

# 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  $^{125}\text{I}$ -labeled G-protein of  $<10^{-6} \text{ M}^{-1}$ . Of 11 antibodies to frog or bovine G-protein tested in solid phase and Western blot assays, all bind to the  $\alpha$  rather than the  $\beta$  or  $\gamma$  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

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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, 1978*b*; 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., 1978*a*; MacKenzie and Molday, 1982; Wong et al., 1982), the 220,000-dalton protein (Papermaster et al., 1978*b*; 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 MgCl<sub>2</sub>, 0.1 mM CaCl<sub>2</sub>, 10 mM HEPES, pH

7.5) and purified on a density gradient of Percoll (M. S. Biernbaum and M. D. Bownds, manuscript submitted for publication).

#### *Preparation of Antigen*

For the fusion in which rhodopsin antibodies were obtained, BALB/c mice were injected intraperitoneally with  $\sim 3 \times 10^4$  Percoll-purified ROS (60  $\mu\text{g}$  protein) in complete Freund's adjuvant, and 3–4 wk later were reimmunized with  $\sim 10^5$  ROS (160  $\mu\text{g}$  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  $\mu\text{g}$  protein in the first injection and 60  $\mu\text{g}$  in the second. The soluble protein fraction ( $\sim 25$  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  $\mu\text{g}$  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  $10^8$  spleen cells was mixed with  $10^7$  NS-1 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  $\mu\text{M}$  hypoxanthine, 0.5  $\mu\text{M}$  amethopterin, 20  $\mu\text{M}$  thymidine, 50  $\mu\text{M}$   $\beta$ -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  $\sim 2$  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 (Immulon I; Dynatech Laboratories, Inc., Alexandria, VA) were coated with 0.3–0.5  $\mu\text{g}$ /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  $\mu\text{l}$  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  $\text{ZnCl}_2$ , 1.0 mM  $\text{MgCl}_2$ , 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  $\mu\text{l}$ /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  $\mu\text{M}$   $\text{ZnCl}_2$ , 1.25 mM  $\text{MgCl}_2$ , 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

$\mu$ l 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  $A_{1\text{cm}}^{1\%} = 13.5 \times 10^{-3}$  (Kirschenbaum, 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 35 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,  $^{125}\text{I}$ -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)  $^{125}\text{I}$ -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  $\mu\text{Ci/ml}$   $^{125}\text{I}$ -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  $^{125}\text{I}$ -protein A, 10 ng/ml antibody was detected, and using the alkaline phosphatase detection system,  $\sim 1$   $\mu\text{g/ml}$  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 radioiodinated as follows (Markwell and Fox, 1978). 20  $\mu$ g of G-protein in 200  $\mu$ l Tris buffer plus 40  $\mu$ M GTP was placed in a 1.0-ml glass centrifuge tube coated with 10  $\mu$ g Iodogen (1,3,4,6-tetrachloro-3 $\alpha$ ,6 $\alpha$ -diphenylglycoluril; Pierce Chemical Co., Rockford, IL) and incubated with 200  $\mu$ Ci of Na<sup>125</sup>I (carrier-free; New England Nuclear) at room temperature for 15 min. Unreacted Na<sup>125</sup>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

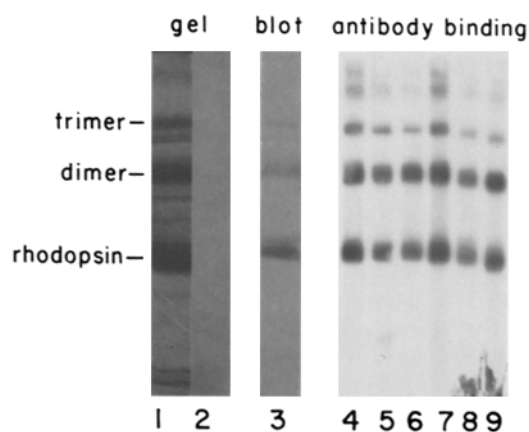


FIGURE 1. Binding of anti-rhodopsin antibodies to replica blots of ROS proteins. The Coomassie-stained polypeptides of ROS are shown before (lane 1) and after (lane 2) electrophoretic blotting to nitrocellulose paper, demonstrating the almost complete transfer of proteins. A replica blot stained with amido black is also shown (lane 3). Blots were incubated with six different anti-rhodopsin antibodies (lanes 4–9) and binding was detected by  $^{125}\text{I}$ -protein A. Antibodies bind to rhodopsin monomers and oligomers and to no other polypeptides.

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-

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

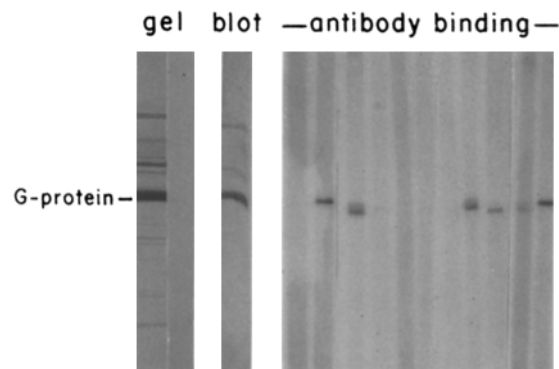


FIGURE 2. Binding of anti-G-protein antibodies to replica blots of soluble and peripheral membrane proteins from frog ROS. On the left, lanes of the Coomassie-stained 10–20% polyacrylamide gradient gels are shown before and after electrophoretic blotting. G-protein is the major polypeptide. The “blot” lane is a replica blot stained with amido black. On the right are 11 replica lanes that were incubated with different antibodies to G-protein. Five strong-binding and one medium-binding antibody to G-protein were detected by  $^{125}\text{I}$ -protein A. The weak binding of the other five antibodies is not visible in the photograph. The slight displacements of the bands are due to misalignments of the blots, and do not indicate staining of  $G_\alpha$  vs.  $G_\beta$  subunits of the G-protein, which are not separated on these gradient gels (see Fig. 3). No binding to other polypeptides was observed.

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 $\alpha$ Subunit*

All antibodies tested bound to the  $\alpha$  subunit rather than the  $\beta$  or  $\gamma$  subunits of G-protein from both frog and bovine sources. In a solid phase assay against purified bovine  $G_\alpha$  or  $G_{\beta\gamma}$  adsorbed to 96-well plates, all 11 antibodies tested bound to the  $G_\alpha$  rather than  $G_{\beta\gamma}$  subunits (data not shown). The purified antibody

(designated 4A) tested in Western blots bound only to the  $G_\alpha$  subunit of purified bovine G-protein and not to the  $\beta$  or  $\gamma$  subunits (Fig. 3, lanes 8 and 9). This antibody also bound to  $G_\alpha$  rather than to the  $G_\beta$  or  $G_\gamma$  subunits on Western blots of frog peripheral membrane proteins separated on 10% polyacrylamide gels, where the three subunits can be clearly distinguished (Fig. 3).

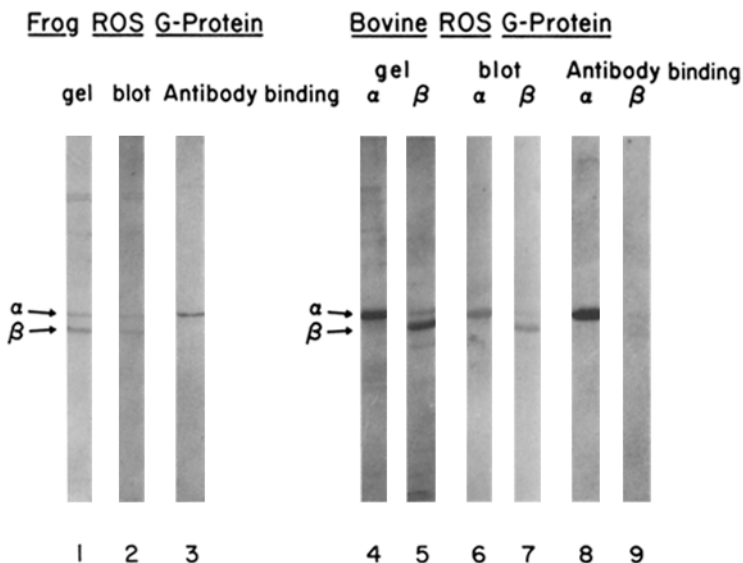


FIGURE 3. Binding of anti-G-protein antibody to replica blots of frog and bovine ROS proteins. Lane 1 shows a Coomassie-stained 10% polyacrylamide gel of frog ROS proteins in which  $G_\alpha$ ,  $G_\beta$ , and  $G_\gamma$  (not visible here) are separated. Lane 2 is a replica blot of lane 1 stained 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  $G_\alpha$ . Lanes 4 and 5 show Coomassie-stained polyacrylamide gels of purified bovine  $G_\alpha$  and  $G_{\beta\gamma}$ , and lanes 6 and 7 are the amido black-stained blots of  $G_\alpha$  and  $G_{\beta\gamma}$ . When identical blots are incubated with antibody 4A and stained as in lane 3, antibody binds strongly to the blot of  $G_\alpha$  (lane 8) but not to  $G_\beta$  (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  $^{125}\text{I}$ -labeled G-protein was quantitated.  $2.5 \times 10^{-7}$  M antibody was reacted with  $5 \times 10^{-7}$ – $1 \times 10^{-8}$  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  $<10^6$   $\text{M}^{-1}$ , which is consistent with the finding that 100% inhibition of phosphodiesterase activity by antibody is obtained at concentrations of  $\sim 10^{-5}$ – $10^{-6}$  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.

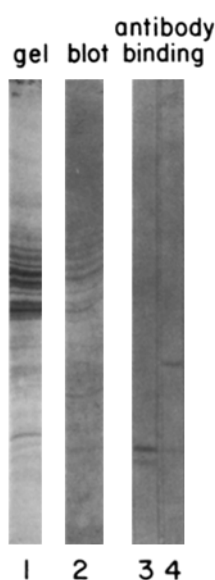


FIGURE 4. Binding of antibodies to replica blots of soluble proteins. The protein pattern of soluble proteins is shown on the gel after Coomassie staining (lane 1) and in the amido black-stained replica blot (2). Lanes 3 and 4 were incubated with two different antibodies to soluble proteins. The antibody shown in lane 3 (designated 5A) binds to polypeptides of ~12,000 and 14,000 daltons, and the antibody shown in lane 4 (5C) binds to polypeptides of ~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, ~150 wells were positive for antibody production in the initial solid phase assay against soluble proteins. Of these, ~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 ~12,000 and 14,000 daltons and the other, 5C, binds to proteins of ~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  $10^3$  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. 5*a*). Two anti-G-protein antibodies (4A and 4H) also stained the ROS (4A is shown in Fig. 5*b*). 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.

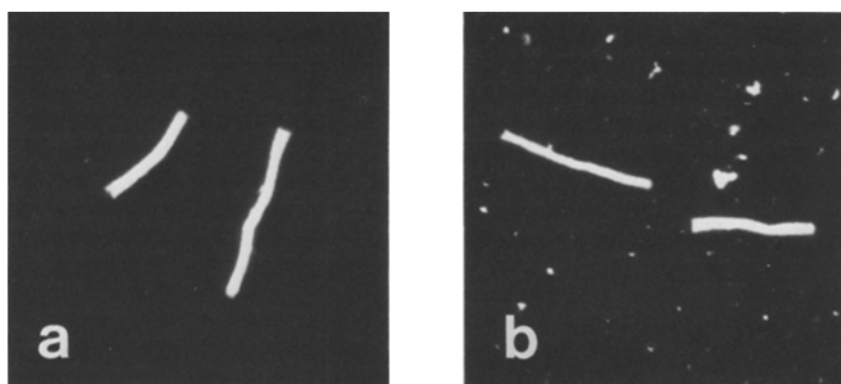


FIGURE 5. Immunofluorescent staining of isolated rod outer segments. Binding of both anti-rhodopsin (*a*) and anti-G-protein (*b*) antibodies to fixed, isolated ROS was detected by FITC second antibody staining. The ROS appear uniformly fluorescent, which indicates a uniform distribution of both antigens. The small fluorescent particles were seen in some preparations (*b*) and are probably aggregated second antibody molecules.  $\times 475$ .

## 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  $G_{\alpha}$  subunit. It is the  $G_{\alpha}$  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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