Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 10882–10886, November 1992
Cell Biology

Retinal rods and cones have distinct G protein β and γ subunits

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Communicated by George Wald, August 17, 1992

ABSTRACT **Guanine nucleotide-binding proteins (G proteins) involved in transmembrane signal-transduction processes are heterotrimers composed of α, β, and γ subunits. The α subunit shows great diversity and is thought to confer functional specificity to a particular G protein. By contrast, the β and γ subunits appear much less diverse; in particular, the β subunit is believed to have no role in G protein specificity. Using immunocytochemistry, we found distinct distribution patterns for different β and γ subunits in the retina. In particular, rod and cone photoreceptors, which both subserve phototransduction but differ in light-response properties, have different β and γ subunits in their outer segments. Thus, the G protein mediating phototransduction shows cell-specific forms of the β and γ subunits in addition to the α subunit. This surprising finding supports the hypothesis that these subunits may also contribute to functional specificity of a G protein.**

The family of guanine nucleotide-binding proteins (G proteins) mediates a wide variety of cellular signal-transduction processes triggered by hormones, neurotransmitters, and sensory stimuli (for review, see refs. 1–5). Structurally these proteins are heterotrimers, consisting of α, β, and γ subunits. The functional diversity of these proteins is thought to derive primarily from the α subunit, of which at least 16 species have been identified, based on biochemical purification or molecular cloning (4, 5). Their functions range from activation or inhibition of various enzymes—such as adenylate cyclase, cGMP phosphodiesterase, and phospholipase C—to modulation of ion channels. Less is known about β and γ subunits. These subunits are tightly bound to each other to form a complex and are only separable under denaturing conditions. The $\beta\gamma$ complex was initially thought to anchor the α subunit to the membrane, thereby facilitating its interaction with the receptor (6). It now appears, however, that the $\beta\gamma$ subunits may have additional or other roles, including regulation of the activated α subunit (1–5) and modulation of such effectors as ion channels (7–10) and the enzymes phospholipase A_2 and adenylyl cyclase (11–14). Nonetheless, the diversities of the β and γ subunits are still believed to be much less than that of the α subunit. Thus, $\beta\gamma$ complexes appear relatively interchangeable among different G proteins (15, 16). Some differences in properties between different $\beta\gamma$ complexes have been noticed and have been attributed largely to the γ subunit (17, 18), for which six species with a molecular size of 6–10 kDa have been identified (4, 5, 19–22). Biochemical studies have identified only two forms of β subunit; these have been designated β_{35} and β_{36} based on their apparent molecular masses of 35 and 36 kDa in gel electrophoresis (23–28). Subsequent molecular cloning has indicated that β_{35} and β_{36} subunits correspond to closely homologous, but distinct, proteins designated β_2 and β_1 (29–34). More recently, however, a third species, β_3 subunit, has been identified in the retina by molecular cloning; this subunit also shows considerable homology to β_1 and β_2 subunits (35). In addition, a β subunit has likewise been identified in *Drosophila* eye; this subunit differs from that in brain (36). To obtain further insight into the functional diversity of β and γ subunits, we have used anti-peptide antibodies to examine the localizations of β_1, β_2, and β_3 subunits as well as of four different γ subunits in the retina, a neural tissue with exceptionally well-understood morphology, physiology, and pharmacology. We find a distinct immunostaining pattern for each of these subunits. In particular, rod and cone photoreceptors have distinct β and γ subunits. This result is unexpected, considering that rods and cones have very similar physiological function, although they show some differences in light-response properties, and suggests that these subunits, in particular the β subunit, may not be as functionally promiscuous as has been believed.

MATERIALS AND METHODS

Antibodies. The anti-β_1 polyclonal antibody U-49 was generated by using a synthetic peptide (CEGNVRVSRELAGHTGY) corresponding to amino acid residues 130–145 of the β_1 molecule and coupled to keyhole limpet hemocyanin; an additional cysteine (underlined) was included at the amino terminus to facilitate coupling. The specificity of this antibody has been reported (33). Polyclonal antisera that react with β_2 (GC-2) and β_3 (B34) subunits were generated by immunization of rabbits with synthetic peptides CGDSTLTQITAGLD and CAELVSGLEVVGR, corresponding to amino acid residues 26–38 of β_2 subunit and 31–42 of β_3 subunit, respectively. The anti-γ antibodies were generated against synthetic peptides CEEFRDYVEERSE (A-4), CASNNTASIAQARK (A-75), CDLMAYCEAHAK (A-25), CKGETPVNSTMSIGQAR (B-53), and CDPLLVGVPASENPF (A-67), respectively. These peptides correspond to amino acid residues 37–49 of γ_1 subunit (transducin γ), 2–14 and 36–46 of γ_2 subunit (also referred to as γ_6 subunit; see refs. 20 and 21), 2–17 of γ_3 subunit (19), and 46–59 of another γ subunit that we term γ_7 subunit (21). We have not examined γ_4 subunit (19), only a partial amino acid sequence of which has been published, or γ_5, another γ subunit reported (22) while the present work was being prepared for publication. The antibodies A-75 and A-25 against the amino and carboxyl terminus of γ_2 subunit, respectively, gave identical results. All results described here were with antibodies that were not affinity-purified. We have obtained qualitatively similar results with affinity-purified antibodies for the β subunits, but the stainings were considerably weaker.

The specificities of all of the above antibodies have been examined with immunoblots of brain tissue. In each case, a single band of appropriate molecular mass for a G protein β subunit (35–36 kDa) or γ subunit (5–8 kDa) was stained (M.A.L. and J.D.R.; J.D.R., unpublished work). We have also attempted immunoblots on total protein preparations from bovine retinas but obtained stainings only with antibodies against β_1 and γ_1 subunits, probably because of the scarcity of the other β and γ subunits in the retina (see

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Results). The specificities of the immunostainings reported here, however, were supported by preadsorption experiments with uncoupled peptides (see *Results*).

The anti-red/green cone (COS-1) and anti-blue cone (OS-2) monoclonal antibodies, which apparently recognize the respective visual pigments (37), were obtained from P. Röhlich (Semmelweis University of Medicine, Budapest). Antibodies against tyrosine hydroxylase and protein kinase C were from Incstar (Stillwater, MN) and Amersham, respectively.

Immunocytochemistry. All immunostaining was done on frozen monkey retinal sections prefixed with 4% (vol/vol) paraformaldehyde (for details, see ref. 38). For immunoperoxidase staining, the sections were first incubated with 5% normal goat serum (Vector Laboratories) in phosphate-buffered saline (PBS) for 1 hr at room temperature to reduce background staining. The sections were then incubated with the primary antibody (1:1000 dilution for anti-β_1 and -β_2, 1:400 dilution for anti-β_3, and 1:1000 for all anti-γ) overnight at 4°C, followed by two washes in PBS for 30 min. Triton X-100 (0.3%) was added to all incubation and wash buffers. The sections were next incubated with a biotin-conjugated goat anti-rabbit secondary antibody (Vector Laboratories; 1:200 dilution) for 2 hr at room temperature and then washed twice in PBS for 30 min, followed by a 1-hr incubation with an avidin–biotin–peroxidase complex (Vector Laboratories; 1:100 dilution) in PBS. After two more washes for 30 min, the stain was developed with a substrate solution of 20 ml of PBS, 0.1 ml of 3% H_2O_2, and 10 mg of diaminobenzidine. The staining reaction was terminated by washing with PBS, and the sections were coverslipped with 50% (vol/vol) glycerol in PBS. In the preadsorption experiments, the primary antibody was incubated with the corresponding synthetic peptide in a peptide/antibody ratio of 1 mg/ml for at least 48 hr at 4°C before dilution and staining.

For double-labeling, the section was, after normal goat serum treatment, incubated with mixed primary antibodies (rabbit anti-β_3 polyclonal and mouse anti-cone monoclonal) overnight at 4°C. After two washes in PBS for 30 min, sections were incubated with mixed secondary antibodies [rhodamine-conjugated goat anti-rabbit IgG secondary antibody (1:50) and fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin secondary antibody (1:50)] for 2 hr at room temperature. The sections were then observed under a light microscope with rhodamine isothiocyanate- or fluorescein isothiocyanate-fluorescence excitation.

Although only observations on monkey retina are reported, we obtained the same results with rat, rabbit, and bovine retinas.

Preparation of Dissociated Retinal Cells. Dissociated retinal neurons were obtained as follows. In dim red light, the eyes of a dark-adapted cynomolgus monkey (*Macaca fascicularis*) were removed under deep anesthesia. The retinas were then dissected out, cut into small pieces, and put in Locke's solution [120 mM NaCl/3.6 mM KCl/1.2 mM $CaCl_2$/2.4 mM $MgCl_2$/20 mM $NaHCO_3$/0.02 mM Na-EDTA/3 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes)/10 mM dextrose, pH 7.4]. Dissociation of a piece of retina into individual cells was done in room light. The retina was first incubated for 45 min at 20°C, with gentle shaking, in Locke's solution minus divalent cations (pH 6.2) and supplemented with papain at 10 units/ml (Worthington), 1.2 mM EDTA, and 5.5 mM cysteine. The tissue was then washed with cold divalent-free Locke's solution (pH 7.4) containing bovine serum albumin (0.1 mg/ml). Dissociation of the treated retina into individual cells was then effected by gentle trituration with a wide-bore transfer pipette. Aliquots of freshly dissociated cells were placed in a test tube and fixed with 4% paraformaldehyde in phosphate buffer overnight at 4°C. The fixed cells were pipetted onto poly(D-lysine)-coated slides and left to settle for 2 hr. The subsequent immunostaining procedures were identical to those described for retinal sections.

RESULTS

β Subunits. Fig. 1*A* shows immunoperoxidase staining of a monkey retinal section with the anti-β_1 peptide antibody. Immunostaining is widespread, being particularly intense at the photoreceptor layer, the outer plexiform layer, and the inner plexiform layer. All immunostaining is abolished after preadsorption of antiserum with excess β_1 peptide (Fig. 1*B*), indicating specificity of the reactivity. The photoreceptor layer contains the outer and inner segments of the rod and cone receptors, but only the rods are stained. A single dissociated rod with intensely stained outer and inner segments is shown in Fig. 1*C*: the cell body and synaptic terminal of the cell are also stained; the latter accounts for most, if not all, of the staining at the (synaptic) outer plexiform layer. We also examined dissociated cone cells and confirmed their lack of immunoreactivity. The intense staining at the inner plexiform layer, which is the second synaptic layer, comes predominantly from the processes of amacrine cells. Fig. 1*D* shows the profuse, stained processes of a dissociated amacrine cell. About 60% of dissociated amacrine cells show β_1-subunit immunoreactivity. Finally, ≈20% or less of ganglion cells are stained (Fig. 1 *A* and *E*). Dissociated bipolar, horizontal, and Müller glial cells show no staining.

Fig. 2*A* shows another monkey retinal section stained with the anti-β_2 peptide antibody. Staining in this case is much weaker and very limited, being confined predominantly to the outer plexiform layer and discrete sublaminae of the inner

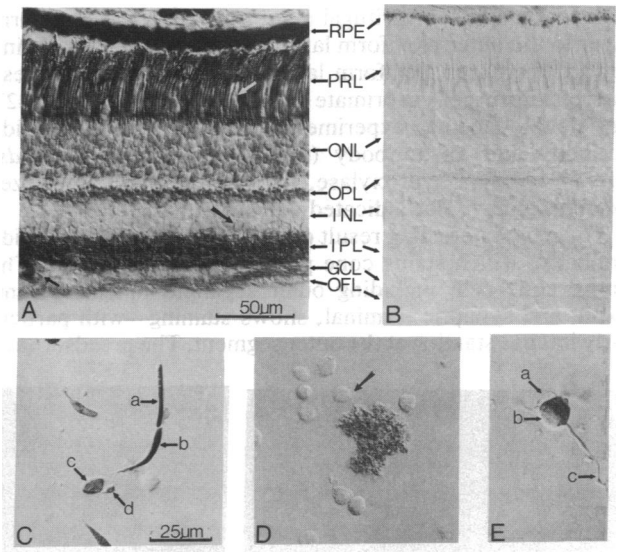

FIG. 1. (*A*) Immunoperoxidase staining of a cross-section of monkey retina with a rabbit polyclonal antibody against G protein β_1 subunit (Nomarski differential-interference-contrast optics; 8-μm frozen section). The anatomical layers are as follows: RPE, retinal pigment epithelium; PRL, photoreceptor layer (containing outer segments and inner segments of receptor cells); ONL, outer nuclear layer (containing cell bodies of photoreceptors); OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; OFL, optic fiber layer. Large black arrow indicates a stained amacrine cell body; small black arrow indicates a stained ganglion cell body; white arrow indicates a cone photoreceptor, which is not stained. (*B*) Absence of staining of monkey retina after adsorbing β_1 peptide to antibody before immunostaining. (*C–E*) Dissociated cells from monkey retina stained with anti-β_1 antibody (Nomarski optics). (*C*) Rod photoreceptor cell (a, outer segment; b, inner segment; c, cell body; d, synaptic terminal). (*D*) Amacrine cell (arrow indicates its cell body). (*E*) Ganglion cell (a, dendrite; b, cell body; c, axon).

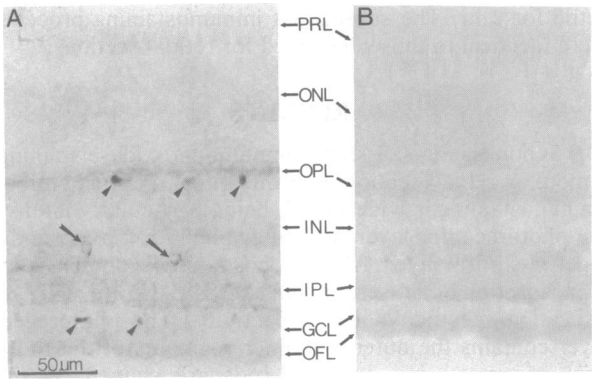

FIG. 2. (*A*) Immunostaining of monkey retinal section with a rabbit polyclonal antibody against G protein β_2 subunit (8-μm frozen section). Anatomical layers are the same as in Fig. 1*A*. Arrows indicate stained amacrine cell bodies. Arrowheads indicate blood vessels. (*B*) Control retinal section stained with anti-β_2 antibody preadsorbed with β_2 peptide.

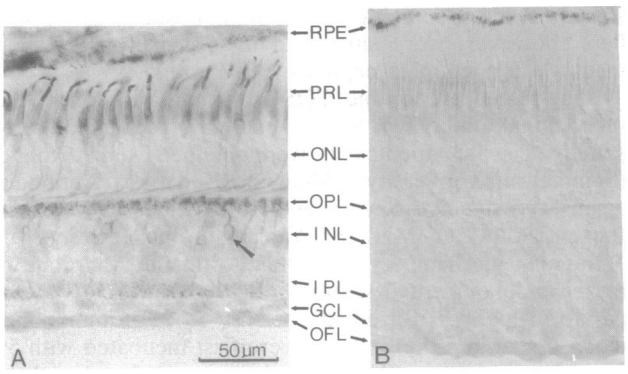

FIG. 3. (*A*) Monkey retinal section stained with a rabbit polyclonal antibody against G protein β_3 subunit (Nomarski optics; 8-μm frozen section). Anatomical layers are the same as in Fig. 1*A*. Arrow indicates a stained bipolar cell. (*B*) Control section stained with anti-β_3 antibody preadsorbed with β_3 peptide (Nomarski optics).

plexiform layer. Preadsorption of β_2 antiserum with excess β_2 peptide again abolishes staining in both locations (Fig. 2*B*). The stained round structures (arrowheads) in Fig. 2*A* are blood vessels. The restricted presence of β_2 subunit in the retina may explain why previous work has detected little or no β_2 mRNA in the retina (39). Staining at the inner plexiform layer appears to arise from a small population of amacrine cells (arrows in Fig. 2*A*). The origin of staining at the outer plexiform layer, on the other hand, is unclear. Stained slender processes can sometimes be seen originating from cell bodies at the inner nuclear layer, possibly interplexiform cells that mediate centrifugal input from the inner plexiform layer to the outer plexiform layer (see ref. 40) and extending toward the outer plexiform layer. One population of these interplexiform cells in primate retina is dopaminergic (41, 42), but double-labeling experiments with the anti-β_2 peptide antibody and an antibody (see *Materials and Methods*) against tyrosine hydroxylase, the enzyme that synthesizes dopamine, have not indicated colocalization.

The most interesting result comes from the anti-β_3 peptide antibody, which stains cone photoreceptors (Fig. 3*A*). The entire cone cell, including outer segment, inner segment, axon, and synaptic terminal, shows staining—with particularly intense staining at the outer segment. The preadsorption experiment (Fig. 3*B*) confirms the specificity. As in humans, the macaque monkey has three classes of cones—namely, red-, green-, and blue-sensitive cones; blue cones are very much in the minority (43, 44). Even though the staining in Fig. 3*A* suggests that all three cone classes react, we used double-labeling immunofluorescence to obtain more convincing evidence. Fig. 4*A Top* shows a monkey retinal section treated with the anti-β_3 peptide antibody and secondarily labeled with a rhodamine-conjugated, goat anti-rabbit antibody. Fig. 4*A Bottom* shows the same section labeled with an anti-red/green cone monoclonal antibody (COS-1, see ref. 37) together with a fluorescein-conjugated, goat anti-mouse secondary antibody. The anti-β_3 peptide antibody labeled all cones recognized by the anti-red/green cone antibody, but in addition it recognized another cone cell (arrow) that the latter did not recognize. This extra cone should be a blue-sensitive cone, a point confirmed by the double-labeling of Fig. 4*B*. Here another monkey retinal section was labeled with anti-β_3 peptide antibody (*Top*) and a mouse monoclonal antibody (OS-2, see ref. 37) specific for blue-sensitive cones (*Bottom*). Clearly the solitary blue cone was recognized by the anti-β_3 peptide antibody. Thus, the β_3 subunit appears present in all three classes of cones. Alternatively, these cells may have closely homologous β subunits that cannot be distinguished by our antibody.

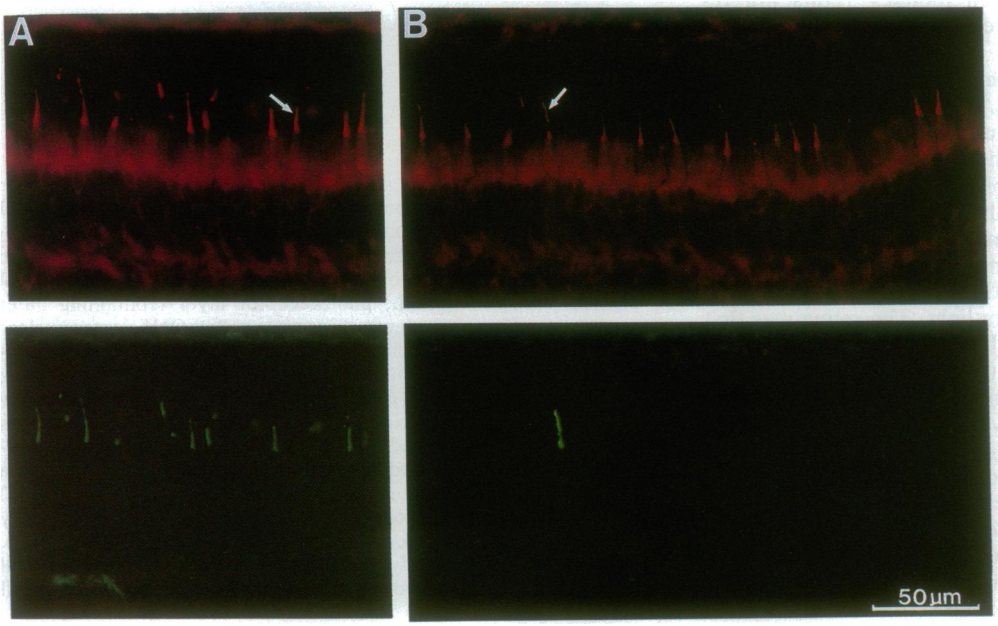

FIG. 4. (*A*) Monkey retinal section double-labeled with rabbit polyclonal antibody against β_3 subunit (*Upper*) and a mouse monoclonal antibody against red/green-sensitive cones (*Lower*) (8-μm frozen section). The arrow in *Upper* indicates a cone recognized by the anti-β_3-subunit antibody but not recognized by the anti-red/green cone antibody, indicating that it is a blue cone. (*B*) Similar double-labeling experiment with the polyclonal antibody against β_3 subunit (*Upper*) and a mouse monoclonal antibody against blue-sensitive cones (*Lower*). Arrow in *Upper* shows a cone recognized by the anti-β_3 antibody and also by the anti-blue cone antibody.

In addition, certain bipolar cells also show β_3 labeling (arrow in Fig. 3*A*). These cells have their synaptic terminals situated in sublamina b of the inner plexiform layer, suggesting that they are rod bipolar or ON-cone bipolar cells (45, 46). Using double-labeling immunofluorescence with a monoclonal antibody against protein kinase C (see *Materials and Methods*) that specifically labels rod bipolar cells (47–49), we have indeed verified β_3-subunit immunoreactivity in these cells (data not shown). Whether this G protein subunit participates in the glutamate-activated, 2-amino-4-phosphonobutyrate (APB)-sensitive synaptic signal-transduction pathway in the outer plexiform layer involving these cells (50–52) remains to be examined.

As further confirmation of these findings, we also used a different set of polyclonal anti-β_1, -β_2, and -β_3 peptide antibodies (LAP 636, LAP 637, and LAP 638, provided by B. Fung and R. H. Lee of the University of California at Los Angeles) for staining. These antibodies recognize the same region of β_2 subunit but recognize different regions of β_1 and β_3 subunits than our antibodies. The findings with these antibodies agree completely with those described above, except that LAP 638, which recognizes β_3 subunit, labels bipolar cells even more strongly than does our B34 antibody.

γ Subunits. Fig. 5 shows monkey retinal sections stained with the different anti-γ antibodies. The antibody against γ_1 subunit (transducin γ) stains rod outer and inner segments strongly and stains synaptic terminals to a lesser degree; outer segments of cone cells are again unstained (Fig. 5*A*). Unlike β_1 subunit, there is no staining of the inner retina. Fig. 5*B* shows results with the anti-γ_2 antibody A-75, which stains the cone outer segments exclusively. All three classes of cone outer segment appear to be stained by this antibody, a conclusion again confirmed by double labeling with the anti-cone antibodies described above (data not shown). A second anti-γ_2 antibody (A-25, see *Materials and Methods*) against a different region of the protein has produced identical results. Thus, like β_3 subunit, γ_2 subunit appears to be in cone cells; but unlike β_3 subunit, it does not appear to be in bipolar cells. Fig. 5*C* shows the staining pattern with the anti-γ_3 antibody. This subunit appears to be located in rod and cone inner segments as well as in the corresponding synaptic terminals; in addition, the inner retinal layers, especially the inner plexiform layer and the optic nerve fiber layer, also show some immunoreactivity. Thus, overall, the combined distribution pattern for γ_1 and γ_3 subunits resembles that for β_1 subunit. The match is not exact, however, in that the anti-β_1 subunit staining in the inner plexiform layer is very intense, whereas the anti-γ_3 subunit staining in the same region has a punctate appearance. Finally, as shown in Fig. 5*D*, the staining pattern with the anti-γ_7 antibody matches quite well that with the anti-β_2 antibody described earlier. The staining is very sparse, being confined to the outer plexiform layer and discrete sublaminae of the inner plexiform layer; the latter fibers arise from specific cell bodies in the amacrine cell layer (Fig. 5*D*). Preadsorption experiments with the respective γ-subunit peptides again confirmed the specificities of these stainings.

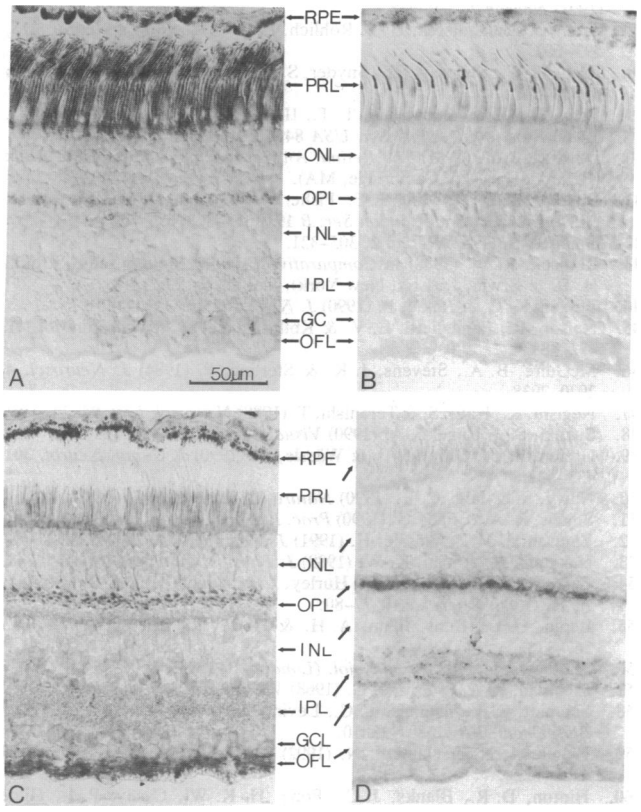

FIG. 5. Immunostainings of monkey retinal sections with rabbit polyclonal antibodies against G protein γ_1 (*A*), γ_2 (*B*), γ_3 (*C*), and γ_7 (*D*) (8-μm frozen sections). See text for details (Nomarski optics in *A* and *B*). Anatomical layers are the same as in Fig. 1*A*.

DISCUSSION

Patterns of immunostaining in the retina for the three β subunits all differ and scarcely overlap; the same is true for the γ subunits. In particular, rod and cone outer segments have different β- and γ-subunit immunoreactivities. Rods and cones both subserve phototransduction, but they differ subtly in light-response properties (see, for example, ref. 53). These differences have generally been thought to arise, at least in part, from the α subunit of transducin, the G protein that mediates phototransduction because rods and cones have different isoforms of this subunit (54, 55). Other proteins in the phototransduction cascade, such as the cGMP phosphodiesterase and the cGMP-gated cation channel, likewise have distinct rod and cone forms. Thus, the distinct G protein β and γ subunits reported here, while surprising, fit this pattern.

The functional significance of the different β and γ subunits in rods and cones remains uncertain. It is nonetheless tempting to think that this difference does impart some specificity to the transduction pathway, as suggested by our finding on the "green" rods of frogs and toads. These cells exhibit the high light sensitivity and the slow response kinetics characteristic of rods (56). On the other hand, their pigment has a peak sensitivity near 430 nm (56, 57), which is more indicative of a blue-sensitive cone pigment than a rod pigment. Using the antibodies described here, we have found that green rods show G protein β_1- and γ_1-subunit immunoreactivities as the rhodopsin-based "red" rods (data not shown), consistent with their rod-response characteristics. Thus, a correlation may indeed exist between light-response properties and G protein-subunit species.

With the many G protein α subunits already identified and the several β and γ subunits now also known, one asks whether rules govern the associations between particular species of these three subunits or whether random combinations between all subunits can occur to potentially generate many G protein species of different functional characteristics. Our findings in the retina represent a beginning of this quest by identifying subunit colocalizations and, hence, possible associations between the various β and γ subunits. Thus, we find the β_1 subunit to colocalize with the γ_1 subunit (in rod outer segment) and possibly the γ_3 subunit (in rod inner segment and elsewhere in retina). The β_2 subunit appears to colocalize with the γ_7 subunit, and the β_3 subunit with the γ_2 subunit (in cone outer segment) and perhaps also

the γ_3 subunit (in inner retina) (see refs. 58 and 59). When distributions of the various α subunits in the retina are also known, a more detailed picture of subunit colocalizations will be possible. Similar information in other parts of the brain and nonneural tissues would be useful.

Considering that the numbers of β and γ subunits identified in any tissue are still relatively small compared with α subunits, it is natural to wonder whether additional species of these subunits exist and remain to be identified. This question is especially interesting should the β and γ subunits be functionally specific. In the retina, at least, additional species may indeed exist. For example, we detect no β_1, β_2, or β_3 immunoreactivity in horizontal cells, despite evidence that phospholipase C is present in these cells (Y.-W.P., S. G. Rhee, and K.-W.Y., unpublished work). Thus, other β-subunit species are probably associated with G protein(s) coupled to the inositolphospholipid signal pathway in these cells. The same conclusion can be drawn for the Müller glial cells, which likewise show phospholipase C immunoreactivity (Y.-W.P., S. G. Rhee, and K.-W.Y., unpublished work). Also, the recently reported cellular localization of the α subunit of G_z in the retina cannot be completely accounted for by the distributions of the three β subunits described here—namely, most retinal ganglion cells immunostain for the α subunit of G_z (60), whereas we find 20% or less of these cells immunostain for β_1 subunit (see *Results*). This result suggests the existence of a β subunit in G_z that is neither β_1, β_2, or β_3. Indeed, most recently another β subunit, β_4, has been reported in brain (61). Additional γ subunits probably occur in the retina as well. For example, the β_1-subunit staining at the inner plexiform layer is far from matched in intensity by the γ-subunit stainings we observed, possibly suggesting another γ subunit in this location—perhaps γ_4 or γ_5 subunit (21, 22), which we have not examined, or a yet-to-be-identified species. Likewise, we have not yet found any obvious γ-subunit immunoreactivity in Müller glial cells. The emergence of additional β and γ subunits would mean an even greater number of functionally distinct G proteins might exist.

We thank Dr. P. Röhlich (Semmelweis University of Medicine, Budapest) for providing us with the monoclonal antibodies (COS-1 and OS-2) against mammalian red/green- and blue-sensitive cones and Dr. L. Johnson (University of Southern California) for another monoclonal antibody (CSA-1) against red/green cones that gave identical results as COS-1. We also thank Drs. B. Fung and R. H. Lee (University of California, Los Angeles) for letting us test their set of polyclonal anti-β_1, β_2, and β_3 peptide antibodies (LAP 636, LAP 637, and LAP 638). Finally, we thank Drs. Y. Koutalos and T. Kurahashi for comments on the manuscript. This work was supported in part by U.S. Public Health Service Grants EY06837 to K.-W. Y., DK34281 to M.A.L., and GM39867 to J.D.R.

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