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Expression of a synthetic bovine rhodopsin gene in monkey kidney cells

(transducin/11-*cis*-retinal/COS-1 cells/transfection/immunoaffinity chromatography)

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ABSTRACT **We report here the high-level expression of a synthetic gene for bovine rhodopsin in transfected monkey kidney COS-1 cells. Rhodopsin is produced in these cells to a level of 0.3% of the cell protein, and it binds exogenously added 11-*cis*-retinal to generate the characteristic rhodopsin absorption spectrum. We describe a one-step immunoaffinity procedure for purification of the rhodopsin essentially to homogeneity. The COS-1 cell rhodopsin activates the GTPase activity of bovine transducin in a light-dependent manner with the same specific activity as that of purified bovine rhodopsin. Electron microscopy of immunogold-stained cells indicates that rhodopsin is located in the plasma membrane of the transfected cells and is oriented with the amino terminus on the extracellular side of the membrane. This orientation is analogous to that of rhodopsin in the disk membranes of photoreceptor cells in the bovine retina.**

Rhodopsin is the photoreceptor protein of vertebrate retinal rod cells (1, 2). Upon absorption of light, rhodopsin undergoes a structural change that allows it to activate the GTP-binding protein, transducin, and thus initiate a sequence of events that results in the hyperpolarization of the rod cell. Light transduction and its regulation is evidently mediated by a number of proteins in the rod outer segment (ROS).

Bovine rhodopsin consists of a polypeptide chain of 348 amino acids whose sequence is known by both protein and DNA sequencing (3–5). 11-*cis*-Retinal linked as a Schiff base to the ε-amino group of Lys-296 serves as the chromophore. The primary event following the capture of a photon by rhodopsin is the isomerization of 11-*cis*-retinal to all-*trans*-retinal. However, little is known about the nature of the structural changes induced in rhodopsin by this isomerization, the consequent interaction with transducin, or the mechanism of light/dark adaptation. We wish to study these questions by carrying out specific amino acid substitutions in the rhodopsin molecule by using recombinant DNA techniques. For site-specific mutagenesis, we have previously synthesized a gene for bovine rhodopsin that contains a suitable number of conveniently placed unique restriction sites (6, 7). These allow the replacement of specific restriction fragments by synthetic counterparts that contain the desired altered codons. The next requirement is the satisfactory expression of rhodopsin in its fully functional form. In this paper, we report on the high-level expression of the synthetic rhodopsin gene in mammalian cells using the expression vector p91023(B) (8, 9). The apoprotein (opsin) produced in these cells can be reconstituted by the addition of exogenous 11-*cis*-retinal. It has been purified essentially to homogeneity by a one-step immunoaffinity procedure and has been characterized.

MATERIALS AND METHODS

Materials. COS-1 monkey kidney cells (10) were obtained from Phillip Sharp (Cancer Center, Massachusetts Institute of Technology). 11-*cis*-Retinal was the gift of Peter Sorter (Hoffman-LaRoche, Nutley, NJ). Bovine retinas were purchased from the J. A. Lawson Co. (Lincoln, NE). Con A-Sepharose 4B was from Sigma. Digitonin, treated according to Bridges (11), was a gift of Tomoko Nakayama (Massachusetts Institute of Technology). 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) was from Calbiochem. Protein A was purchased from Miles. $Na^{125}I$ (16.4 mCi per μg of iodine; 1 mCi = 37 GBq) and [γ-^{32}P]GTP (30 Ci/mmol) were from Amersham.

Buffers and Media. Medium A was Dulbecco's modified Eagle's medium containing D-glucose (4.5 g/liter), streptomycin (100 mg/ml), penicillin (100 mg/ml), a supplement of 2 mM L-glutamine, and 10% heat-inactivated (56°C for 30 min) fetal calf serum; medium B was the same as medium A but without the serum. Phosphate-buffered saline (PBS) was 10 mM sodium phosphate buffer, pH 7/150 mM NaCl. Buffer A was 21 mM Hepes buffer, pH 7/140 mM $CaCl_2$/5 mM KCl/0.7 mM Na_2HPO_4/5.5 mM dextrose/10% dimethylsulfoxide; buffer B was 50 mM 3-[*N*-morpholino]propanesulfonate, pH 7.0/100 mM NaCl/1 mM $CaCl_2$/1 mM $MnCl_2$/1% CHAPS containing phenylmethylsulfonyl fluoride (0.1 mg/ml); buffer C was the same as buffer B but without phenylmethylsulfonyl fluoride; buffer D was the same as buffer C except that it contained 1% digitonin instead of 1% CHAPS; and buffer E was 10 mM Tris, pH 7.4/150 mM NaCl.

Antibodies. The polyclonal rabbit anti-rhodopsin IgG antibody used for rhodopsin immunoblots was the generous gift of Paul A. Hargrave (University of Florida). Rho 4D2, the monoclonal antibody specific for the amino terminus of rhodopsin, has been described (12). The monoclonal antibody, rho 1D4, specific for the carboxyl terminus of rhodopsin, has also been described (13). Rho 1D4 was purified from mouse ascites fluid by precipitation with 50% saturated ammonium sulfate followed by ion-exchange chromatography on DEAE-Sephacel. The antibody was >95% pure as determined by $NaDodSO_4$ gel electrophoresis. Peptide I (Asp-Glu-Ala-Ser-Thr-Thr-Val-Ser-Lys-Thr-Glu-Thr-Ser-Gln-Val-Ala-Pro-Ala, corresponding to the carboxyl-terminal 18 amino acids of bovine rhodopsin), which inhibits the binding of rhodopsin to the antibody rho 1D4 (14), was the generous gift of Michael Kopczynski and Chon Vo (Massachusetts General Hospital, Boston).

Rhodopsin and Transducin from Bovine Retina. ROS were prepared from frozen bovine retina by the procedure of Hong and Hubbell (15) as modified by Fung *et al.* (16). Rhodopsin

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Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; ROS, rod outer segment(s).
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was purified by chromatography on a Con A-Sepharose 4B column (17) in 1% digitonin. Transducin was isolated from bleached ROS as described by Fung et al. (16).

Coupling of the Monoclonal Antibody rho 1D4 to Sepharose 2B (18). Ten milliliters of packed Sepharose 2B was suspended in 10 ml of distilled water and was treated with 1 g of CNBr at pH 10–11. The mixture was stirred gently at 23°C, and the pH was maintained at 10–11 by dropwise addition of 1 N NaOH (over a period of 30 min). The Sepharose was then washed four times with 40 ml of cold 0.01 M sodium borate (pH 8.5). Approximately 14 mg of rho 1D4 was added to 7 ml of the packed CNBr-treated Sepharose 2B in 10 ml of 0.01 M borate buffer (pH 8.5). The mixture was shaken overnight at 4°C. Rho 1D4-Sepharose 2B was then washed with PBS containing 0.01 M glycine and stored in PBS containing 0.01 M NaN_3. Greater than 95% of the antibody was bound to the Sepharose as estimated by the protein remaining in the supernatant fraction.

Cloning of the Synthetic Rhodopsin Gene into the Expression Vector p91023(B). The vector p91023(B) (8, 9) contains a unique *Eco*RI restriction site for cloning downstream from the adenovirus major late promoter. For cloning into this site and expression, the synthetic rhodopsin gene was modified at the termini as follows (Fig. 1). The *Nar* I–*Bam*HI fragment at the 3′ end (nucleotides 1036 to 1048) was replaced with synthetic duplex II (Fig. 1). This substitution preserved the nucleotide sequence of the gene, including the *Bam*HI site immediately following the stop codon, TAA, at position 1045 and extended the sequence by the four bases AATT, which would allow the ligation of the gene into an *Eco*RI site. However, this ligation would not generate the full recognition sequence for *Eco*RI. Therefore, excision of the gene would require cleavage by *Bam*HI in addition to *Eco*RI.

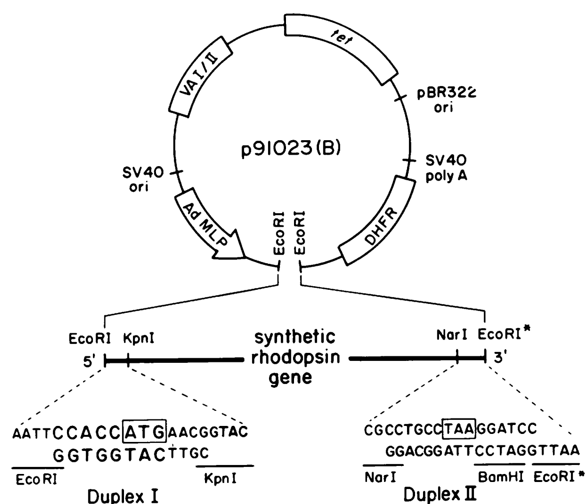

FIG. 1. A schematic diagram showing the main features of the expression vector p91023(B) and the strategy used to clone the synthetic rhodopsin gene into the vector. Ad MLP, major late promoter from adenovirus; DHFR, coding sequences for dihydrofolate reductase; SV40 poly A, polyadenylylation signal from simian virus 40 early region; pBR322 ori, pBR322 origin of replication; *tet*, tetracycline resistance gene from pBR322; VA I/II, viral-associated genes from adenovirus; SV40 ori, simian virus 40 origin of replication. For cloning of the synthetic rhodopsin gene into the unique *Eco*RI site of the vector, the termini of the gene were modified by ligating the synthetic duplexes I and II at the 5′ and 3′ ends of the gene, respectively. Duplex I was an *Eco*RI–*Kpn* I restriction fragment that contained a CCACC consensus sequence (ref. 19; bold-faced letters) immediately upstream of the translation initiation codon (boxed letters). Duplex II, an *Nar* I–*Eco*RI* restriction fragment containing the termination codon (boxed letters), was used to make the 3′ end of the gene compatible with the *Eco*RI site in the vector.

The *Eco*RI–*Kpn* I restriction fragment (nucleotides −6 to 11) at the 5′ end of the synthetic gene was replaced by the synthetic duplex I (Fig. 1). This placed the consensus sequence, CCACC (20), immediately upstream of the translation initiation codon in the gene with the expectation that this change would promote translation of the mRNA.

The synthetic rhodopsin gene was cloned into p91023(B) in a four-component ligation mixture that contained (*i*) the vector digested with *Eco*RI and then treated with calf intestinal phosphatase, (*ii*) and (*iii*) synthetic duplexes I and II with their 5′ ends phosphorylated, and (*iv*) a *Kpn* I–*Nar* I restriction fragment comprising the nucleotide sequences for codons Gly-3 to Ala-346 of the rhodopsin gene. The ligation mixture was used to transform $CaCl_2$-treated *Escherichia coli* DH1 cells. Plasmid DNA was prepared from tetracycline-resistant colonies (tetracycline at 5 μg/ml) and screened for the presence of the rhodopsin gene by endonuclease digestion with *Eco*RI and *Bam*HI. Positive clones were characterized by restriction enzyme digestion and DNA sequence analysis to confirm that the correct orientation of the gene in relation to the promoter had been achieved and that only a single copy each of duplex I and duplex II had been inserted into the plasmid. One of these clones was selected for transfection of COS-1 cells and expression of the rhodopsin gene.

Transfection of COS-1 Cells. COS-1 cells were grown under an atmosphere of 5% CO_2 at 37°C in medium A. Eighteen hours before treatment with DNA, the cells were plated at a density of 5×10^6 cells per 10-cm tissue culture plate and were grown overnight in medium A. Each plate of cells was washed twice with 5 ml of medium B and then transfected with 4 ml of medium B containing 8 μg of DNA, 0.1 M Tris (pH 8.0), and DEAE-dextran at 0.25 mg/ml (19). Following incubation for 6 hr at 37°C, the cells were washed with 5 ml of medium B and then incubated for 3 min at room temperature in 2 ml of buffer A (21). The cells were then treated with 0.1 mM chloroquine (22) in medium A (5 ml/plate) for 2 hr at 37°C. They were washed twice with medium B and then incubated overnight in medium A. Fresh medium A was provided, and the incubation was continued for 48 hr before harvesting the cells.

Preparation of Cell Extracts. Cells from a single 10-cm culture plate were washed twice with 5 ml of PBS while they were still attached to the culture dish, then dislodged by scraping with a rubber policeman in the presence of 1 ml of PBS, and collected by centrifugation in an Eppendorf microfuge for a few seconds. The cell pellet was washed once with 1 ml of PBS and resuspended in 1 ml of PBS containing 40 μM 11-*cis*-retinal. This addition and all subsequent manipulations were done in the dark under light from a 15-watt bulb filtered through a Kodak no. 2 safelight filter. After incubation with 11-*cis*-retinal for 1 hr at room temperature, the cells were collected by centrifugation. The cell pellet, containing about 10^7 cells, was resuspended in 1 ml of buffer B at 4°C. Following a 10-min incubation on ice, the nuclei were removed by centrifugation, and the postnuclear supernatant fraction was used for the purification of rhodopsin.

Purification of Rhodopsin. To the combined postnuclear supernatant fractions (about 10 ml) from 10 culture plates ($\approx 10^8$ cells) was added 0.2 ml of packed rho 1D4-Sepharose 2B (1.4 mg of rho 1D4 per ml). The mixture was agitated by gentle rotation overnight at 4°C. The gel was collected by centrifugation, transferred to a 1.5-ml Eppendorf centrifuge tube, and washed four times with 1 ml of buffer B followed by four washes with 1 ml of either buffer C or buffer D. After addition of 0.4 ml of 50 μM peptide I in either buffer C or buffer D, the gel suspension was incubated for 1 hr at room temperature while being agitated. The peptide I eluate was then separated by centrifugation, and the gel was washed with an additional 0.4 ml of elution buffer. Peptide I was separated from the eluted protein either by selective absorp-

tion of rhodopsin on concanavalin A-Sepharose 4B or by filtration on Sephadex G-50.

Functional Assay of Rhodopsin. Rhodopsin was assayed for light-dependent activation of the GTPase activity of bovine transducin (23). The assay mixture (100 μl) contained 0.1 M NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 0.1% digitonin, 145 pmol of transducin, 1–8 pmol of rhodopsin (5.5 pmol of rhodopsin purified from transfected COS-1 cells), 1.32 μM [γ-^{32}P]GTP (1–20 Ci/mmol), and 20 mM Tris·HCl (pH 7.5). The reaction was initiated (25°C) by the addition of GTP. Four 20-μl aliquots were removed from the reaction mixture at appropriate time points and were assayed for [γ-^{32}P]phosphate released (24). Reaction rates were corrected for a 0.053 min^{-1} first-order decay of activated rhodopsin, which was measured independently under identical assay conditions (Roland Franke and H.G.K., unpublished data). Light reactions were initiated 1 min after exposure to room light, whereas dark reactions were conducted under light from a 15-watt incandescent bulb passed through a Kodak no. 2 safelight filter.

Immunogold-Dextran Labeling of COS-1 Cells. Transfected COS-1 cells grown on glass coverslips were rinsed in PBS and fixed in 1.25% glutaraldehyde/PBS for 1 hr at 25°C. The cells were then washed in PBS and incubated in PBS containing 0.01 M glycine for 1 hr. For indirect labeling, the cells were treated with hybridoma culture fluid containing the antibody rho 4D2. After 1 hr, the cells were washed in buffer E and treated for 1 hr with an immunogold-dextran conjugate consisting of goat anti-mouse immunoglobulin coupled to gold-dextran particles (11, 25). The cells were then washed in buffer E, refixed in 1.25% glutaraldehyde/PBS, postfixed in 1% OsO_4, dehydrated in ethanol, and embedded in Epon/Araldite for electron microscopy.

Other Methods. Procedures for oligonucleotide synthesis, cloning, and sequencing of DNA fragments were as described (6). Polyacrylamide gel electrophoresis of proteins was performed according to Laemmli (26). Silver staining of proteins on polyacrylamide gels was according to Wray *et al.* (27). Protein was determined by the method of Lowry *et al.* (28) as modified by Bensadoun and Weinstein (29). Immunoblotting was performed according to Burnette (30). Protein A was labeled with $Na^{125}I$ using chloramine T (31).

RESULTS

Expression of the Synthetic Rhodopsin Gene. Cell extracts were prepared 72 hr after transfection, and the postnuclear supernatant fraction was examined by immunoblotting. As shown in Fig. 2*A*, only the cells transfected with the vector containing the rhodopsin gene gave a positive response (lane 3), whereas cells that had been mock-transfected (lane 1) or transfected with the vector without the rhodopsin gene (lane 2) did not react with the anti-rhodopsin antibody. COS-1 cell rhodopsin migrated slightly slower than ROS rhodopsin and could form a smear. The tendency of the protein to smear on $NaDodSO_4$ gels was variable and sometimes much less evident (Fig. 2*B*, lane 2).

The amount of rhodopsin expressed in transfected cells was 5–10 μg per 10^7 cells as judged from a comparison of the immunoblot signal from the COS-1 cell extract and that from a known amount of ROS rhodopsin on the same gel.

Purification of Rhodopsin from COS-1 Cells. Transfected cells ($\approx 10^8$ from ten 10-cm tissue-culture plates) were harvested and incubated in the dark with 10 ml of PBS containing 20 μM 11-*cis*-retinal. After solubilization of the cells with 10 ml of buffer B, rhodopsin was purified from the postnuclear supernatant fraction by treatment with rho 1D4-Sepharose 2B (0.28 mg of rho 1D4) (see *Materials and Methods*). Rhodopsin was eluted from the antibody matrix by incubation for 1 hr with 0.4 ml of 50 μM peptide I. The total protein

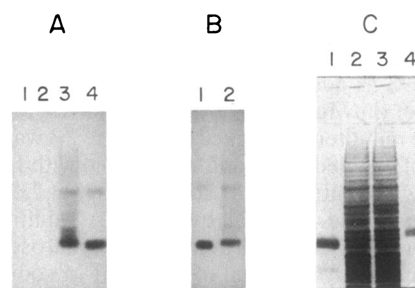

FIG. 2. (*A*) Immunoblot analysis of rhodopsin expressed in COS-1 cells transfected with the vector p91023(B) containing the synthetic rhodopsin gene. Postnuclear supernatant fractions (30 μl, corresponding to about 3×10^5 cells) from cells transfected without DNA (mock transfected; lane 1), with vector alone (lane 2), or with vector containing the synthetic rhodopsin gene (lane 3) were subjected to $NaDodSO_4$/10% polyacrylamide gel electrophoresis and then immunoblotted. Lane 4 contained 107 ng of rhodopsin from ROS as a marker. (*B*) Same as *A*, but showing results from another experiment. Lanes: 1, 107 ng of rhodopsin from ROS; 2, 30 μl of postnuclear supernatant fraction from COS-1 cells transfected with the synthetic rhodopsin gene. (*C*) Analysis of fractions obtained during purification of rhodopsin from transfected COS-1 cells by $NaDodSO_4$/polyacrylamide gel electrophoresis. Protein was visualized by silver stain. Lanes: 1, 100 ng of bovine rhodopsin; 2, postnuclear supernatant fraction ($\approx 1 \times 10^5$ cells); 3, proteins that did not bind to rho 1D4-Sepharose 2B (about 1×10^5 cells); 4, fraction eluted with peptide I (about 1×10^6 cells). The sample applied to lane 4 contained ten times more protein than that applied in lanes 2 and 3.

in the postnuclear supernatant, the protein fraction eluted by the peptide, and the proteins not bound to the antibody matrix were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis and visualized by silver staining (Fig. 2*C*). Rhodopsin as eluted by peptide I was essentially homogeneous, migrating slightly slower than rhodopsin from ROS.

Spectral Characterization of Expressed Rhodopsin. The absorption spectrum of a typical rhodopsin preparation from COS-1 cells is shown in Fig. 3. The absorption maximum is at 500 nm, which is characteristic of the pigment isolated from ROS. Based on the absorbance at 500 nm and using an absorption coefficient of 42,700 $M^{-1}\cdot cm^{-1}$ at 498 nm (15), the total recovery was 50 μg from 10^8 cells. The ratio of the absorbance at 280 nm to that at 500 nm was 3.2 for this sample, whereas a lower ratio (2.9) was observed for another preparation. Rhodopsin prepared from ROS has a ratio of 1.6–1.7 (32). The data would suggest that 50% of the protein in our preparation has been reconstituted with 11-*cis*-retinal to form rhodopsin. This agrees with the specific content of 19 nmol of 11-*cis*-retinal per mg of protein determined for this preparation. A specific content of 26 nmol/mg would indicate theoretical purity of rhodopsin. The interpretation that the protein is pure but only 50% has combined with the retinal is supported by the fact that after a second absorption to and elution from rho 1D4-Sepharose 2B or chromatography on a Con A-Sepharose 4B, the spectral ratio was unaltered.

Light Activation of the COS-1 Cell Rhodopsin. COS-1 cell rhodopsin activated the GTPase activity of bovine transducin in a light-dependent manner. Using limiting concentrations of the rhodopsin, its specific activity was determined and compared to that of bovine rhodopsin. Thus, in the experiment of Fig. 4*A*, 5.5 pmol of COS-1 cell rhodopsin was used in the presence of 145 pmol of bovine transducin. The reaction was initiated by the addition of [γ-^{32}P]GTP either in the dark or 1 min after exposure to room light and was assayed for the release of ^{32}P-labeled inorganic phosphate. As seen in Fig. 4*A*, the hydrolysis of [γ-^{32}P]GTP was light-dependent; the activity in light (hydrolysis of 4.3 pmol of GTP per min) was about 10-fold greater than the dark reaction (0.45 pmol of GTP per min). The rates for the light-dependent

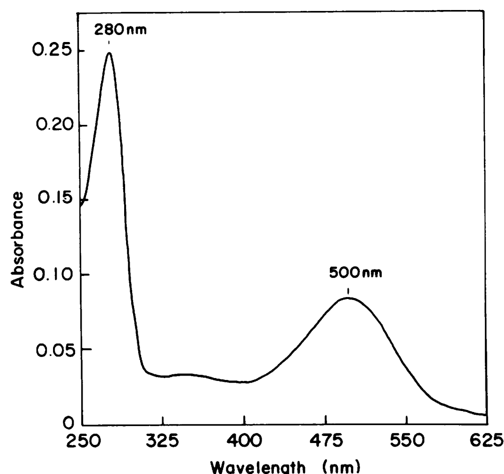

FIG. 3. Absorption spectrum of rhodopsin expressed in and purified from COS-1 cells. The sample (29 μg) used to record the spectrum was 58% of the total amount of rhodopsin that was obtained from ten 10-cm plates of COS-1 cells. The ratio of absorbance at 280 nm to that at 500 nm was 3.2.

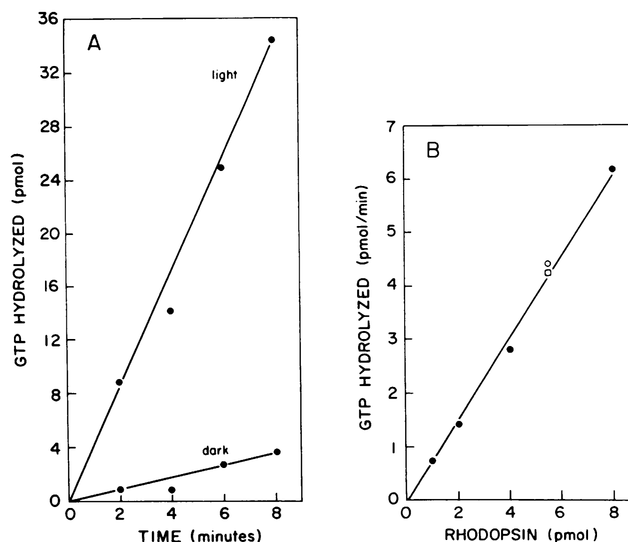

FIG. 4. COS-1 cell rhodopsin activates the GTP hydrolysis activity of bovine transducin. (*A*) Time course for the hydrolysis of GTP by bovine transducin as catalyzed by rhodopsin purified from transfected COS-1 cells. GTP hydrolysis was measured as described in *Materials and Methods* for the reaction catalyzed by ROS rhodopsin. Both the light and dark reactions were performed with 5.5 pmol of COS-1 cell rhodopsin. The reaction rate for the light reaction was 4.3 pmol of GTP hydrolyzed per min, whereas that for the dark reaction was 0.45 pmol of GTP per min. (*B*) Specific activity of COS-1 cell rhodopsin. The reaction rate for hydrolysis of GTP by transducin (light reaction) was plotted as a function of the amount of ROS rhodopsin used in the assay (●). The specific activity for rhodopsin was determined to be 0.75 min^{-1} from the slope of the line. The reaction rate determined in *A* for the COS-1 cell rhodopsin (○) was also plotted in *B* and falls exactly on the line defined for reactions with the ROS protein. Therefore, the specific activity of the COS-1 cell rhodopsin is identical to that of the naturally occurring protein isolated from ROS. Since the COS-1 cell rhodopsin assays contained residual peptide I (5 μM) from the purification procedure, a control was performed to demonstrate that the peptide had no effect on the reaction under these conditions. The rate of reaction determined for 5.5 pmol of ROS rhodopsin in the presence of 5 μM peptide I (□) falls on the line observed for the reaction in the absence of peptide.

activation of transducin by bovine rhodopsin plotted as a function of the amount of rhodopsin are shown in Fig. 4*B*. The rate of GTP hydrolysis was a linear function of the rhodopsin concentration under the conditions used. The ROS and COS-1 rhodopsin showed identical specific activity in this reaction.

Immunochemical Localization of Rhodopsin to the Plasma Membrane of Transfected COS-1 Cells. The transfected COS-1 cells were labeled by the indirect immunogold-dextran labeling method. As visualized by transmission electron microscopy, approximately 15% of the cells were labeled. (One labeled and one unlabeled cell are shown in the micrograph in Fig. 5). This would indicate that only 15% of the cells were expressing the synthetic rhodopsin gene. Although we cannot rule out the possibility that the protein was produced in unlabeled cells but not translocated to the cell surface, the deduced percentage of labeled cells agrees well with the expected transfection efficiency using the above-described procedure (19). The gold particles were randomly distributed along the surface of both the microvilli and cell body as illustrated in Fig. 5. COS-1 cells transfected with the vector without the rhodopsin gene did not show any labeling with the immunogold-dextran particles.

DISCUSSION

Expression of rhodopsin at high levels has been achieved by transfection of COS-1 monkey kidney cells with an expression plasmid p91023(B) (8, 9) harboring a synthetic rhodopsin gene. The protein produced in the cells binds exogeneously supplied 11-*cis*-retinal and generates the absorption spectrum characteristic of rhodopsin. This spontaneous reconstitution *in vivo* seems to be advantageous for the purification and maintenance of the protein in the correctly folded state. We have described a simple one-step immunoaffinity procedure for the purification of the rhodopsin produced in a fully functional form. The efficient purification seems to have resulted from a combination of the specific, high-affinity binding of rhodopsin to the antibody-Sepharose and its selective elution from the antibody with the synthetic peptide. The purified protein gave a single major band as judged by silver staining and immunoblotting.

Rhodopsin expressed in the COS-1 cells has been characterized in three different ways. First, on immunoblots the COS-1 cell protein gave a positive response with anti-rhodopsin antibody. Second, the visible absorption spectrum of the protein was the same as that of ROS rhodopsin. Third, in a functional assay, purified COS-1 cell rhodopsin showed light-dependent activation of bovine transducin in a manner indistinguishable from that of rhodopsin from bovine ROS.

The yield of COS-1 cell rhodopsin as determined from the absorbance of the purified protein at 498 nm using an absorption coefficient of 42,700 $M^{-1} \cdot cm^{-1}$ (15) was 5 μg from 10^7 cells. From this, we estimate that about 0.3% of the COS-1 cell protein in the postnuclear supernatant fraction is rhodopsin. However, this is a low estimate because it neglects the losses during purification and the protein that may not have combined with 11-*cis*-retinal. Indeed, as discussed above, our present interpretation is that only about 50% of the opsin present in the COS-1 cells reconstitutes with 11-*cis*-retinal. Further, we have determined that about 15% of the cells were successfully transfected with the synthetic rhodopsin gene. Therefore, it is reasonable to conclude that in the COS-1 cells expressing the synthetic rhodopsin gene about 4% of the cell protein is rhodopsin.

Polyacrylamide gel electrophoresis showed that the COS-1 cell rhodopsin migrated slightly more slowly than bovine rhodopsin. This may reflect differences in oligosaccharide structure or perhaps some other posttranslational modifications, such as covalent attachment of a fatty acid or acetylation of the amino-terminal NH_2 group. These possibilities need further study. Significantly, none of the COS-1 cell

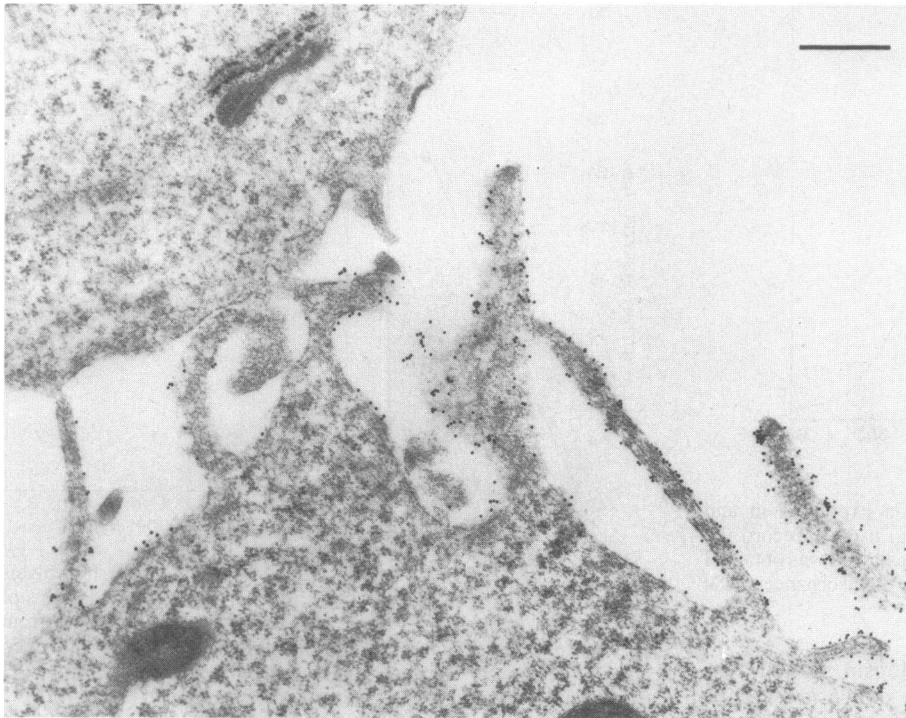

FIG. 5. Transmission electron micrograph of COS-1 cells indirectly labeled for rhodopsin with immunogold-dextran conjugates. COS-1 cells transfected with the p91023(B) vector containing the synthetic gene for rhodopsin were fixed in glutaraldehyde and labeled with monoclonal antibody rho 4D2 followed by goat anti-mouse immunogold-dextran particles (average diameter of 12 nm). The lower cell exhibits random labeling of gold particles on its surface; the upper cell shows no labeling. (Bar represents 0.5 μm.)

rhodopsin moves faster than bovine rhodopsin on electrophoresis indicating that glycosylation has taken place.

The opsin in the COS-1 cells probably integrates into the cell membrane(s). This is indicated, first, by the observation that it binds to concanavalin A-Sepharose quantitatively (data not shown); therefore, it must have been glycosylated in the lumen of the endoplasmic reticulum, which requires insertion into the membrane. Second, electron microscopy using immunogold labeling also indicates that rhodopsin resides in the plasma membrane of the COS-1 cells. The labeling experiments that use anti-rhodopsin antibody specific for the amino terminus also suggest that the latter is exposed on the extracellular surface of the COS-1 cells. This orientation is analogous to that of rhodopsin in the plasma membrane of the ROS (12).

Our previous attempts to obtain satisfactory levels of rhodopsin expression in *E. coli* and yeast systems have had little success. The rhodopsin gene expression and immunoaffinity purification procedure described here is highly encouraging for structure–function studies.

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