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Production of Antibodies against Rhodopsin after Immunization with $\beta\gamma$-Subunits of Transducin: Evidence for Interaction of $\beta\gamma$-Subunits of Guanosine 5′-Triphosphate Binding Proteins with Receptor†

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ABSTRACT: The light-detecting system of retinal rod outer segments is regulated by a guanyl nucleotide binding (G) protein, transducin, which is composed of α-, β-, and γ-subunits. Transducin couples rhodopsin to the intracellular effector enzyme, a cGMP phosphodiesterase. The $\beta\gamma$ complex ($T_{\beta\gamma}$) is required for the α-subunit (T_α) to interact effectively with the photon receptor rhodopsin. It is not clear, however, whether $T_{\beta\gamma}$ binds directly to rhodopsin or promotes T_α binding to rhodopsin only by binding to T_α. We have found that serum from rabbits immunized with $T_{\beta\gamma}$ contained a population of antibodies that were reactive against rhodopsin. These antibodies could be separated from $T_{\beta\gamma}$ antibodies by adsorbing the latter on immobilized transducin. Binding of purified rhodopsin antibodies was inhibited by $T_{\beta\gamma}$, suggesting that the rhodopsin antibodies and $T_{\beta\gamma}$ bound to the same site on rhodopsin. We propose that the rhodopsin antibodies act both as antiidiotypic antibodies against the idiotypic $T_{\beta\gamma}$ antibodies and as antibodies against rhodopsin. This hypothesis is consistent with the conclusion that $T_{\beta\gamma}$ interacts directly with the receptor. It is probable that in an analogous way, $G_{\beta\gamma}$ interacts directly with receptors of the adenylate cyclase system.

In the light-detecting system of retinal rod outer segments, the photon receptor rhodopsin is coupled with the effector enzyme cGMP phosphodiesterase through transducin, a GTP-binding regulatory (G)[1] protein (Stryer et al., 1981). Activation of rhodopsin by light stimulates binding of GTP to transducin (Fung & Stryer, 1980) which can then activate the phosphodiesterase. With hydrolysis of the bound GTP, transducin returns to the inactive state. Transducin has significant structural and functional homology with the stimulatory and inhibitory G proteins, G_s and G_i, respectively, that regulate the hormone-sensitive adenylate cyclase (Gilman, 1984; Lefkowitz et al., 1984). These G proteins are heterotrimers with α-, β-, and γ-subunits (Hildebrandt et al., 1984; Bokoch et al., 1984; Fung et al., 1981). The α-subunits bind and hydrolyze GTP (Gilman, 1984; Fung et al., 1981). Examination of cDNA clones for T_α, $G_{i\alpha}$, and $G_{s\alpha}$ has revealed striking homology (Medynski et al., 1985; Tanabe et al., 1985;

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[1] Abbreviations: G proteins, guanyl nucleotide binding regulatory proteins; G_s and G_i, stimulatory and inhibitory G proteins, respectively, of adenylate cyclase; $G_{s\alpha}$ and $G_{i\alpha}$, α-subunits, respectively, of G_s and G_i; $G_{\beta\gamma}$, β- and γ-subunits of G_s and G_i; T_α and $T_{\beta\gamma}$, α- and $\beta\gamma$-subunits of transducin; ELISA, enzyme-linked immunosorbent assay; TBS, Tris-buffered saline; TTBS, Tris-buffered saline with 0.05% Tween-20; buffer A, 0.15 M Na_2HPO_4, pH 8.5; buffer B, 2.7 mM KCl/1.5 mM KH_2PO_4/150 mM NaCl/8 mM Na_2HPO_4; buffer C, buffer B containing 0.001% Triton X-100, 2 mM EDTA, and 1% horse serum; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate.

Yatsunami & Khorana, 1985; Robishaw et al., 1986; Itoh et al., 1986; Nukada et al., 1986), reflecting the parallel functions of these proteins. The β-subunits of different G proteins appear to be highly conserved both immunologically and functionally. $G_{\beta\gamma}$ can replace $T_{\beta\gamma}$ in reconstituting the light-dependent GTPase activity with T_α and rhodopsin (Kanaho et al., 1984). Antibodies raised against $T_{\beta\gamma}$ cross-react with G_β, but not G_γ (Gierschik et al., 1985). A cDNA clone for T_β has been used to demonstrate the presence of related mRNAs in other mammalian tissues (Fong et al., 1986), further supporting the hypothesis that β-subunits of different G proteins are closely related.

Activation of G proteins as a consequence of receptor-promoted GTP binding is believed to result from dissociation of α- and $\beta\gamma$-subunits (Gilman, 1984). In this activated conformation, the G protein can act upon the effector enzyme. Reassociation of α with $\beta\gamma$ results in termination of activation. Fung demonstrated that $T_{\beta\gamma}$ is essential for rhodopsin stimulation of guanyl nucleotide binding and GTP hydrolysis by T_α (Fung, 1983), most likely because T_α is unable to interact effectively with rhodopsin in the absence of $T_{\beta\gamma}$. The finding that both subunits are required for either to bind to rhodopsin raises the possibility that both subunits possess a binding site for rhodopsin. Alternatively, $T_{\beta\gamma}$ could increase the affinity of T_α for rhodopsin without directly interacting with the photon receptor. If $T_{\beta\gamma}$ does possess a binding site for rhodopsin, the antigen-binding site of antibodies directed against this region might be expected to resemble the $T_{\beta\gamma}$ binding site on rhodopsin. In turn, an antibody against this idiotope of the first antibody (an antiidiotypic antibody) would resemble, or carry an "internal image" of, the interaction site on $T_{\beta\gamma}$ (Jerne, 1974) and bind to rhodopsin. To test the hypothesis that $T_{\beta\gamma}$ binds directly to rhodopsin, serum samples from rabbits immunized with $T_{\beta\gamma}$ were screened for the presence of anti-rhodopsin antibodies. As reported here, anti-rhodopsin antibodies were produced in these rabbits, consistent with the conclusion that $T_{\beta\gamma}$ interacts directly with rhodopsin.

Experimental Procedures

Preparation of Antibodies. Transducin, T_α, $T_{\beta\gamma}$, and rhodopsin were purified and stored as described (Kühn, 1980; Shinozawa et al., 1980; Hong & Hubbell, 1973). Preparations of rhodopsin showed no evidence of contaminating transducin subunits on silver-stained SDS–polyacrylamide gels. Rabbits were immunized with 500 μg of $T_{\beta\gamma}$ or other proteins emulsified in complete Freund's adjuvant injected subcutaneously at multiple sites. Booster injections (100–200 μg of protein in incomplete Freund's adjuvant) were given at intervals of 3–5 weeks. Serum samples were obtained 7–10 days after each injection. IgG fractions were purified by protein A–Sepharose chromatography (Ey et al., 1978):, recovery was ~2 mg of IgG/mL serum.

Affinity Purification of Anti-$T_{\beta\gamma}$ Antibodies. Transducin (6 mg) was reacted with 2 mL of CNBr-activated Sepharose 4B (Pharmacia) in 0.1 M $NaHCO_3$/0.5 M NaCl, pH 8.3, for 5 h at room temperature according to the manufacturer's instructions. Coupling efficiency was 95%. After unreacted groups were blocked with ethanolamine, the resin was washed and stored in buffer A. The IgG fraction was applied to transducin–Sepharose (2 mL), and the effluent was recycled through the column for 2 h. The column was washed extensively with buffer A, and bound protein (~70 μg) was eluted with 0.1 M sodium acetate, pH 4.0. After reequilibration of the column in buffer A, the initial effluent was cycled over the column for an additional 5 h in an attempt to maximize removal of IgG capable of reacting with transducin or $T_{\beta\gamma}$.

Table I: Inhibition of Binding of Purified Anti-rhodopsin IgG to Rhodopsin by Transducin or Transducin Subunits[a]

additions	reaction with rhodopsin (absorbance 495 nm)
none	0.31
transducin (2 μg)	0.18
$T_{\beta\gamma}$ (1 μg)	0.16
T_α (1 μg)	0.22
$T_\alpha + T_{\beta\gamma}$ (1 μg of each)	0.16
none[b]	0.14

[a] Anti-rhodopsin IgG fraction purified on transducin-coated microtiter dishes was diluted 1/80 and incubated for 4 h at 25 °C in rhodopsin-coated wells containing the indicated additions. This experiment was repeated 3 times with IgG from two separate purifications. [b] No rhodopsin on wells.

For some experiments (e.g., Table I), this fraction was further incubated with transducin coated on vinyl microtiter dishes as described below. The IgG fraction was diluted 20-fold in buffer B containing bovine serum albumin (1 mg/mL). Samples (100 μL) were added to transducin-coated wells and after 2 h at 23 °C transferred to a new transducin-coated well. This procedure was repeated until each sample had been incubated sequentially in five separate wells.

ELISA. Microtiter dishes (96 wells, Falcon) were coated with transducin or its subunits (10 μg/mL, 50 μL/well) for 2 h at 23 °C or with rhodopsin (20 μg/mL, 25 μL/well) by drying at 37 °C. Wells were incubated for 1 h with buffer B containing bovine serum albumin, 5 mg/mL, and then rinsed twice with buffer B before addition of 50 μL of antiserum of IgG diluted in buffer B with bovine serum albumin, 1 mg/mL. (Dilutions of IgG fractions are reported relative to the volumes of serum from which they were derived). After 12 h at 4 °C, wells were rinsed 3 times with buffer C (buffer B containing 0.001% Triton X-100, 2 mM EDTA, and 1% horse serum) and 3 times with buffer B. Goat anti-rabbit IgG conjugated with horseradish peroxidase (Bio-Rad) diluted 1/2000 in buffer B with bovine serum albumin, 1 mg/mL, was added (100 μL/well), and after 1 h at 23 °C, wells were rinsed 3 times with buffer C and 3 times with buffer B. Peroxidase substrate (100 μL/well of 0.012% phenylenediamine and 0.04% H_2O_2 in citrate buffer, pH 5.0) was added, and 15–30 min later, the absorbance at 495 nm was measured with an ELISA plate reader (Flow Laboratories). All experiments were replicated at least twice.

Immunoblotting on Nitrocellulose Paper. Proteins separated on discontinuous SDS–polyacrylamide gels (Laemmli, 1970) were transferred electrophoretically to nitrocellulose paper using a Bio-Rad Trans-blot apparatus. After transfer, the nitrocellulose blot was first incubated for 1 h in TBS containing 3% gelatin and then rinsed twice in TTBS. Blots were then incubated for 4 h with different sera diluted in TTBS containing 1% gelatin, rinsed twice with TTBS, and incubated for 2 h with horseradish peroxidase conjugated goat anti-rabbit IgG diluted 1/2000 in TTBS containing 1% gelatin. After two washes with TTBS and one wash with TBS, the blot was developed with the peroxidase substrate 4-chloro-1-naphthol.

Results

Generation of Antibodies against $T_{\beta\gamma}$ and T. Serum samples from rabbits immunized with $T_{\beta\gamma}$ were tested for the presence of anti-$T_{\beta\gamma}$ antibodies by using a solid-phase ELISA as described under Experimental Procedures. Reactivity with transducin subunits was also assessed by immunoblotting after separation by polyacrylamide gel electrophoresis. As shown in Figure 1, the anti-$T_{\beta\gamma}$ serum reacted with both β- and

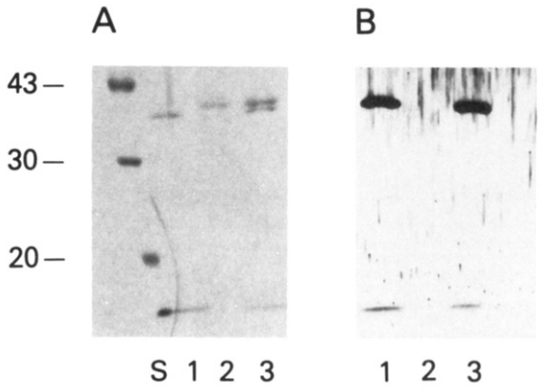

FIGURE 1: Reaction of anti-$T_{\beta\gamma}$ serum with transducin subunits. Approximately 5 μg of $T_{\beta\gamma}$ (lane 1), T_α (lane 2), or transducin (lane 3) was electrophoresed on a discontinuous SDS–polyacrylamide gel (12% separating gel) and then electroblotted from the gel to nitrocellulose paper. Duplicate blots were (A) stained for protein with amido black and (B) immunostained with anti-$T_{\beta\gamma}$ serum used for other experiments reported here (1/1000 dilution).

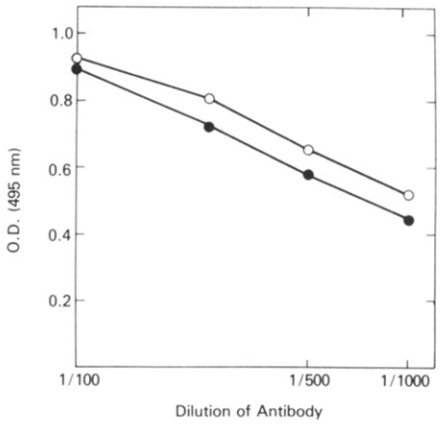

FIGURE 2: Binding of anti-$T_{\beta\gamma}$ serum to rhodopsin and $T_{\beta\gamma}$. Anti-$T_{\beta\gamma}$ serum was assayed for reactivity with $T_{\beta\gamma}$ (●) or rhodopsin (○).

γ-subunits. In agreement with results from the ELISA (data not shown), no reactivity toward T_α was seen.

Detection of Anti-rhodopsin Antibodies. Numerous serum samples from five rabbits immunized with $T_{\beta\gamma}$ were tested for their reactivity with rhodopsin at a 1/500 dilution using an ELISA. One sample exhibited considerable reactivity with both rhodopsin and transducin (0.70 and 0.74 units, respectively; also see Figure 2). Five other serum samples from the same rabbit failed to react appreciably with rhodopsin; preimmune serum likewise did not react. Reactivity of the anti-$T_{\beta\gamma}$ serum with rhodopsin was similar to that of sera from rabbits immunized with rhodopsin or transducin (data not shown). The anti-$T_{\beta\gamma}$ serum reacted comparably with holotransducin and $T_{\beta\gamma}$ (Figure 3); reaction with T_α was no greater than that with uncoated wells (data not shown).

Separation of Anti-rhodopsin from Anti-$T_{\beta\gamma}$ Antibodies. The IgG fraction from anti-$T_{\beta\gamma}$ serum that did not bind to transducin–Sepharose (see Experimental Procedures) was essentially unaltered in rhodopsin-binding activity whereas reactivity with $T_{\beta\gamma}$ was reduced 50% (Figure 4A vs. Figure 2). IgG eluted from transducin–Sepharose reacted with $T_{\beta\gamma}$ but had relatively little rhodopsin-binding activity (Figure 4B). From the IgG that did not bind to transducin–Sepharose, a fraction apparently free of transducin-binding activity was obtained after further incubation with transducin coated on microtiter dishes (Figure 5). Binding of this purified anti-rhodopsin IgG was inhibited ~90% by addition of $T_{\beta\gamma}$ and 80% by transducin; T_α inhibited binding by ~50% (Table I).

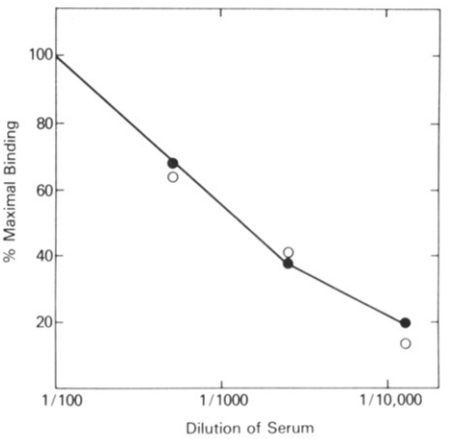

FIGURE 3: Binding of anti-$T_{\beta\gamma}$ serum to $T_{\beta\gamma}$ and transducin. Serum from a rabbit immunized with $T_{\beta\gamma}$ was assayed for reactivity with $T_{\beta\gamma}$ (○) or transducin (●) as described under Experimental Procedures. Binding in the absence of antigen was 5% of total binding. Maximal binding (absorbance 495 nm) was 0.28 with $T_{\beta\gamma}$ and 0.34 with transducin.

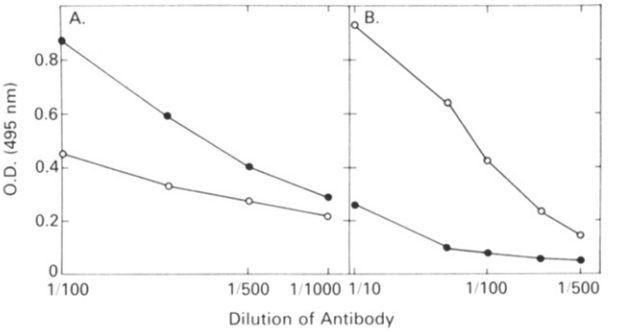

FIGURE 4: Reaction of affinity-purified anti-$T_{\beta\gamma}$ and anti-rhodopsin IgG fractions with rhodopsin and $T_{\beta\gamma}$. IgG from anti-$T_{\beta\gamma}$ serum was separated into a fraction that did not bind to transducin–Sepharose (A) and a fraction that did (B). These fractions diluted as indicated were assayed for reactivity with $T_{\beta\gamma}$ (○) or rhodopsin (●).

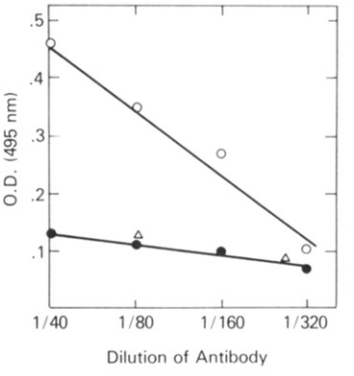

FIGURE 5: Reaction of purified anti-rhodopsin IgG fraction with rhodopsin and transducin. Anti-rhodopsin IgG fraction purified on transducin-coated microtiter dishes as described under Experimental Procedures was diluted as indicated and assayed for reactivity with rhodopsin (○), transducin (●), or uncoated wells (△).

In the absence of $T_{\beta\gamma}$, T_α can bind to rhodopsin to some extent (Fung, 1983), and by doing so could interfere with antibody binding to the $T_{\beta\gamma}$ site.

Discussion

Anti-rhodopsin antibodies found in serum from a rabbit immunized with $T_{\beta\gamma}$ were separated from anti-$T_{\beta\gamma}$ antibodies by adsorbing the latter on immobilized transducin, demonstrating that they were a distinct population of antibodies. As they were not present in preimmune serum, but were generated

after immunization with $T_{\beta\gamma}$, it seems likely that these anti-rhodopsin antibodies are antiidiotypic antibodies directed against the idiotypic anti-$T_{\beta\gamma}$ antibodies. Definitive proof of this interpretation, however, will require direct demonstration of interaction of these antibodies with the anti-$T_{\beta\gamma}$ antibodies. The hypothesis of the idiotypic–antiidiotypic network predicts that an antiidiotypic antibody may have conformational similarity with an epitope on the original antigen (Jerne, 1974). Since binding sites of both the antiidiotypic antibody and antigen are complementary to the idiotypic antibody, antibodies which interact with the original antigen can also interact with the antiidiotypic antibody. Presumably, the antiidiotypic antibodies described here recognize rhodopsin because they resemble the site on transducin that interacts with rhodopsin in the rod outer segments and are binding to this site.

There are several reports of production of antiidiotypic antibodies that bind to a hormone receptor following immunization with the hormone or other receptor ligand. Immunization with anti-insulin antibodies (Sege & Peterson, 1978) as well as with insulin (Schecter et al., 1984) has resulted in generation of insulin receptor antibodies. Antiidiotypic antibodies that interact with the β-adrenergic receptor and mimic hormone action have also been described (Schreiber et al., 1980). In these cases, the epitope recognized by the antiidiotypic antibodies is a portion of the receptor that is accessible on the cell surface whereas the anti-rhodopsin antibodies produced in rabbits immunized with $T_{\beta\gamma}$ presumably react with a site(s) on the receptor that is (are) involved with signal transduction rather than signal detection.

Using purified transducin subunits, Fung demonstrated that $T_{\beta\gamma}$ stimulated both the Gpp(NH)p binding and GTPase activity of T_α, probably by facilitating the interaction of T_α with photolyzed rhodopsin (Fung, 1983). Purified $T_{\beta\gamma}$ bound poorly to rhodopsin in the absence of T_α, leaving open the possibility that $T_{\beta\gamma}$ interacts with rhodopsin only indirectly through its binding to T_α. The generation of anti-rhodopsin antibodies in response to immunization with $T_{\beta\gamma}$, as reported here, is consistent with the conclusion that the function of $T_{\beta\gamma}$ in the visual cascade system involves its interaction with rhodopsin as well as with T_α. The finding that $T_{\beta\gamma}$ inhibited binding of these anti-rhodopsin antibodies to immobilized rhodopsin is in agreement with this interpretation. Functional and structural similarities between the $\beta\gamma$ complex of transducin and those of other G proteins support the idea that the $\beta\gamma$ complex plays a similar role in the adenylate cyclase and the retinal photoreceptor systems (Gilman, 1984). $G_{i\alpha}$ and $G_{\beta\gamma}$ can replace T_α and $T_{\beta\gamma}$, respectively, in reconstituting light-stimulated GTPase activity with rhodopsin (Kanaho et al., 1984). In that system, photolyzed rhodopsin mimics the action of agonist-occupied inhibitory receptors, and it seems probable that $G_{\beta\gamma}$ interacts with $G_{i\alpha}$ and inhibitory receptor in a manner analogous to the interaction of $T_{\beta\gamma}$ with T_α and rhodopsin.

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