981).

In other systems, antibodies have been used to alter the function of their target proteins, thus confirming their function (Frackelton and Rotman, 1980; Jay et al., 1981; Clark et al., 1981; Tzartos et al., 1981; Gabay and Schwartz, 1982; Kiehart et al., 1982). Ultimately, proteins can be purified on antibody affinity columns, making possible definitive biochemical characterization. We intend to use antibody inhibition of ROS proteins to explore their role in visual transduction. By noting other reactions affected by inhibition of a given protein, we hope to discern the sequential order of reactions in the transduction pathway.

METHODS

Preparation of ROS

ROS were prepared by gently shaking retinas from dark-adapted frogs in isotonic Ringer's solution (115 mM NaCl, 2.5 mM KCl, 2 mM MgCl₂, 0.1 mM CaCl₂, 10 mM Hepes, pH
Preparation of Antigen
For the fusion in which rhodopsin antibodies were obtained, BALB/c mice were injected intraperitoneally with $3 \times 10^4$ Percoll-purified ROS (60 µg protein) in complete Freund's adjuvant, and 3-4 wk later were reimmunized with $10^5$ ROS (160 µg protein) in incomplete Freund's adjuvant. To prepare the soluble and peripheral membrane fraction that was the antigen for the fusion in which G-protein antibodies were obtained, Percoll-purified ROS were hypotonically treated with distilled water, and the membrane was sedimented at 100,000 g for 30 min. Mice were immunized with 20 µg protein in the first injection and 60 µg in the second. The soluble protein fraction (~25 proteins) was prepared by mechanically disrupting purified ROS in an isotonic Ringer's solution and removing the membranes by sedimentation at 100,000 g for 30 min. Mice were immunized with 60 µg protein in both injections.

Fusion
The fusion to obtain antibody-producing hybridomas was done by modifications of the method of Kohler and Milstein (1975). 4 d after the second immunization, a suspension of $10^6$ spleen cells was mixed with $10^7$ NS-I myeloma cells and fused using polyethylene glycol (40%, 1,000 mol wt; Baker Chemical Co., Phillipsburg, NJ). The cell mixture was washed and then resuspended in 200 ml Dulbecco's modified Eagle's medium supplemented with 20% fetal calf serum, 60 µM hypoxanthine, 0.5 µM amethopterin, 20 µM thymidine, 50 µM β-mercaptoethanol, penicillin, and streptomycin (HAT medium), and 1% (vol/vol) mouse red blood cells. Cells were then pipetted into 96-well plates ($10^5$ cells in 0.2 ml medium/well) and were tested for antibody production after ~2 wk.

Cell Culture and Subcloning
Antibody-producing cells were grown in culture and maintained at a density of $10^5$-$10^6$ cells/ml in the above medium without amethopterin (HT medium). Cells were subcloned at least once by the method of limiting dilution.

Solid Phase Antibody Binding Assay
The solid phase antibody assay using a fluorogenic detection system was performed following the method of L. Kahan (personal communication). 96-well microtiter plates (Immulet I; Dynatech Laboratories, Inc., Alexandria, VA) were coated with 0.3-0.5 µg/well of ROS proteins in Tris buffer (0.1 M Tris, 0.5 M KCl, pH 9.0). For rhodopsin antibody assays, ROS proteins were used in the wells; for G-protein assays, the soluble plus peripheral membrane fraction was used; for soluble protein assays, the soluble fraction was used. To assay antibody, 50 µl of culture supernatant or ascites fluid diluted in culture medium was incubated in antigen-coated wells for 3-15 h, and then washed with buffer A (150 mM NaCl, 50 mM Tris, 0.25% gelatin, 0.02 mM ZnCl₂, 1.0 mM MgCl₂, 0.25% Nonidet P-40, pH 7.4). For detection of antibody binding, goat anti-mouse immunoglobulins linked to alkaline phosphatase (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) at a 1:100 dilution in buffer A including 10% fetal calf serum was incubated in wells for 3-15 h. After washing, 200 µl/well of a fluorogenic alkaline phosphatase substrate (naphthol AS-MX phosphate; Sigma Chemical Co., St. Louis, MO) in AMP buffer (1 M aminomethyl propanol, 25 µM ZnCl₂, 1.25 mM MgCl₂, pH 9.9) was incubated for several hours at 37°C. Using this assay method, a minimum of 10 ng/ml antibody, or 500 pg/50
μl well, could be detected. Under conditions of antibody excess, the lower limit of antigen detection was 1 ng.

**Titer**

Antibody titer was determined in the solid phase assay using serial dilutions of antibody, and the titer stated is the maximum dilution at which there was a positive signal.

**Concentration**

The concentration of purified antibody was determined from the absorbance at 280 nm, using $A_{280}^\text{nm} = 13.5 \times 10^{-4}$ (Kirschbaum, 1973).

**Protein Blotting**

The protein specificity of monoclonal antibodies was determined by the binding of antibodies to nitrocellulose blots of electrophoretically separated ROS proteins. Proteins were separated on 0.75-mm-thick, one-dimensional polyacrylamide gels (Polans et al., 1979) and then transferred electrophoretically to nitrocellulose paper (Towbin et al., 1979) for 3 h at 55 V in a transfer buffer of 25 mM Tris, 192 mM glycine, 20% (vol/vol) methanol, and 0.2% sodium dodecyl sulfate, pH 6.5. The blot was then cut into a number of identical strips for antibody assay. To locate polypeptide bands on the blot, one strip was stained in amido black (naphthol blue black [Sigma Chemical Co.], 0.1% in 45% methanol, and 10% acetic acid in distilled water) and destained in 90% methanol and 2% acetic acid. Remaining strips were incubated 1 h at 37°C in saline solution (0.9% NaCl, 10 mM Tris-HCl, pH 7.4) containing 0.5% gelatin to eliminate nonspecific protein binding. Blots were then washed in saline, incubated overnight in culture supernatant or ascites fluid diluted in saline plus gelatin, and washed again.

At this point, three different detection systems were compared to determine the best sensitivity: alkaline phosphatase, $^{125}$I-protein A, and horseradish peroxidase. (a) Alkaline phosphatase: blot strips were incubated 3–12 h in alkaline phosphatase-labeled goat anti-mouse immunoglobulin (Kirkegaard & Perry Laboratories, Inc.) at a 1:100 dilution in saline plus gelatin and washed in saline. Blot strips were then laid onto a 5-mm-thick 1.2% agar gel containing the alkaline phosphatase substrate naphthol AS-MX phosphate. After 5–60 min, fluorescent bands were visible in the agar layer under ultraviolet illumination.

(b) $^{125}$I-protein A: to increase sensitivity, blots were incubated with rabbit anti-mouse antibody at a 1:100 dilution in saline plus gelatin. Blots were washed in saline and then incubated with 100 μCi/ml $^{125}$I-protein A (New England Nuclear, Boston, MA) in saline plus gelatin, and washed in saline. The dried blot was then exposed to Kodak X-Omat AR film (Eastman Kodak Co., Rochester, NY) for several days. The band(s) on the blot to which antibody bound was detected as a dark band. (c) Horseradish peroxidase: blots were incubated 1 h in rabbit anti-mouse immunoglobulins diluted 1:100 in TBS (Tris-buffered saline: 20 mM Tris-HCl, 500 mM NaCl, pH 7.5) and then washed in TTBS (TBS plus 0.05% Tween-20). Blots were then incubated in horseradish peroxidase-labeled goat anti-rabbit IgG (Bio-Rad Laboratories, Richmond, CA; 1:3,000 dilution in TBS plus 1% gelatin) for 1 h, washed in TTBS, and then immersed in the horseradish peroxidase color development solution (Bio-Rad Laboratories). A purple band on the blot strip, indicating the location of antibody binding, was visible within 1–30 min.

**Sensitivity of Blotting Methods**

Using $^{125}$I-protein A, 10 ng/ml antibody was detected, and using the alkaline phosphatase detection system, ~1 μg/ml antibody could be detected. The horseradish peroxidase
method was the most sensitive, detecting as little as 1 ng/ml antibody. When antibody was in excess, ~10 ng antigen was the detection limit with this method.

**Antibody Purification**

Antibodies were purified from culture supernatant or ascites fluid on protein A-Sepharose 4B affinity columns (Pharmacia Fine Chemicals, Piscataway, NJ) using the procedure of Ey et al. (1981). After elution from the column, purified antibody was dialyzed into Ringer's solution and concentrated using an Amicon (Lexington, MA) ultrafiltration unit and PM filter (10,000 mol wt cut-off). A solid phase assay confirmed that the pH 3 fraction contained antibody of the same titer as the original ascites fluid.

**Determination of Binding Affinities**

For determination of the binding affinities of anti-G-protein antibodies, relatively pure G-protein was prepared from Percoll-purified ROS by the method of Kuhn (1981), which uses Tris buffer and the light-dependent binding of G-protein to ROS membranes. The G-protein fraction was radiiodinated as follows (Markwell and Fox, 1978). 20 μg of G-protein in 200 μl Tris buffer plus 40 μM GTP was placed in a 1.0-ml glass centrifuge tube coated with 10 μg Iodogen (1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril; Pierce Chemical Co., Rockford, IL) and incubated with 200 μCi of Na$_{125}$I (carrier-free; New England Nuclear) at room temperature for 15 min. Unreacted Na$^{125}$I was removed by dialysis against Ringer's solution.

The extent of G-protein-antibody binding was determined by a modification of the method of Soos and Siddle (1982). Various concentrations of iodinated G-protein between $5 \times 10^{-7}$ and $1 \times 10^{-8}$ M were incubated with $2.5 \times 10^{-7}$ M antibody in Ringer's solution for 30 min at room temperature. To precipitate the antibody, formalin-fixed Staphylococcus aureus (Immunoprecipitin; Bethesda Research Laboratories, Gaithersburg, MD) was then added in fivefold excess in a buffer containing 0.5% Nonidet P-40, 0.1% bovine serum albumin, and 0.1% sodium azide. After 1 h incubation, the S. aureus, bound antibody, and G-protein were precipitated (6,000 g, 15 min) and the pellet was washed once with Ringer's. Supernatant, wash, and pellet fractions were counted in a scintillation counter. A solid phase assay of a control sample lacking G-protein confirmed that antibody was absent from the supernatant. To monitor nonspecific adsorption of G-protein to S. aureus, control samples of G-protein minus antibody were incubated and counted as above and subtracted from the counts for the pellet fractions. To confirm the specificity of antibody binding, the supernatant and pellet fractions were analyzed by polyacrylamide gel electrophoresis.

**Immunofluorescence Microscopy**

Direct binding of antibodies to fixed ROS was observed by immunofluorescence microscopy. After preparation in the dark under infrared illumination and after subsequent bleaching of some samples by exposure to room illumination for 5 min, ROS were fixed by mixing 1:1 with 8% paraformaldehyde (Osborn and Weber, 1982), and a drop was placed on a coverslip. After 10–15 min, coverslips were immersed in 0.2% Nonidet P-40 in Ringer's for 2 min and washed in Ringer's. Coverslips were then incubated in various anti-rhodopsin or anti-G-protein antibodies (culture medium or dilutions of ascites fluid in culture medium) for 30–45 min. After washing, coverslips were incubated with a second antibody, a 1:25–1:50 dilution in Ringer's of rabbit anti-mouse immunoglobulin labeled with fluorescein isothiocyanate (FITC; Cappel Laboratories, Cochranville, PA). The second antibody solution was centrifuged before use at 8,000 g for 10 min to remove
aggregated material. Coverslips were then washed well in Ringer's, mounted on slides in 50% glycerol, and observed on a Zeiss Universal microscope (Carl Zeiss, Inc., New York) with epifluorescence optics. (Note: antibodies can be made available to interested researchers upon request.)

RESULTS

Antibodies to Rhodopsin

In the first fusion, in which purified ROS was the antigen, all antibodies obtained were found to be against rhodopsin. Six different antibody-producing cell lines were subcloned, and the antibody obtained from ascites fluid was purified on an affinity column of protein A, which binds strongly to some subclasses of mouse IgG (Ey et al., 1978). All six antibodies bound to rhodopsin monomer and oligomers on Western replica blots of ROS proteins separated by polyacrylamide gel electrophoresis (Fig. 1). To eliminate the possibility that these antibodies cross-reacted with G-protein, which co-migrates with rhodopsin monomer on polyacrylamide 10–20% gradient gels, antibodies were assayed on blots of the protein fraction containing only soluble and peripheral membrane proteins from hypotonically treated ROS. No antibody binding to these blots was observed, which indicates no cross-reaction with any other ROS proteins in this fraction (data not shown). The antibody concentrations in ascites fluid ranged from 6.5 to 15 mg/ml, and the titers ranged from $10^{-4}$ to $10^{-7}$.

As a control for nonspecific binding by mouse immunoglobulins and other proteins that might bind to protein A, ascites fluid from mice injected with NS-
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1 myeloma cells (nonsecretors of antibody) was purified on protein A affinity columns and incubated with Western blots of ROS proteins. No detectable binding was observed.

Antibodies to G-Protein

Antibodies to G-protein were obtained in one fusion where soluble and peripheral membrane proteins from hypotonic-treated ROS were the antigen. Of the 100 different wells initially positive for antibody production in the first solid phase assay, 85 bound to G-protein when assayed in Western blots, and the remaining 15 were negative. Fig. 2 shows binding to G-protein in Western blots by 11 supernatants from cell culture wells that had been positive in the first solid phase assay. Five cell lines showed strong antibody binding, one showed moderate binding, and five showed weak binding, which is not visible in Fig. 2. Cell lines producing the 50 antibodies that gave the strongest response were frozen for subsequent use, and of these, 11 have been subcloned and purified.

G-Protein Antibodies Bind to the α Subunit

All antibodies tested bound to the α subunit rather than the β or γ subunits of G-protein from both frog and bovine sources. In a solid phase assay against purified bovine Ga or Gβ, adsorbed to 96-well plates, all 11 antibodies tested bound to the Ga rather than Gβ subunits (data not shown). The purified antibody
(designated 4A) tested in Western blots bound only to the Gα subunit of purified bovine G-protein and not to the β or γ subunits (Fig. 3, lanes 8 and 9). This antibody also bound to Gα rather than to the Gβ or Gγ subunits on Western blots of frog peripheral membrane proteins separated on 10% polyacrylamide gels, where the three subunits can be clearly distinguished (Fig. 3).

![Figure 3](image)

**Figure 3.** Binding of anti-G-protein antibody to replica blots of frog and bovine ROS proteins. Lane 1 shows a Coomassie-stained 10% polyacrylamide gel of frog ROS proteins in which Gα, Gβ, and Gγ (not visible here) are separated. Lane 2 is a replica blot of lane 1 stained with amido black to indicate the proteins transferred, and lane 3 is an identical blot, which was incubated with the anti-G-protein antibody 4A and stained using the horseradish peroxidase detection system. The antibody binds only to Gα. Lanes 4 and 5 show Coomassie-stained polyacrylamide gels of purified bovine Gα and Gβ, and lanes 6 and 7 are the amido black-stained blots of Gα and Gβ. When identical blots are incubated with antibody 4A and stained as in lane 3, antibody binds strongly to the blot of Gα (lane 8) but not to Gβ (lane 9).

**Binding Affinity of G-Protein Antibodies**

To determine the binding affinities of the two protein A-purified anti-G-protein antibodies (designated 4A and 4H), their binding to 125I-labeled G-protein was quantitated. 2.5 × 10^-7 M antibody was reacted with 5 × 10^-7 to 1 × 10^-6 M purified G-protein. Antibody was precipitated by Immunoprecipitin, and ~20% of the G-protein was bound by antibody 4A and ~10% by antibody 4H, regardless of the G-protein concentration. This result allows a rough estimate for the binding affinity of <10^6 M^-1, which is consistent with the finding that 100% inhibition of phosphodiesterase activity by antibody is obtained at concentrations of ~10^-5 to 10^-6 M G-protein and antibody (Hamm and Bownds, 1984). A more accurate determination of the binding affinity would require using higher anti-
body concentrations (Trucco and dePetris, 1981), as well as determining whether iodination affects the availability of G-protein for antibody binding. The antibody titers were $10^{-5}$ for 4A and $10^{-3}$ for 4H, which also indicates a difference in binding between the two antibodies. Analysis of immunoprecipitated proteins by gel electrophoresis and autoradiography showed that while a number of iodinated bands were visible in the supernatant fraction, only G-protein was present in the antibody-precipitated pellet fraction, which indicates specific binding to G-protein by both antibodies.

![Figure 4](image)

**Figure 4.** Binding of antibodies to replica blots of soluble proteins. The protein pattern of soluble proteins is shown on the gel after Coomassie staining (lane 1) and in the amido black-stained replica blot (2). Lanes 3 and 4 were incubated with two different antibodies to soluble proteins. The antibody shown in lane 3 (designated 5A) binds to polypeptides of $\sim 12,000$ and $14,000$ daltons, and the antibody shown in lane 4 (5C) binds to polypeptides of $\sim 35,000$ and $14,000$ daltons.

**Antibodies to Cytoplasmic Proteins**

In the fusion in which the proteins of the soluble fraction were injected as antigen, $\sim 150$ wells were positive for antibody production in the initial solid phase assay against soluble proteins. Of these, $\sim 50$ remained stable and showed binding to Western blots of soluble proteins, and the cell lines were frozen for subsequent study. To date, two of these lines have been subcloned, and both produced antibodies that bound to two bands, as shown in Fig. 4. One antibody, 5A, binds to proteins of $\sim 12,000$ and $14,000$ daltons and the other, 5C, binds to proteins of $\sim 35,000$ and $14,000$ daltons. None of these polypeptides has been identified as to function. The antibody titers are $10^7$ for 5A and $10^5$ for 5C. These antibodies will be further characterized.
**Immunofluorescent Staining of Isolated ROS**

Binding of both anti-rhodopsin and anti-G-protein antibodies to ROS was demonstrated using an FITC-labeled second antibody staining technique. Fig. 5 shows ROS fixed onto coverslips, incubated with antibodies, and then labeled with FITC anti-mouse antibody. All six anti-rhodopsin antibodies fluoresced brightly after staining (one is shown in Fig. 5a). Two anti-G-protein antibodies (4A and 4H) also stained the ROS (4A is shown in Fig. 5b). Although four other anti-G-protein antibodies tested did not stain ROS, these antibodies were used in a lower concentration as tissue culture supernatant rather than as ascites fluid, which may be responsible for the difference in result. Alternatively, these four antibodies may not bind to G-protein in fixed ROS. No differences in staining were observed between ROS fixed in the dark compared with those bleached by room illumination before fixation. In control preparations, ROS showed no fluorescence after fixation or incubation with the second antibody alone.

**DISCUSSION**

This report describes initial efforts toward developing a number of monoclonal antibodies to ROS proteins, especially those involved in light-dependent reactions. Current efforts in studying phototransduction have been limited because pharmacological or other biochemical reagents have nonspecific effects in addition to their primary effect, and because they react with classes of compounds (Wells and Kramer, 1981). Since monoclonal antibodies react with a single antigenic site, a higher level of specificity is attainable. Furthermore, antibodies can provide direct correlation of several different types of information, including identification of an antigen’s molecular weight by Western blotting, localization...
in the cell by immunocytochemistry, and determination of its function in solution by antibody inhibition or enhancement of its activity.

To date, we have obtained antibodies to several different ROS proteins and have used them for identification, localization, and inhibition. The first group, six anti-rhodopsin antibodies, bound to all oligomers of frog rhodopsin on Western blots, where the antigen is denatured. By immunocytochemical means, it was also demonstrated that they bound to isolated frog ROS fixed in either the light or dark. In an enzyme-linked immunosorbent assay, synthetic N-terminal peptides from bovine rhodopsin have been found to compete with the binding of the anti-rhodopsin antibodies to bovine rhodopsin, which indicates that all six antibodies bind to the N-terminal segment of the molecule, and that they cross-react with bovine rhodopsin (G. Adamus and P. A. Hargrave, personal communication).

The second group, ~50 anti-G-protein antibodies, bound to G-protein on Western blots. All antibodies tested (11) bound specifically to the Gα subunit. It is the Gα subunit that binds GTP and is thought to interact with the phosphodiesterase (Fung et al., 1981). Two of the antibodies were shown by immunofluorescent staining to bind to intact ROS, fixed in either the light or the dark, while the other nine did not. These two antibodies bound to both native and denatured forms of the antigen, since they bound to G-protein in solution as well as on blots, on solid phase assay plates, and on fixed ROS. The differences in a given antibody’s binding strength detected by these three different assay methods might be accounted for by differences in the form of the antigen and/or the assay method itself.

The third group consists of antibodies to cytoplasmic proteins, none of which has yet been identified. The antibodies will be further characterized and may help elucidate the role of these proteins.

The results to date have shown that antibodies to ROS proteins can successfully be used to identify the molecular weights and to localize the proteins in isolated ROS. Use of antibodies in inhibiting one light-dependent reaction, G-protein activation by light, is described in the accompanying paper (Hamm and Bownds, 1984). Additional antibodies will be generated by the methods described here and applied to further studies of the phototransduction pathway.

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REFERENCES

Barnstable, C. J. 1980. Monoclonal antibodies which recognize different cell types in the rat retina. Nature (Lond.). 286:231–235.
Clark, R., D. P. Lane, and R. Tjian. 1981. Use of monoclonal antibodies as probes of simian virus 40 T antigen ATPase activity. J. Biol. Chem. 256:11854–11858.

Clark, S. P., and R. S. Molday. 1979. Orientation of membrane glycoproteins in sealed rod outer segment disks. Biochemistry. 18:5868–5873.

Dewey, M. M., P. K. Davis, J. K. Blasie, and L. Barr. 1969. Localization of rhodopsin antibody in the retina of the frog. J. Mol. Biol. 39:395–405.

Ey, P. L., S. J. Prowse, and C. R. Jenkin. 1978. Isolation of pure IgG1, IgG2a, and IgG2b immunoglobulins from mouse serum using protein A-Sepharose. Immunochemistry. 15:429–436.

Frackelton, A. R., Jr., and B. Rotman. 1980. Functional diversity of antibodies elicited by bacterial β-D-galactosidase. J. Biol. Chem. 255:5286–5290.

Fung, B. K.-K., J. B. Hurley, and J. Styer. 1981. Flow of information in the light-triggered cyclic nucleotide cascade of vision. Proc. Natl. Acad. Sci. USA. 78:152–156.

Gabay, J., and M. Schwartz. 1982. Monoclonal antibody as a probe for structure and function of an Escherichia coli outer membrane protein. J. Biol. Chem. 257:6627–6630.

Hamm, H. E., and M. D. Bownds. 1984. A monoclonal antibody to guanine nucleotide binding protein inhibits the light-activated cyclic GMP pathway in frog rod outer segments. J. Gen. Physiol. 84:265–280.

Hermolin, J., M. A. Karell, H. E. Hamm, and M. D. Bownds. 1982. Calcium and cyclic GMP regulation of light-sensitive protein phosphorylation in frog photoreceptor membranes. J. Gen. Physiol. 79:633–655.

Hubbell, W. L., and M. D. Bownds. 1979. Visual transduction in vertebrate photoreceptors. Annu. Rev. Neurosci. 2:17–34.

Jan, L. Y., and J.-P. Revel. 1974. Ultrastructural localization of rhodopsin in the vertebrate retina. J. Cell Biol. 62:257–273.

Jay, G., G. Khoury, A. B. DeLeo, W. G. Dippold, and L. J. Old. 1981. p53 transformation-related protein: detection of an associated phosphotransferase activity. Proc. Natl. Acad. Sci. USA. 78:2932–2936.

Kiehart, D. P., I. Mabuchi, and S. Inoue. 1982. Evidence that myosin does not contribute to force production in chromosome movement. J. Cell Biol. 94:165–178.

Kirschbaum, D. M. 1975. Molar absorptivity and A1% values for proteins at selected wave lengths of the ultraviolet and visible regions. Anal. Biochem. 56:237–263.

Kohler, G., and C. Milstein. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature (Lond.). 256:495–497.

Kremer, N. E., R. F. Hixon, W. L. Pak, and F. Wong. 1982. Monoclonal antibodies against squid rhodopsin. Invest. Ophthalmol. Visual Sci. 22:66. (Abstr.)

Kuhn, H. 1978. Light-regulated binding of rhodopsin kinase and other proteins to cattle photoreceptor membranes. Biochemistry. 17:4389–4395.

Kuhn, H. 1980. Light- and GTP-regulated interaction of GTPase and other proteins with bovine photoreceptor membranes. Nature (Lond.). 283:587–589.

Kuhn, H. 1981. Interactions of rod cell proteins with the disc membrane: influence of light, ionic strength, and nucleotides. Curr. Top. Membr. Transp. 15:174–201.

Lee, R. H., B. M. Brown, and R. N. Lolley. 1981. Protein kinases of retinal rod outer segments: identification and partial characterization of cyclic nucleotide dependent protein kinase and rhodopsin kinase. Biochemistry. 20:7532–7538.

MacKenzie, D., and R. S. Molday. 1982. Organization of rhodopsin and a high molecular
weight glycoprotein in rod photoreceptor disc membranes using monoclonal antibodies. J. Biol. Chem. 257:7100–7105.

Mancillas, J. R., J. F. McGinty, A. I. Selverston, H. Karten, and F. E. Bloom. 1981. Immunocytochemical localization of enkephalin and substance P in retina and eyestalk neurones of lobster. Nature (Lond.). 293:576–578.

Markwell, M. A. K., and C. F. Fox. 1978. Surface-specific iodination of membrane proteins of viruses and eucaryotic cells using 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril. Biochemistry. 17:4807–4817.

Molday, R. S., and D. MacKenzie. 1983. Monoclonal antibodies to rhodopsin: characterization, cross-reactivity, and application as structural probes. Biochemistry. 22:653–660.

Osborn, M., and K. Weber. 1982. Immunofluorescence and immunocytochemical procedures with affinity purified antibodies: tubulin-containing structures. Methods Cell Biol. 24:97–125.

Papermaster, D. S., B. G. Schneider, M. A. Zorn, and J. P. Kraehenbuhl. 1978a. Immunocytochemical localization of opsin in outer segments and Golgi zones of frog photoreceptor cells. J. Cell Biol. 77:196–210.

Papermaster, D. S., B. G. Schneider, M. A. Zorn, and J. P. Kraehenbuhl. 1978b. Immunocytochemical localization of a large intrinsic membrane protein to the incisures and margins of frog rod outer segment disks. J. Cell Biol. 78:415–425.

Pober, J. S., and M. W. Bitensky. 1979. Light-regulated enzymes of vertebrate retinal rods. Adv. Cyclic Nucleotide Res. 11:265–301.

Polans, A. S., J. Hermolin, and M. D. Bownds. 1979. Light-induced dephosphorylation of two proteins in frog rod outer segments. Influence of cyclic nucleotides and calcium. J. Gen. Physiol. 74:595–613.

Shichi, H., and R. L. Somers. 1978. Light-dependent phosphorylation of rhodopsin. Purification and properties of rhodopsin kinase. J. Biol. Chem. 253:7040–7046.

Soos, M., and K. Siddle. 1982. Characterization of monoclonal antibodies directed against human thyroid stimulating hormone. J. Immunol. Methods. 51:57–68.

Stryer, L., J. B. Hurley, and B. K.-K. Fung. 1981. First stages of amplification in the cyclic-nucleotide cascade of vision. Curr. Top. Membr. Transp. 15:93–108.

Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA. 76:4350–4354.

Trisler, G. D., M. D. Schneider, and M. Nirenberg. 1981. A topographic gradient of molecules in retina can be used to identify neuron position. Proc. Natl. Acad. Sci. USA. 78:2145–2149.

Trucco, M., and S. dePetris. 1981. Determination of equilibrium binding parameters of monoclonal antibodies specific for cell surface antigens. In Immunological Methods. Vol. II. I. Lefkovits and B. Permis, editors. Academic Press, Inc., New York. 1–26.

Tzartos, S. J., D. E. Rand, B. L. Einarson, and J. M. Lindstrom. 1981. Mapping of surface structures of Electrophorus acetylcholine receptor using monoclonal antibodies. J. Biol. Chem. 256:8635–8645.

Wells, J. N., and G. L. Kramer. 1981. Phosphodiesterase inhibitors as tools in cyclic nucleotide research: a precautionary comment. Mol. Cell. Endocrinol. 23:1–9.

Wong, F., R. B. Leonard, N. E. Kremer, and R. M. Denney. 1982. Photoreceptor specific monoclonal antibodies. Invest. Ophthalmol. Visual Sci. 22:205. (Abstr.)