A Third Form of the G Protein \( \beta \) Subunit

1. IMMUNOCHEMICAL IDENTIFICATION AND LOCALIZATION TO CONE PHOTORECEPTORS*

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Vertebrate retinal cones play a major role in both photopic vision and color perception. Although the molecular mechanism of visual excitation in the cone is not as well understood as in the rod, it is generally thought to involve a cone-specific G protein (cone transducin) that couples the cone visual pigment to a cGMP phosphodiesterase. Like all other G proteins, cone transducin is most likely a heterotrimer consisting of \( \alpha \), \( \beta \), and \( \gamma \) subunits. A \( \alpha \) subunit of cone transducin has been localized to the outer segment of bovine cones, but its associated \( \beta \) and \( \gamma \) subunits are unknown. To identify the \( \beta \) subunit involved in the phototransduction process of cones, we have developed a panel of antipeptide antisera against the most diverse region of the amino acid sequences encoded by \( \beta_1 \), \( \beta_2 \), and \( \beta_3 \) cDNAs and used them to determine the distribution of the \( \beta \) isoforms in different retinal preparations. We found that the \( \beta \) subunit is present in bovine retinal transducin and phosducin-TB complex preparations which were previously thought to contain only \( \beta_3 \). Analysis of its subcellular distribution indicated that \( \beta_3 \) is predominantly cytoplasmic. Immunocytochemical staining of bovine retinal sections with the anti-\( \beta_3 \) antisera further revealed a specific localization of \( \beta_3 \) in cones but not in rods. In contrast, anti-\( \beta_3 \) antisera stained only the rods. These results suggest that \( \beta_3 \) is the \( \beta \) subunit of cone transducin and confirms the proposition that rods and cones utilize distinct signaling proteins for phototransduction.

G proteins are a large group of structurally related regulatory proteins that serve as transducers of signals between cell surface receptors and intracellular effectors (Stryer and Bourne, 1986; Gilman, 1987; Neer and Clapham, 1988; Birnbauer, 1990). Members of this family are all heterotrimeric composed of \( \alpha \), \( \beta \), and \( \gamma \) subunits. The GTP-binding \( \alpha \) subunits are most unique and generally define the difference in specificity among various forms of G proteins. Currently, at least 16 distinct \( \alpha \) subunits have been identified in various mammalian tissues (Simon et al., 1991). They have been shown to function as signal carriers in the regulation of intracellular adenylate cyclase and cyclic GMP phosphodiesterase activities, ion channel permeability, and phosphoinositide turnover (Stryer and Bourne, 1986; Gilman, 1987; Birnbauer, 1990). Multiple forms of the \( \beta \) and \( \gamma \) subunits have also been detected by a combination of biochemical, immunological, and cloning techniques (Simon et al., 1991).

The four known mammalian \( \beta \) isoforms, designated \( \beta_1 \), \( \beta_2 \), \( \beta_3 \), and \( \beta_4 \), are all 340 amino acid residues long and share more than 80% sequence identity (Sugimoto et al., 1985; Codina et al., 1986; Fong et al., 1986, 1987; Gao et al., 1987; Levine et al., 1990; Weizsäcker et al., 1992). In contrast, the \( \gamma \) subunits are more divergent and evidence for seven distinct isoforms has thus far been found (Hurley et al., 1984; Yatsunami et al., 1985; Ovchinnikov et al., 1985; Robishaw et al., 1989; Gautam et al., 1989, 1990; Tamir et al., 1991). The \( \beta \) and \( \gamma \) subunits are isolated as tightly associated \( \beta \gamma \) complexes with diverse functions. They have been shown to interact with the \( \alpha \) subunits to facilitate binding to receptors (Fung, 1983; Florian and Sternweis, 1985) and attenuate adenylate cyclase activity (Katada et al., 1984; Cerione et al., 1986).

Additionally, specific \( \beta \gamma \) complexes have been implicated in the stimulation of adenylate cyclase (Tang and Gilman, 1991; Federman et al., 1992) and phospholipase \( A_2 \) (Jelsma and Axelrod, 1987; Kim et al., 1989), as well as the regulation of pheromone-mediated mating in yeast (Whiteway et al., 1989) and the interaction with a phosphoprotein, phosducin, in retinal photoreceptors (Lee et al., 1987; Ho et al., 1990).

Of the four known \( \beta \) isoforms, only the \( \beta \gamma \) complexes consisting of \( \beta_1 \) and \( \beta_2 \) have been purified and characterized (Fung, 1983; Sternweis and Robishaw, 1984; Evans et al., 1986, 1987; Woolkalis and Manning, 1987). The \( \beta_3 \) polypeptide is identical to the \( \beta \) subunit of rod transducin, the G protein that couples photolysed rhodopsin to cyclic GMP phosphodiesterase in rod retinas (Roof et al., 1985; Codina et al., 1986). It migrates with an apparent molecular weight of 36,000 on SDS-polyacrylamide gels (Sugimoto et al., 1985; Fong et al., 1986). Immunochimical studies and Northern blot analysis indicate that \( \beta_3 \) is widely distributed and is present...
at various levels in nearly all purified G protein preparations (Roof et al., 1988; Fong et al., 1987; Woollakis and Manning, 1987). The Gβ peptide has an apparent molecular weight of 35,000 (Mumby et al., 1986; Gao et al., 1987; Amatrusa et al., 1988). This peptide is abundant in preparations of G protein but is absent in transfusion purified from bovine retinas (Evans et al., 1986, 1987; Fong et al., 1987; Woollakis and Manning, 1987). The Gβγ complex containing the Gβ or Gγ polyepetide have not yet been identified and characterized. Northern blot analysis shows that the mRNAs of these two Gβ isoforms are abundant in the retina (Levine et al., 1990; Weizsäcker et al., 1992). This observation points to the existence of novel retinal G proteins which may have a specific function in vision.

As a first step to characterize the Gβ subunit and to determine its cellular distribution in vertebrate retinas, we have developed a panel of antipeptide antibodies directed against three of the four Gβ isoforms. Using these specific antisera, we show that Gβ2 is localized in cones, in agreement with the retinal staining pattern observed by Yau et al. (1992) using a different antipeptide antisera directed against Gβ2. This Gβ isoform is also present in purified bovine retinal transfusion and the phosducin-Tβγ complex. In the second paper of this series (Fung et al., 1992), we describe the purification and characteriization of a Tβγ complex composed of Gβ2 and a novel Gγ. Our results suggest that this Tβγ complex may play an important role in the regulation of the visual excitatory process in cone photoreceptors.

**EXPERIMENTAL PROCEDURES**

**Materials**—Frozen bovine retinas were purchased from J. A. Lawson Co., Lincoln, NE. Bovine Gβ and human Gβ2 and Gγ2 CDNA clones were the generous gift of Dr. Henry Fong, University of Southern California. The purification of phosducin-Tβγ complex (Lee et al., 1987), transfudacin (Fung, 1983), and brain Gβγ (Sternweis and Robishaw, 1984; Fung et al., 1990a) were performed as described previously. All purified proteins were stored in 40% glycerol at -20 °C.

Peptides LAP-636 (ADATLSQITNNIDP), LAP-637 (GDSTLSAQPITNNIDP), and LAP-638 (ADYTLAELVSGLEV), corresponded in the density gradient of individual coomassie Blue-stained polyepetide bands using bovine serum albumin (Sigma) as a standard. For Western blot analysis, proteins separated by SDS-PAGE were transferred to Immobilon-P membranes according to a modified procedure (Sternweis et al., 1988) of Towbin et al. (1979). The immunoreactive bands were detected by incubation with 125I-protein A, followed by autoradiography. The radioactivity of each immunoreactive band was quantified either by densitometric scanning of the autoradiogram or scintillation counting.

**Immunostaining of Bovine Retinas**—Fresh bovine eyes were obtained from a local slaughterhouse and the cornea, iris, lens, and vitreous were removed. The remaining eyecup was fixed at 4 °C for 4 days by immersion in 4% formaldehyde freshly prepared from paraformaldehyde. Strips of retina attached to the underlying choriocapillary layer were dissected from the scleral layer and infiltrated for 12 h in 30% sucrose. The infiltrated strips were then immersed in Tissue-Tec O.C.T. Compound (Miles Scientific), frozen in liquid nitrogen, and stored at -80 °C.

Frozen sections of the tissue were cut at 7–9 μm on a Reichert-Jung 2800 Frigcut cryostat, transferred to slides coated with amonpropyl triethoxysilane (Aldrich), and stained using the Avidin Biotinylated Enzyme Complex (ABC) technique according to instructions provided by the supplier (Pierce Chemical Co.). All staining was performed at 21 °C in a humidified chamber, and preimmune controls were treated alongside the experimental sections. The experimental and control sections were incubated for 45 min in protein A-purified IgG at a concentration of 0.1 μg/ml, 30 min in goat anti-rabbit IgG, and 30 min in avidin-biotin complex to visualize the cell bodies. The sections were counterstained for 1 min in Mayer's hematoxylin. Sections were viewed in a Zeiss PM III photomicroscope and photographed with Kodak Ektachrome 50 film.

**RESULTS**

**Specificity of Antiser-a**—To develop a panel of specific antibodies directed against the different isoforms of Gβ subunits, we immunized rabbits with synthetic peptides corresponding to residues 26–39 of the deduced amino acid sequences of Gβ1, Gβ2, and Gβ3 CDNA (Levine et al., 1990). We then tested the immunochemical specificities of the antisera against individual peptides by slot blot analysis. In this experiment, peptides immobilized on Immobilon-P membranes were incubated with antisera diluted between 15- and 3000-fold, followed by 125I-protein A binding to detect the amount of bound antibodies. As shown in Fig. 1, each of these antisera was specific for the immunizing peptide, with little or no cross-reactivity against the other two.

To further evaluate the specificity of these antisera toward the Gβ isoforms, we synthesized all three Gβ polyepetides by in vitro translation of the Gβ1, Gβ2, and Gβ3 mRNA in the presence of [35S]methionine. We then tested the selectivity of each antisera by immunoprecipitation (Fig. 2). Panel A

**Immunoprecipitation of Gβ1, Gβ2, and Gβ3 Polyepetides**—Normal rabbit serum and β-363, β-367, and β-383 antisera (200 μl) were preincubated with 40 μl of washed protein A-Sepharose (Sigma; IgG binding capacity = 2.1 mg/ml) for 3 h at room temperature with constant shaking. Immunoglobulin-protein A-Sepharose complexes were washed twice with the addition of 75 μl of immunoprecipitation buffer (20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 0.05% Tween 20) and pelleted by centrifugation at 18,000 × g for 5 min. The supernatants were discarded, and pellets were resuspended to 200 μl with immunoprecipitation buffer. Fifty microliters of the immunoglobulin-protein-A-Sepharose complex was then incubated at room temperature with 100 μl of translation mixture. After 3 h, 0.75 ml of immunoprecipitation buffer was added and the mixture centrifuged for 5 min. Supernatants were discarded and the pellets resuspended in 60 μl of electrophoresis sample buffer (Laemmli, 1970), heated at 90 °C for 5 min, and centrifuged. Samples of the supernatant (40 μl) were analyzed by SDS-PAGE and the radiolabeled polyepetide bands visualized by fluorography.

**Analytical Methods**—SDS-PAGE was performed according to Laemmli (1970) (12.5% slab gels, 1.5-mm thickness). Protein content of Gβ in the phosducin-Tβγ complex, transfudacin, and brain Gβγ was determined from the densitometric intensity of individual Coomassie Blue-stained polyepetide bands using bovine serum albumin (Sigma) as a standard. For Western blot analysis, proteins separated by SDS-PAGE were transferred to Immobilon-P membranes according to a modified procedure (Navon et al., 1987) of Towbin et al. (1979). The immunoreactive bands were detected by incubation with 125I-protein A, followed by autoradiography. The radioactivity of each immunoreactive band was quantified either by densitometric scanning of the autoradiogram or scintillation counting.

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Slot blot analysis was carried out as described under "Experimental Procedures" using antisera diluted between 15- and 3000-fold and scanning of individual slots. Immobilon-P membranes containing 2 pg of bound peptides. Following 125I-protein A binding and autoradiography, the relative immunoreactivity of each antiserum was determined by densitometric procedures. The immunoprecipitates were analyzed by SDS-PAGE and visualized by fluorography.

Immunoechemical Evidence for the Gβ subunit—Although a significant amount of Gβ3 mRNA has been detected in vertebrate retinas and a number of cell lines (Levine et al., 1990), the Gβ3 poly peptide has never been identified in any tissue. To further search for evidence of Gβ3 in various G protein preparations, we purified the bovine retinal phosducin-Tpy complex and transducin, and bovine brain Gβγ (lane 3) and probed each protein preparation with antisera β-636, β-637, and β-638 (Fig. 3). As shown in the Western blot presented in panel B of Fig. 3, all three protein preparations contain a high level of Gβ3, consistent with the ubiquitous distribution of this isoform in all G protein preparations (Roof et al., 1985; Fong et al., 1987; Woolkalis and Manning, 1987). In contrast, Gβ2 appeared to have a more restricted distribution. Only the brain Gβγ preparation was found to contain a significant amount of Gβ3 subunit immunoreactive to antisera β-637. Gβ2 was not detected in either the phosducin-Tpy or transducin preparation. This result confirms earlier observations that the phosducin-Tpy complex contains Gβ3, but not Gβ2 (Lee et al., 1987). When these three protein preparations were tested against antiserum β-638, all were found to contain a minor population of Gβ3 subunit, with the highest amount associating with phosducin-Tpy complex and the least with brain Gβγ. This result revealed a hitherto undetected mixture of Gβ1 and Gβ3 subunits in these highly purified preparations of phosducin-Tpy complex and transducin.

We have also detected a minute amount of Gβ3 isoform in isolated outer segment preparations and total retinal homogenate. Western blot analysis of total retinal homogenate with precipitated in an amount substantially greater than that observed with the normal rabbit serum (panel B). The β-637 and β-638 antisera demonstrated similar specificities toward the Gβ isoforms against which they were directed. The small amount of radiolabeled Gβ1 precipitated with β-637 and β-638 antisera is equivalent to that observed with normal rabbit serum (panel B) and most likely reflects a property of the in vitro expressed Gβ1. The ability of each antiserum to selectively immunoprecipitate the Gβ poly peptide against which it was generated clearly illustrates that specificity is conserved in the transition to the complete Gβ poly peptides and allows us to immunochemically probe the Gβ compositions of various protein preparations.
antiserum β-638 revealed two faint immunoreactive protein bands: a 37- and a 30-kDa band (data not shown). The 37-kDa band, which comigrated with the GB3 subunit, was presumed to be the GB3 isofrom. Based on the relative amount of radioactivity associated with this immunoreactive band, we estimated that the amount of GB3 in retinas is at least an order of magnitude less than GB4. The 30-kDa band was present in both brain homogenates and 5-day-old mouse retinas lacking mature outer segments, indicating that it is not specific to photoreceptors. Since it is also absent in all our G protein preparations, we believe that this 30-kDa band is most likely derived from a low level of cross-reactivity of antiserum β-638 with another protein.

**Distribution of the GB3 Subunit**—While the majority of the Tβγ subunit of transducin is membrane-bound under physiological conditions, those that copurify with phosducin have been shown to be cytoplasmic. Since the majority of the GB3 subunit is found in the soluble phosducin-Tβγ preparation, we suspected that it might also be cytoplasmic. In order to establish whether this is the case, we separated the retinal proteins into cytoplasmic, crude outer segments, and particulate fractions based on the protocol described previously (Lerea et al., 1986). We then quantified the amounts of Ta, phosducin, Gβ1, and Gβ3 in each of these fractions by Western blot analysis. The results of this experiment are summarized in Table I. As expected, we found that the majority of Ta and phosducin were in the rod outer segment membranes and soluble fractions, respectively. The Gβ3 subunit was found to distribute equally among both the soluble and membrane fractions, reflecting its association with both the Ta subunit of transducin and phosducin. In contrast, the majority of the Gβ3 subunit was found in the soluble fractions throughout the course of the fractionation. This result suggested that Gβ3 is cytosolic, perhaps in association with a soluble G protein or phosducin.

We next examined the cellular distribution of the Gβ1 and Gβ3 subunits in bovine retinas by immunostaining with antisera β-636 and β-638. As shown in Fig. 4, panel A, formaldehyde-fixed frozen sections of bovine retina stained with antiserum β-636 showed a fairly intense staining of rod outer and inner segments and synaptic terminals. The rod somata and axons in the outer nuclear layer were not stained above background. Cone outer segments, inner segments, and nuclei appeared to be unstained, but due to the thickness of the frozen sections (7–9 μm) and masking by numerous rod outer segments, we were unable to tell whether all the cones were unstained. Sections of bovine neurosensory retina treated with antiserum β-638 showed a staining pattern distinct from that with antiserum β-636 (Fig. 4, panel C). Cone outer segments, the myloid region of inner segments, cone cell bodies, axons, and synaptic terminals were stained. The most intense staining was observed in cone outer segments. In contrast, all components of rod photoreceptors were unstained. Control sections incubated in preimmune sera were also not stained (Fig. 4, panels B and D). The addition of 50-fold molar excess of peptides LAP-638 to antiserum β-638 completely abolished the selective staining of cones. This finding demonstrated that the majority of the Gβ3 subunit is localized in cone photoreceptors and raises the possibility that Gβ3 subunit of cone transducin is of the Gβ3 isofrom.

**TABLE I**

| Subcellular distribution of Ta, Gβ3, Gβ3, and phosducin |
|---------------------------------------------------------|
| Frozen bovine retinas were homogenized in Ringer's buffer (10 mM MOPS, pH 7.5, 60 mM KCl, 30 mM NaCl, 2 mM MgCl2, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfon fluoride) containing 65% sucrose and fractionated by centrifugation at 37,000 × g for 30 min. The dense retinal membranes recovered at the bottom of the tube were homogenized again in Ringer's buffer and separated by centrifugation into a supernatant and a particulate fraction (designated "retinal particulate"). The sucrose supernatant containing the detached rod outer segments (ROS) was diluted with an equal volume of Ringer's buffer and similarly separated by centrifugation into a supernatant and a membrane pellet (designated "crude ROS"). The two supernatants containing the soluble proteins were then combined and designated as the "soluble" fraction of the bovine retinas. To determine the relative amount of each protein shown in the table, portions of these three preparations (0.4% equivalent of a retina) were subjected to quantitative Western blot analysis using antibodies directed against Gβ3 (β-636), Gβ3 (β-638), Ta (TF15), and phosducin (Gerty-AP) as described under "Experimental Procedures." The relative amount of each protein in the three retinal preparations was determined either by densitometric scanning of the autoradiogram and by direct scintillation counting of the immunoreactive band. Both methods gave essentially the same results. The sum of each protein in the three retinal preparations was taken as 100%.

| Protein | Retinal preparations | Soluble | Crude ROS | Retinal particulate | % of total |
|---------|----------------------|---------|-----------|---------------------|-----------|
| Gβ3     | 64                   | 30      | 7         |                     | 100%      |
| Gβ3     | 86                   | 11      | 3         |                     | 100%      |
| Ta      | 26                   | 59      | 15        |                     | 100%      |
| Phosducin | 87               | 12      | 1         |                     | 100%      |

**DISCUSSION**

Vertebrate retinas contain two types of photoreceptors, rods and cones. Rods are exquisitely sensitive but easily become overloaded and incapable of signaling under high light levels. Cones, on the other hand, are 2 orders of magnitude less sensitive than rods but respond and turn off faster to light stimulation (Pugh and Cobbs, 1986). Despite the differences in sensitivity and response kinetics, rods and cones appear to share a very similar mechanism for visual excitation on the molecular level (Attwell, 1985). Rods are known to use a retina-specific G protein (transducin) to transduce the light signal detected by rhodopsin into an increase in the enzymatic activity of a cGMP phosphodiesterase (Fung, 1986; Lolley and Lee, 1990; Stryer, 1991). The decrease in intracellular level of cGMP leads to the closure of many cGMP-regulated cation channels in the plasma membrane, resulting in a decrease in Na⁺ conductance and the graded hyperpolarization of the cell. Although the mechanism of visual excitation in cones is not as well understood, compelling electrophysiological evidence indicates a G protein-mediated pathway very similar to that in rods is also involved in lowering cGMP concentration in cones following light stimulation (Pugh and Cobbs, 1986).

In an earlier study, Lerea et al. (1986) reported the immunocytochemical localization of a cone-specific Ta subunit to the outer segment of bovine cones but did not identify the associated cone transducin Tβγ and Ty subunits. We now show by immunostaining of bovine retinas with isofrom-specific antibodies that Gβ3 subunits are differentially localized in rods and cones. At the level of resolution provided by light microscopy, Gβ3 has been shown previously to be identical to the rod Tβ subunit, is found predominantly in the outer segments, the inner segments, and the synaptic terminals of rods. In contrast, Gβ3 is not present in rod photoreceptors, but is distributed throughout cone photoreceptors, with the highest concentration in the cone outer segments. These results strongly imply that Gβ3 is the Tβ subunit of cone transducin, although its role in cone phototransduction remains to be confirmed biochemically.

Our immunostaining of the bovine retinal sections also shows a distinct distribution of Gβ3 subunit in the inner segments,
the cell bodies, axons, and the synaptic terminals. Since the cone Ta subunit is detected only in the outer segment (Lerea et al., 1986), this observation raises the possibility that GB3, in addition to complexing with cone Ta in the outer segment, may also be interacting with another protein component. If this is the case, what might be the identity of this cellular component? In earlier studies, we have shown that the Tp-y complex in rods is tightly associated with a cytosolic phosphoprotein, phosducin (Lee et al., 1987; Lee et al., 1990). By analogy, it seems likely that a similar type of complex may exist in cones. In agreement with this hypothesis, we note that the Go3 isoform is most abundant in the purified preparation of the phosducin-Tp-y complex. More concrete evidence in support of this hypothesis will be presented in the second paper (Fung et al., 1992).

In additional studies, we have also examined the distribution of Go3 in subcellular fractions and in several purified protein preparations. We found that the GB3 complex containing Go3 is predominantly cytoplasmic under normal isotonic conditions. This result is of particular interest in view of reports that both the cone-specific Ta and cyclic GMP phosphodiesterase are found primarily in the soluble fractions (Lerea et al., 1986; Gillespie and Beavo, 1988). In rods, transducin is loosely bound to the disc membranes in the outer segment. Upon light stimulation, it becomes tightly associated with photoactivated rhodopsin and is released only upon binding GTP (Kühn, 1980). In this context, it will be of considerable interest to determine whether the soluble cone transducin behaves similarly to light and GTP. The differences in cytoplasmic distribution and membrane binding property of cone transducin may well be able to account for the reduced sensitivity and increase in photoreponse kinetics characteristics of cones.

The results of our study provide a glimpse at how signals can be specifically channelled via G proteins to different second messenger pathways. In the phototransduction process, a high degree of signal specificity at the cellular level is achieved by restricting one specific set of rhodopsin, transducin, and phosphodiesterase to a particular subtype of photoreceptors. The interactions of these proteins, in turn, produce the appropriate photoreponses essential for the proper functioning of rods and cones. Another mechanism for ensuring specificity of G protein activity within a cell is by compartmentalization. By confining a specific set of transduction machinery to a local region of the cell, potential interference between several incoming signals can be minimized. In the case of phototransduction, the three key components, rhodopsin, transducin, and phosphodiesterase are all sequestered in...
the outer segment of the photoreceptors, where the processing of the light signal can be achieved with precision and high degree of specificity. Finally, in some cells where several functionally interchangeable receptors and G proteins are colocalized at the plasma membrane, excessive cross-talks between various G protein-mediated pathways are likely further limited through the preferential interaction of a specific G protein subtype with a particular receptor (Neer and Clapham, 1988). Because each photoreceptor subtype possesses distinct signaling proteins, this mechanism may not be significant in the case of the phototransduction process.

In the retina, the segregation of each one of the many possible G protein-mediated pathways at the cellular, compartmental, and molecular levels help to maintain the specificity of cellular responses to light. The challenge in the future will be to determine if this general principle also applies to other biological systems such as the brain, where numerous signals concomitantly enter neuronal cells through a diversity of G protein-coupled receptors.

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