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G Protein–Effector Coupling: Interactions of Recombinant Inhibitory γ Subunit with Transducin and Phosphodiesterase†

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ABSTRACT: A bacterial expression vector for the inhibitory γ subunit of retinal rod phosphodiesterase has been constructed by inserting a mouse γ cDNA into pUC19. *Escherichia coli* 222 transformed with this plasmid produces a 12-kDa recombinant protein consisting of 18 additional amino acids attached to the amino terminus of γ. The fusion protein, designated β-gal-γ, has been refolded into an active form in formic acid and partially purified by gel filtration chromatography. Despite a large extended sequence at the amino terminus, β-gal-γ is able to inhibit the activity of trypsin-activated phosphodiesterase, bind tightly to the catalytic $\alpha\beta$ subunits, and interact with the α subunit of transducin in a nucleotide-dependent manner. The availability of large quantities of active bacterial γ, together with the ability to change its primary structure by site-directed mutagenesis, promises to provide considerable new information on the interaction between transducin and phosphodiesterase, as well as insights into the molecular mechanism of G protein–effector coupling.

One of the best studied biological processes involving G protein interaction with an effector enzyme is visual excitation of vertebrate retinal rods, where the direct activation of a cGMP-specific phosphodiesterase (PDE)[1] by transducin has been conclusively demonstrated (Chabre, 1985; Fung, 1986; Stryer, 1986; Hurley, 1987; Liebman et al., 1987). In this system, photolyzed rhodopsin catalyzes the exchange of GTP for GDP bound to transducin (Fung & Stryer, 1980). The transducin–GTP complex, in turn, stimulates the PDE activity (Fung et al., 1981), leading to a transient reduction of the intracellular level of cGMP (Yee & Liebman, 1978; Woodruff & Bownds, 1979; Blazynski & Cohen, 1986; Cote et al., 1986) and the closure of many cGMP-sensitive cation channels (Fesenko et al., 1985; Yau & Nakatani, 1985). As a result, the influx of Na^+ through the plasma membrane of the rod outer segment (ROS) decreases (Hagins et al., 1970; Baylor et al., 1979) and the rod hyperpolarizes (Tomita, 1970).

cGMP-specific PDE, the key enzyme involved in the regulation of intracellular cGMP concentration of ROS, is a peripheral membrane protein consisting of α (M_r = 90 000), β (M_r = 86 000), and γ (M_r = 10 000) polypeptides (Baehr et al., 1979). In this multimeric form, the catalytic activity associated with the $\alpha\beta$ subunits is inhibited by γ (Hurley & Stryer, 1982). The inhibition can be relieved by limited tryptic digestion, which selectively destroys the γ subunit (Hurley & Stryer, 1982), or alternatively, by interaction with the GTP-bound form of T_α (Fung et al., 1981; Wensel & Stryer, 1986). A clue that T_α may stimulate the phosphodiesterase activity by binding to the inhibitory γ subunit was first noted by

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[1] Abbreviations: GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); mAb, monoclonal antibody; PDE, retinal cGMP phosphodiesterase; T_α, α subunit of transducin; α, β, and γ, subunits of phosphodiesterase; β-gal-γ, β-galactosidase-γ fusion protein.

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Yamazaki et al. (1983), who reported the elution of an inhibitory factor from frog ROS with Gpp(NH)p. Deterre et al. (1986) subsequently detected by ion-exchange chromatography a population of the γ–T_α–GTPγS complex in a protein preparation extracted from the ROS membranes at low ionic strength. In a more recent study, we have demonstrated by reconstitution with purified protein components that the T_α–GTPγS complex interacts with γ, but not with $\alpha\beta$ (Fung & Griswold-Prenner, 1989). These findings, taken together, provide a very convincing argument for the release of the γ–T_α–GTP complex from the catalytic $\alpha\beta$ subunits as the mechanism of PDE activation.

Although the primary structures of both the bovine (Ovchinnikov et al., 1986) and mouse γ (Tuteja & Farber, 1988) have been deduced from the cDNA sequences, the location of the binding domain for T_α is not known. Neither is there any structural information about the mechanism of inhibition exerted by γ upon binding to $\alpha\beta$. While the purification of γ from bovine rod PDE has been achieved (Hurley & Stryer, 1982; Fung & Griswold-Prenner, 1989), the small quantities of γ obtained do not allow extensive structural analysis of the molecule. As a first step toward studying the structure–function relationship of γ by site-directed mutagenesis and producing a larger amount of γ for future crystallographic investigation, we have constructed an *Escherichi coli* expression plasmid that produces a fusion protein (β-gal-γ) consisting of 18 additional residues attached to the amino terminus of mouse γ. We have further shown that the β-gal-γ, similar to native bovine γ, is capable of interacting with transducin and the catalytic subunits of PDE.

Experimental Procedures

Materials. *E. coli* strain 222, Δcya-854 trp his ilv rpsL crp-222 (Puskas et al., 1983), was a generous gift from Dr. Henry Bourne (Department of Pharmacology, University of California, San Francisco). Plasmid vector pUC19 was obtained from Boehringer Mannheim. Pansorbin was a product of Calbiochem. Immobilized TPCK–trypsin was purchased from Pierce. ^{125}I protein A was prepared by iodination with chloramine T. Monoclonal antibodies were immobilized on cyanogen bromide activated Sepharose according to the procedure described by Pharmacia. ROS membranes were isolated as described previously (Hong & Hubbell, 1973). Transducin was eluted from photolyzed ROS membranes with GTP and purified by column chromatography (Fung et al., 1981). Activated transducin was prepared by incubating the purified protein with GTPγS in the presence of photolyzed rhodopsin in reconstituted membrane vesicles (Fung, 1983). Rod PDE was extracted from photolyzed ROS membranes at low ionic strength and purified by column chromatography (Baehr et al., 1979; Fung & Nash, 1983). Trypsin-activated PDE consisting only of the $\alpha\beta$ polypeptides was prepared by allowing purified PDE to pass slowly through a small column of immobilized TPCK–trypsin according to a modified procedure (Fung & Griswold-Prenner, 1989) of Hurley and Stryer (1982). The activated enzyme exhibited approximately 95% of maximal phosphodiesterase activity. The compositions of the buffered solutions were as follows: buffer A, 10 mM MOPS, pH 7.5, 2 mM $MgCl_2$, 1 mM dithiothreitol, and 200 mM NaCl; buffer B, 20 mM Tris, pH 7.5, 0.5 M NaCl, and 0.05% Tween 20; buffer C, 10 mM MOPS, 1 mM EDTA, 5 mM dithiothreitol, and 1 mM phenylmethanesulfonyl fluoride, pH 7.5.

Preparation of Anti-Peptide and Monoclonal Antibodies. Peptides with sequences corresponding to residues 2–16 (Asn-Leu-Glu-Pro-Pro-Lys-Ala-Glu-Ile-Arg-Ser-Ala-Thr-Arg-Val) and 73–87 (Phe-Asn-His-Leu-Glu-Leu-His-Glu-Leu-Ala-Gln-Tyr-Gly-Ile-Ile) of γ (Ovchinnikov et al., 1986) were synthesized by the Merrifield solid-phase method at the Peptide Synthesis Facility at UCLA. Each peptide was purified by gel filtration and conjugated to keyhole limpet hemocyanin with glutaraldehyde. The resulting conjugate was used to immunize rabbits by subcutaneous injection at multiple points every 4 weeks. Subunit specificity of the antisera was confirmed on Western blot analysis with resolved PDE subunits (Fung & Griswold-Prenner, 1989). The anti-peptide antibodies were designated anti-γ_{2-16} and anti-γ_{73-87}, respectively.

The method for the production of monoclonal antibodies was reported previously (Navon & Fung, 1988). The mAb PDE812 used in this study recognizes the α subunit of PDE and can immunoprecipitate the holoenzyme.

Extraction and Partial Purification of the β-gal-γ Fusion Protein. *E. coli* strain 222 cells carrying the recombinant plasmid were grown to the stationary phase at 37 °C in LB medium containing 50 μg/mL ampicillin. Cells were harvested by centrifugation, resuspended in buffer C, and disrupted by sonication. The soluble materials were removed by centrifugation at 13000*g*. The insoluble β-gal-γ fusion protein and cell debris were resuspended in buffer C and sonicated. The centrifugation and sonication were repeated several times until all the soluble and peripheral membrane proteins of the cells were removed. The final pellet was then solubilized in 0.2 M formic acid at 70 °C. β-gal-γ, along with other acid-solubilized proteins, was separated from the insoluble materials by centrifugation at 13000*g*. The cleared supernatant was concentrated to approximately 25 mg/mL and chromatographed on a Bio-Gel P-100 gel filtration column (0.6 × 75 cm) with 0.2 M formic acid. Fractions containing β-gal-γ were identified on a Western blot by using anti-γ peptide antibodies and by assaying for inhibitory activity. Peak protein fractions were pooled and stored in formic acid at 4 °C.

Assays for β-gal-γ Inhibitory and Binding Activities. Phosphodiesterase activity was determined by measuring proton release due to cyclic GMP hydrolysis (Yee & Liebman, 1978). The ability of β-gal-γ to inhibit the phosphodiesterase activity of trypsin-activated PDE was determined by the following procedure. An aliquot (1 μL) of partially purified β-gal-γ in 0.2 M formic acid was first placed in the well of a microtiter plate. An equal volume of 0.2 M NaOH was then carefully added to a separate area of the same well. The two samples were then rapidly mixed by adding 38 μL of buffer A, followed immediately by 80 μL of 0.02 mg/mL trypsin-activated phosphodiesterase. After a 3-min incubation an aliquot of 80 μL of 5 mM cGMP was added. The change in pH was monitored with a pH microelectrode (Microelectrodes, Inc., Londonderry, NH) and displayed on a strip chart recorder.

The binding of β-gal-γ to the catalytic $\alpha\beta$ subunit of PDE was assayed by immunoprecipitation with mAb PDE812 bound to Pansorbin. Briefly, trypsin-activated PDE in buffer A was recombined with an amount of β-gal-γ sufficient to completely inhibit all the phosphodiesterase activity under the same conditions as described above. After a 3-min incubation at 21 °C, mAb PDE812 bound to Pansorbin in buffer A containing 4 mg/mL bovine serum albumin was added. Bound PDE was pelleted by centrifugation, washed twice with buffer B to remove unbound β-gal-γ, and eluted from the immunoprecipitate with gel electrophoresis sample buffer (Fung & Griswold-Prenner, 1989). The samples were then analyzed on a Western blot by using rabbit anti-PDE antisera, together with a mixture of anti-γ_{2-16} and anti-γ_{73-87} antibodies to en-

hance the detection of the γ subunit in the immunoprecipitates.

Interaction between T_α–GTPγS and β-gal-γ was measured by coimmunoprecipitation with T_α-specific mAb TF16 (Navon & Fung, 1987; Fung & Griswold-Prenner, 1989). Recombinant β-gal-γ in the immunoprecipitates were identified on a Western blot by using a mixture of anti-γ_{2-16} and anti-γ_{73-87} antibodies and visualized with ^{125}I protein A.

Analytical Methods. Protein concentrations were routinely determined by the method of Coomassie Blue binding (Bradford, 1976) using γ-globin from Bio-Rad Laboratories as the standard. SDS–polyacrylamide gel (13%) electrophoresis was performed by the method of Laemmli (1970). Lysozyme (14 400 kDa), soybean trypsin inhibitor (21 500 kDa), carbonic anhydrase (31 000 kDa), ovalbumin (45 000 kDa), bovine serum albumin (68 000 kDa), and phosphorylase B (92 500 kDa) were used as molecular weight standards. Western blot analysis was carried out as described previously (Navon et al., 1986).

Results

Expression and Partial Purification of Active β-gal-γ. As reported previously, a cDNA clone of the γ subunit was obtained by screening a mouse retinal λgt10 library (Tuteja & Farber, 1988). To construct an expression vector, the entire coding region of mouse γ was excised by digestion with *Pst*I and *Eco*RI endonucleases and inserted in-frame into the *Pst*I/*Eco*RI sites within the *lacZ′* gene of pUC19. Clones were selected, and those containing the inserts with the desired orientation were confirmed by restriction analysis and DNA sequencing. As shown in Figure 1, the cDNA construct encodes a fusion protein containing 18 additional amino acids at the amino-terminal end of mouse γ; the first 11 residues originate from the *lacZ′* gene and the polylinker of pUC19 and the next 7 residues from the 5′ untranslated sequence of γ. When the proteins of *E. coli* 222 carrying the recombinant plasmid were analyzed by SDS–polyacrylamide electrophoresis (lower left panel of Figure 1) and on a Western blot by using a mixture of anti-γ_{2-16} and anti-γ_{73-87} antibodies (lower right panel of Figure 1), an immunoreactive band at an apparent molecular weight of 12 000 was detected (lane 3). This band migrates slightly slower than the authentic γ of PDE (lane 1) and is absent in *E. coli* 222 carrying the control pUC19 plasmid (lane 2). This result indicates that the inserted γ cDNA is expressed in *E. coli* 222 to produce a β-gal-γ fusion protein. The amount of β-gal-γ produced varies in different clones. On the average, the level of the recombinant β-gal-γ was approximately 0.1% of the total bacterial proteins.

The β-gal-γ fusion protein synthesized in *E. coli* 222 was found to be in the insoluble fraction of the bacterial homogenates. By repeated washing at low ionic strength with buffer C to remove the soluble and peripheral membrane proteins, the amount of the β-gal-γ fusion protein could be enriched to approximately 1% of the total protein. The protein was then solubilized in 0.2 M formic acid and further fractionated by gel filtration chromatography (Figure 2). As detected by Western blot analysis using anti-γ peptide antibodies, the majority of the β-gal-γ fusion protein was eluted in fractions 23–25 (panel B), which contain proteins of molecular weights ranging from 8000 to 25 000 (panel A). While the majority of the β-gal-γ fusion protein is monomeric, a small percentage appears to form dimers. When the proteins in these fractions were assayed for the ability to inhibit the phosphodiesterase activity of trypsin-activated PDE, all the fractions with β-gal-γ fusion protein exhibit potent inhibitory activity (panel C). The peak fractions containing β-gal-γ were pooled and stored at 4 °C. This preparation contained approximately 5% of β-gal-γ

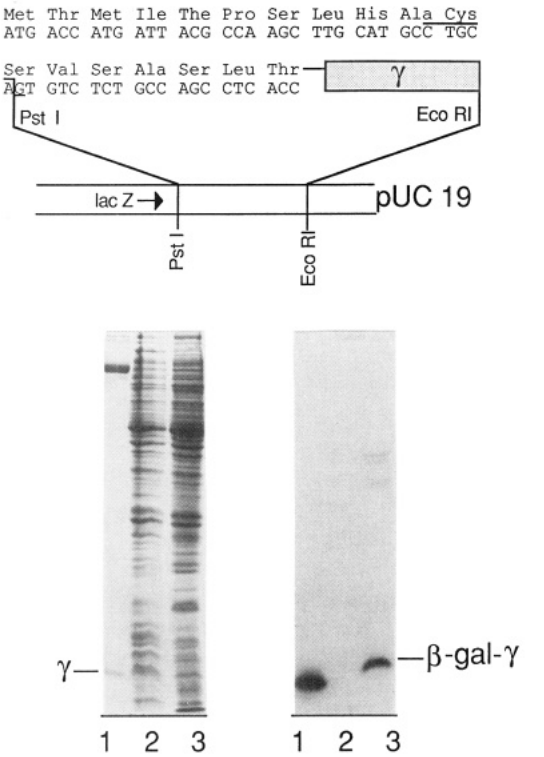

FIGURE 1: Construction and expression of the β-gal-γ fusion protein. Upper panel: β-gal-γ construct showing the entire coding region of γ (shaded area) and the nucleotide sequence encoding 18 additional amino acids at the amino terminus of γ. The expression vector was constructed by ligating the γ cDNA to the *Pst*I/*Eco*RI sites within the *lazZ′* gene of pUC19. Lower panels: Analyses of the β-gal-γ recombinant protein synthesized in *E. coli* 222 by SDS–polyacrylamide gel electrophoresis (left) and Western blot (right). The lower left panel shows the Coomassie Blue stained pattern of 5 μg of rod PDE (lane 1), 50 μg of the protein homogenate of *E. coli* 222 containing pUC19 (lane 2), and 50 μg of the protein homogenate of *E. coli* 222 containing the β-gal-γ expression vector (lane 3). The lower right panel is the Western blot of the corresponding gel except only 1 μg of rod PDE was applied to lane 1. The β-gal-γ fusion protein was detected with a mixture of anti-γ_{2-16} and anti-γ_{73-87} antibodies and visualized with ^{125}I protein A.

as estimated by quantitative Western blot analysis using purified PDE as a standard, and the overall yield was approximately 40–50% of the amount of β-gal-γ in the total bacterial homogenate. In 0.2 M formic acid, the recombinant β-gal-γ fusion protein is stable for at least a month.

Inhibitory and Binding Properties of β-gal-γ. As shown previously by Hurley and Stryer (1982), PDE can be maximally activated by proteolysis with immobilized trypsin, which selectively removes the γ subunit. Upon recombination with a stoichiometric amount of γ, the activity associated with the αβ catalytic subunits is again inhibited. The ability of β-gal-γ to similarly inhibit the trypsin-activated PDE is illustrated in the left panel of Figure 3. The phosphodiesterase activity was progressively inhibited with the addition of increasing amounts of partially purified β-gal-γ. The activity of 9 pmol of trypsin-activated PDE was completely inhibited with the addition of 4.2 μg of partially purified β-gal-γ, which contains an estimated 5% or 18 pmol of β-gal-γ. Thus, similar to native γ (Chabre, 1988; Griswold-Prenner et al., 1988), roughly two molecules of β-gal-γ were needed to fully inhibit the activity of each molecule of αβ. This result shows that β-gal-γ is as active as γ.

To further ascertain that β-gal-γ is capable of binding to αβ, inhibited PDE was immunoprecipitated with mAb PDE812 and the amount of β-gal-γ and αβ in the immunoprecipitate was quantified on a Western blot by using a mixture

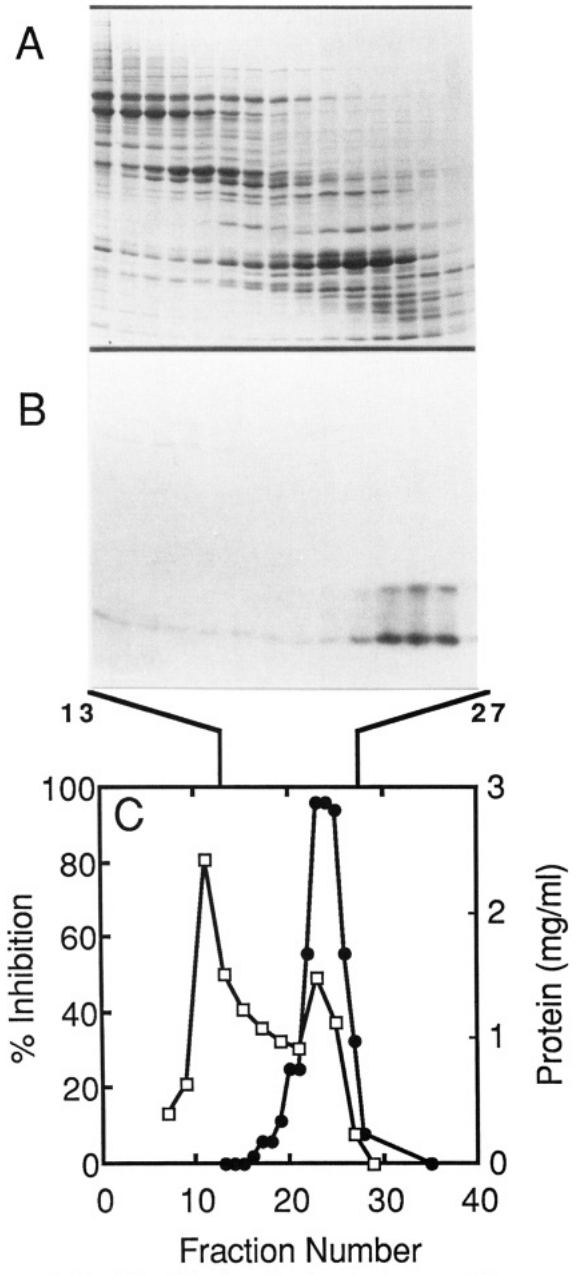

FIGURE 2: Partial purification of active β-gal-γ recombinant protein by gel filtration. Panel A: SDS–polyacrylamide gel electrophoresis analysis of 10 μL of the peak column fractions obtained by gel filtration chromatography of the solubilized bacterial proteins on a 0.6 × 70 cm Bio-Gel P-100 column. Panel B: Western blot analysis of 10 μL of the peak column fractions. The blot was incubated with a mixture of anti-γ_{2-16} and anti-γ_{73-87} antibodies and visualized with ^{125}I protein A. Panel C: Protein concentration (□) and inhibitory activity (●) of column fractions.

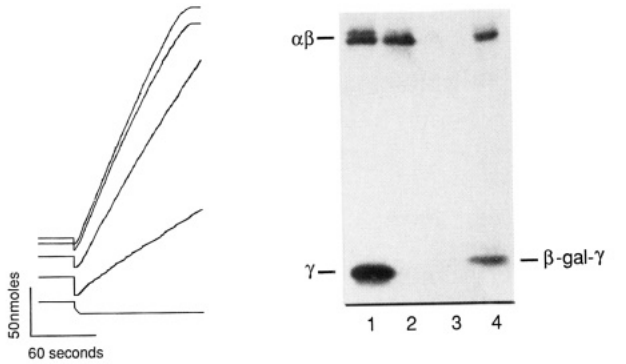

FIGURE 3: Interaction of β-gal-γ with $\alpha\beta$. Left panel: Inhibition of trypsin-activated PDE by β-gal-γ. The phosphodiesterase activity was determined by measuring the rate of proton release due to cGMP hydrolysis as described under Experimental Procedures. Traces, from top to bottom, correspond to the phosphodiesterase activities of trypsin-activated PDE (1.6 μg) after the addition of 0, 0.5, 1.0, 2.0, and 4.2 μg of partially purified β-gal-γ. Right panel: Binding of β-gal-γ to trypsin-activated PDE. Native PDE (lane 1), trypsin-activated PDE (lane 2), β-gal-γ (lane 3), and β-gal-γ recombined with trypsin-activated PDE (lane 4) were immunoprecipitated with α-specific mAb PDE812 according to the method described under Experimental Procedures. Proteins in the immunoprecipitates were extracted with electrophoresis sample buffer and identified on Western blot with a mixture of anti-γ_{2-16}, anti-γ_{73-87}, and rabbit anti-PDE antisera.

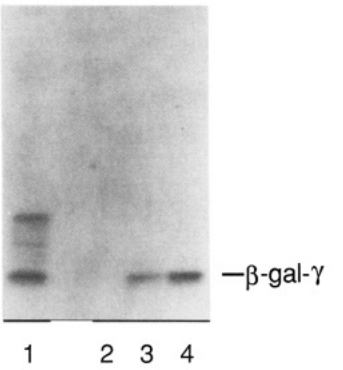

FIGURE 4: Nucleotide-dependent coimmunoprecipitation of β-gal-γ with T_α. The procedure for preparing nucleotide-bound T_α and the conditions of immunoprecipitation were identical with those described previously (Fung & Griswold-Prenner, 1989). Proteins coimmunoprecipitated with TF16 (lane 2), the TF16–T_α–GDP complex (lane 3), or the TF16–T_α–GTPγS complex (lane 4) were extracted from the immunoprecipitates with electrophoresis sample buffer and identified on Western blot with a mixture of anti-γ_{2-16} and anti-γ_{73-87} antibodies. Lane 1 contains 2.5 μg of the partially purified β-gal-γ standard.

of anti-PDE antibodies and anti-γ antibodies. As shown in the right panel of Figure 3, native PDE containing bound γ (lane 1) and trypsin-activated PDE (lane 2) were quantitatively precipitated by mAb PDE812. In contrast, β-gal-γ was not precipitated (lane 3). Upon reconstitution with trypsin-activated PDE, however, the monomeric form of β-gal-γ was coimmunoprecipitated as part of the holoenzyme (lane 4). Morevoer, the relative amounts of $\alpha\beta$ to γ in both native and reconstituted PDE immunoprecipitates, as determined by densitometric scanning of the autoradiogram, were comparable (data not shown). This analysis confirms that β-gal-γ binds tightly to $\alpha\beta$.

Using a T_α-specific monoclonal antibody TF16, we have demonstrated previously that purified γ can be selectively coimmunoprecipitated with the T_α subunit of transducin (Fung & Griswold-Prenner, 1989). Moreover, the binding of γ to T_α–GTPγS is 4-fold higher than to T_α–GDP. To determine whether β-gal-γ, like native γ, is capable of interacting with T_α in a nucleotide-dependent manner, partially purified β-gal-γ was incubated with either the T_α–GTPγS–TF16 or the T_α–GDP–TF16 complex and then precipitated with Pansorbin. As shown by Western blot analysis of the immunoprecipitates (Figure 4), monomeric β-gal-γ coimmunoprecipitates with T_α–GDP–TF16 (lane 3) and T_α–GTP–TF16 (lane 4), but not with TF16 alone (lane 2). Furthermore, the interaction of β-gal-γ with T_α is nucleotide-dependent, as indicated by a 2.5-fold increase in the amount of coimmunoprecipitated β-gal-γ in the presence of bound GTPγS. Most interestingly, only the 12-kDa β-gal-γ fusion protein was capable of interacting with T_α, as indicated by the absence of higher molecular weight aggregates of β-gal-γ (lane 1). This result again demonstrated that the properties and activities of the β-gal-γ

fusion protein are very similar to those of native γ.

Discussion

Of the many properties of G proteins, their interactions with the effectors are probably the least understood. To date, only adenylate cyclase and rod cGMP PDE have been purified and reconstituted with their respective G proteins to form active systems with characteristics very similar to those in intact cells (Gilman, 1987). Using the interactions between PDE and transducin as a model system, we have recently initiated a series of studies intended to elucidate the molecular basis of G protein–effector coupling. We first established that the inhibitory γ subunit of PDE, but not the catalytic $\alpha\beta$, interacts with T_α–GTP (Fung & Griswold-Prenner, 1989). This finding greatly simplified the analysis of the T_α binding domain of PDE and allowed us to focus our investigation exclusively on γ.

In this paper, we present the first description of the expression and characterization of a β-galactosidase-γ fusion protein that interacts with both transducin and the catalytic subunits of PDE. This recombinant protein is produced constitutively in *E. coli* 222, which we found to give the highest level of expression among several strains of bacteria tested. Induction with isopropyl β-D-thiogalactopyranoside did not further increase the yield, even though the expression of β-gal-γ is controlled by the *lac* promoter. Although the recombinant β-gal-γ fusion protein was found in the particulate fraction after cell lysis, it can be solubilized in formic acid and correctly refolded into an active conformation upon neutralization of the pH. Taking advantage of this unique property of β-gal-γ, we were able to isolate milligram quantities of the β-gal-γ fusion protein, which represent approximately 5% of total protein. We did not make any further attempt to purify the β-gal-γ fusion protein to homogeneity by column chromatography, because we suspected that, at the present level of protein expression, it would be difficult to achieve. Instead, we concentrated on increasing the level of β-gal-γ expression. Preliminary results have shown that a 10-fold increase in β-gal-γ expression can be obtained by using the phagemid vector pUC119 (Vieira & Messing, 1987).

Although the β-gal-γ fusion protein has biochemical properties very similar to that of the authentic γ, there are two major differences in their primary sequences. First, the γ portion of the recombinant β-gal-γ fusion protein is derived from a mouse cDNA encoding an amino acid sequence differing at positions 8 and 17 from bovine γ. In mouse γ these amino acids are Gly 8 and Ile 17, while in the bovine γ they are Ala 8 and Met 17 (Tuteja & Farber, 1988). Second, 18 amino acids have been added to the amino terminus of the mouse γ. Despite such marked differences in primary structures, the β-gal-γ fusion protein appears to possess inhibitory activity very similar to that of bovine γ and, like native γ, is capable of binding selectively to the $\alpha\beta$ catalytic subunits, as well as to the T_α–GTPγS complex. Thus, by all known criteria, β-gal-γ functions in a manner totally analogous to that of native bovine γ. Moreover, the fact that β-gal-γ derived from mouse γ interacts with bovine $\alpha\beta$ catalytic subunits and T_α indicates that the function of the inhibitory γ subunit is conserved among the two species.

The γ subunit of PDE is a key protein component involved in the regulation of cGMP-mediated visual excitation in retinal rods. The high-affinity binding of γ to the catalytic $\alpha\beta$ subunits suppresses the phosphodiesterase activity and serves to maintain a high cGMP level in dark-adapted rods. During light activation, γ is removed by the interaction with T_α–GTP. The increase in phosphodiesterase activity rapidly reduces the cytosolic cGMP concentration, leading to the closure of many cGMP-regulated cation channels. Hence, the dual interactions of γ with transducin and $\alpha\beta$ mediate respectively a positive and a negative effect on the cGMP level of ROS and indirectly control the electrical activity of the rod. Since the discovery of the inhibitory γ subunit many years ago, the major limiting factor in delineating the interactions of γ with $\alpha\beta$ and T_α–GTP has been the limited supply of retinal PDE, the only known source of γ. The successful production of large quantities of active β-gal-γ in *E. coli*, coupled with the capability to alter the primary structure of the recombinant protein by site-directed mutagenesis, not only will facilitate the identification of the domains responsible for T_α and $\alpha\beta$ bindings but also will provide an unique opportunity to resolve the three-dimensional structure of γ by X-ray crystallography and 2-D NMR.

Registry No. PDE, 9068-52-4.

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Overcoming the Overlap Problem in the Assignment of ^1H NMR Spectra of Larger Proteins by Use of Three-Dimensional Heteronuclear ^1H-^{15}N Hartmann-Hahn-Multiple Quantum Coherence and Nuclear Overhauser-Multiple Quantum Coherence Spectroscopy: Application to Interleukin 1β†

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ABSTRACT: The application of three-dimensional (3D) heteronuclear NMR spectroscopy to the sequential assignment of the ^1H NMR spectra of larger proteins is presented, using uniformly labeled (~95%) [^{15}N]interleukin 1β, a protein of 153 residues and molecular mass of 17.4 kDa, as an example. The two-dimensional (2D) 600-MHz spectra of interleukin 1β are too complex for complete analysis, owing to extensive cross-peak overlap and chemical shift degeneracy. We show that the combined use of 3D ^1H-^{15}N Hartmann-Hahn-multiple quantum coherence (HOHAHA-HMQC) and nuclear Overhauser-multiple quantum coherence (NOESY-HMQC) spectroscopy, designed to provide the necessary through-bond and through-space correlations for sequential assignment, provides a practical general-purpose method for resolving ambiguities which severely limit the analysis of conventional 2D NMR spectra. The absence of overlapping cross-peaks in these 3D spectra allows the unambiguous identification of $C^\alpha H(i)$-$NH(i+1)$ and $NH(i)$-$NH(i+1)$ through-space nuclear Overhauser connectivities necessary for connecting a particular $C^\alpha H(i)$-$NH(i)$ through-bond correlation with its associated through-space sequential cross-peak. The problem of amide NH chemical shift degeneracy in the ^1H NMR spectrum is therefore effectively removed, and the assignment procedure simply involves inspecting a series of 2D ^1H-^1H slices edited by the chemical shift of the directly bonded ^{15}N atom. Connections between residues can be identified almost without any knowledge of the spin system types involved, though this type of information is clearly required for the eventual placement of the connected residues within the primary sequence. Strategies for obtaining identification of spin system types include traditional analysis of the spectrum of nonlabile protons, site-directed mutagenesis, and specific labeling of selected amino acids. It is envisaged that the intrinsic simplicity of the 3D heteronuclear spectra, even for proteins of 150-200 residues, will permit the development of efficient computer-assisted or automated sequential assignment methods.

The extension of nuclear magnetic resonance (NMR)[1] experiments from one to two dimensions [see Ernst et al. (1987) for a review] has played a key role in the development of methods for the determination of three-dimensional structures of macromolecules in solution. The first step of such structure determinations is the sequential assignment of the ^1H NMR spectrum by means of experiments that identify through-bond and sequential through-space connectivities. By spreading the resonances into two frequency dimensions, the concomitant increase in both resolution and information content enables these connectivities to be readily analyzed. To date, the spectra of a substantial number of small proteins (<100 residues) have

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[1] Abbreviations: NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser enhancement spectroscopy; HOHAHA, homonuclear Hartmann-Hahn spectroscopy; HMQC, heteronuclear multiple quantum coherence spectroscopy.

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