0026-895X/90/060797-04$02.00/0

MOLECULAR PHARMACOLOGY, 37:797–800

Immunological Characterization of Guanine Nucleotide-Binding Proteins: Effects of a Monoclonal Antibody Against the γ Subunit of Transducin on Guanine Nucleotide-Binding Protein-Receptor Interactions

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Received August 17, 1989; Accepted February 6, 1990

SUMMARY

Guanine nucleotide-binding proteins (G proteins) transduce signals from agonist- and light-sensitive receptors. In the visual excitation system, the photon receptor rhodopsin is coupled to the G protein G_t (transducin). G_t is composed of α, β, and γ subunits; the α subunit binds guanine nucleotide, whereas the β and γ subunits, which are tightly associated, appear to facilitate interaction of α with receptor and pertussis toxin-catalyzed ADP-ribosylation of α. To study the function of transducin, monoclonal antibodies were developed against the purified protein. Monoclonal antibody 2H3 reacted with G_{t_γ} but not G_{t_γ} from bovine brain or rabbit liver. In the absence of photolyzed rhodopsin, both intact 2H3 and Fab fragments of 2H3 were able to inhibit completely, in a concentration-dependent manner, ADP-ribosylation of transducin by pertussis toxin. 2H3 had no effect on ADP-ribosylation in the presence of photolyzed rhodopsin. The GTPase activity of transducin, which is dependent on rhodopsin, was inhibited only 50% by 2H3. These data are consistent with the hypotheses that an epitope recognized by 2H3 may be important in the formation of the $\alpha\beta\gamma$ complex or that interaction of 2H3 with γ may alter conformation of the latter and, thereby, inhibit complex formation. Further, reactions of γ with 2H3 appear to be prevented by interaction with rhodopsin, suggesting that its interaction either shields or alters the epitope recognized by 2H3.

The G proteins are a family of membrane-associated signal-transducing proteins that couple receptors to intracellular effectors (1–4). The well characterized G proteins include G_t, which couples rhodopsin to a cGMP phosphodiesterase in retinal rod outer segments, and G_i and G_s, which couple inhibitory and stimulatory receptors, respectively, to the adenylyl cyclase catalytic unit (1–4). G proteins are heterotrimers consisting of α, β, and γ subunits (1–4). The α subunits, which for each G protein are unique, bind guanine nucleotides and are probably responsible for some actions on the effector (1–4). It appears that dissociation of the α subunit, with GTP bound, from βγ is necessary for activation of the G protein (1–4). Several functions have been proposed for the βγ complex. It has been postulated to serve as a membrane anchor for the α subunit (5) and to be indirectly responsible for inactivation of adenylyl cyclase, due to its ability to bind and inactivate the G_{s_α} subunit (3). It has been proposed by some investigators to regulate ion channels and activate phospholipase A_2 (6–8). The βγ complex of transducin is necessary for G_{t_α} binding to rhodopsin and enhances both rhodopsin-stimulated GTP hydrolysis by G_{t_α} and Gpp(NH)p binding (9).

The β and γ subunits remain tightly associated throughout purification and cannot be separated without denaturation. Because of this, no activities have been ascribed to the individual subunits. Based on deduced amino acid sequences derived from cDNA clones, it appears that there are at least two forms of G_β that are very similar in primary structure but are derived from different genes (10–13). In contrast, G_{t_γ} is clearly different from G_γ, based on immunoreactivity (14), two-dimensional peptide maps (15), and sequence (16). Although both $G_{t_{\beta\gamma}}$ and $G_{\beta\gamma}$ can promote rhodopsin-stimulated GTP hydrolysis by G_{t_α} or G_{i_α} (17), there are functional differences between the two βγ complexes. For example, $G_{\beta\gamma}$ is more potent than $G_{t_{\beta\gamma}}$ in its ability to inhibit G_{s_α}-stimulated adenylyl cyclase activity (18). Because the differences between complexes may lie in the γ subunit, we investigated possible functions of the γ subunit of transducin, using a monoclonal antibody against G_{t_γ}, as reported here.

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ABBREVIATIONS: G protein, guanine nucleotide-binding protein; G_t, transducin; G_{t_α} and $G_{t_{\beta\gamma}}$, α and β_γ subunits of G_t; G_i and G_s, inhibitory and stimulatory guanine nucleotide-binding proteins; Gpp(NH)p, guanyl-5′-yl-β,γ-imido-diphosphate; EGTA, ethylene glycol bis(β-aminoethyl ether,-*N*,*N*,*N*′-*N*′-tetraacetic acid; SDS, sodium dodecyl sulfate.

Experimental Procedures

Monoclonal antibodies. The cell line that secretes antibody 2H3 was derived from spleen cells from a BALB/c mouse that was immunized with bovine holotransducin. Details of the immunization, cell fusion, and screening are published elsewhere (19). 2H3 was subcloned in agar repeatedly to ensure monoclonality. Protein A-Sepharose-purified IgG from cells grown in serum-free medium was used in all experiments, unless otherwise noted. Fab fragments were prepared using a published procedure (20). Briefly, Protein A-Sepharose-purified 2H3 and papain (24 units/mg; Sigma, St. Louis, MO) in a ratio of 100:1 (w/w) were incubated for 4 hr at 37° in 20 mM phosphate buffer, pH 7.5, containing 10 mM cysteine and 2 mM EDTA. Fab fragments were separated from Fc fragments and intact IgG on a Protein A-Sepharose column.

ADP-ribosylation. Pertussis toxin (List Biological Laboratories, Campbell, CA) was activated with dithiothreitol for 10 min immediately before use (21). Transducin or its subunits were incubated at 30° with 50 mM potassium phosphate (pH 7.5), 20 mM thymidine, 0.1 μM Gpp(NH)p, 2 mM $MgCl_2$, 20 μM ATP, 10 μM [α]^{32}P]NAD (1000 Ci/mol; New England Nuclear), pertussis toxin (0.25 μg/tube), and rhodopsin (1.5 μg) or an equivalent amount of phospholipid (total volume, 0.1 ml). After 1 hr, samples were placed on ice, diluted to 2.5 ml with buffer A (20 mM Tris·HCl, pH 7.5, 0.5 mM $MgCl_2$, 1 mM dithiothreitol, 0.05 mM EDTA, 0.5 mM NaN_3), and transferred to 0.45-μm type HA filters (Millipore, Bedford, MA). Filters were washed four times with 3.0 ml of buffer A before radioassay. The accuracy of this method for measuring ADP-ribosylation of G_{t_α} was determined in one experiment by electrophoresing identical samples on SDS-polyacrylamide gels. Autoradiography of this gel revealed only one radioactive band, corresponding to G_{t_α}.

Protein A-Sepharose precipitation. Purified 2H3, with other additions as indicated, was incubated for 1 hr at room temperature (total volume, 0.5 ml). Protein A-Sepharose (Pharmacia-LKB, Piscataway, NJ) equilibrated in buffer B (50mM Tris·HCl, pH 8.5, 0.15M NaCl) (0.5 ml) was added and then, following incubation for 20 min, samples were transferred to 2-ml Econo-columns (Bio-Rad, Richmond, CA), followed by 1 ml of buffer B; the flow-through plus wash was collected and, after a further wash with 3 ml of buffer B (discarded), columns were eluted with 1.5 ml of 0.05 M glycine-HCl, 0.15 M NaCl, pH 2.3. Samples of the eluted fractions were analyzed by SDS-polyacrylamide gel electrophoresis (22).

GTPase assays. GTPase activity was assayed in a total volume of 0.1 ml using [γ-^{32}]GTP, as described (17). An irrelevant monoclonal antibody derived from the same myeloma cell line was used to maintain a constant amount of IgG in all assays.

Immunoblots. Immunoblotting was performed by a modification of published methods (23), except that the second antibody was a goat anti-mouse IgG coupled to alkaline phosphatase (Bio-Rad), and blots were developed as described by the manufacturer.

Protein purification. Rhodopsin (24), transducin (25), and the transducin α and βγ subunits (26) were purified from bovine retina as described (19). Rhodopsin was reconstituted into liposomes as described by Jackson and Litman (27); the rhodopsin preparation contained rhodopsin (0.6 mg/ml) and dimyristoyl phosphatidylcholine, in a molar ratio of 100:1 phospholipid to rhodopsin. Before use in experiments, rhodopsin was activated by exposure to room light for 10 min.

Results

Monoclonal antibody 2H3 reacted with holotransducin and $G_{t_{\beta\gamma}}$ in an enzyme-linked immunosorbent assay (data not shown) and reacted specifically with G_{t_α} on immunoblot (Fig. 1). On immunoblots, 2H3 did not react with γ subunits purified from bovine brain or rabbit liver or with any other protein present in homogenates of total brain or liver protein (data not shown).

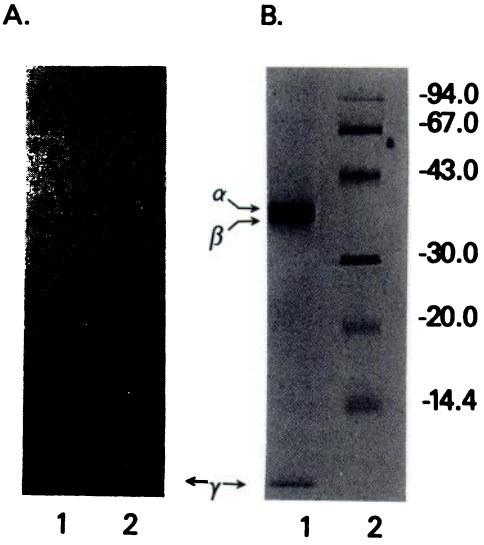

Fig. 1. Immunoblot of transducin with monoclonal antibody 2H3. Transducin (10 μg) (*lane 1*) and standard proteins (*lane 2*) were subjected to electrophoresis (15%), transferred to nitrocellulose paper and reacted with 2H3 (A) or stained with amido black (B).

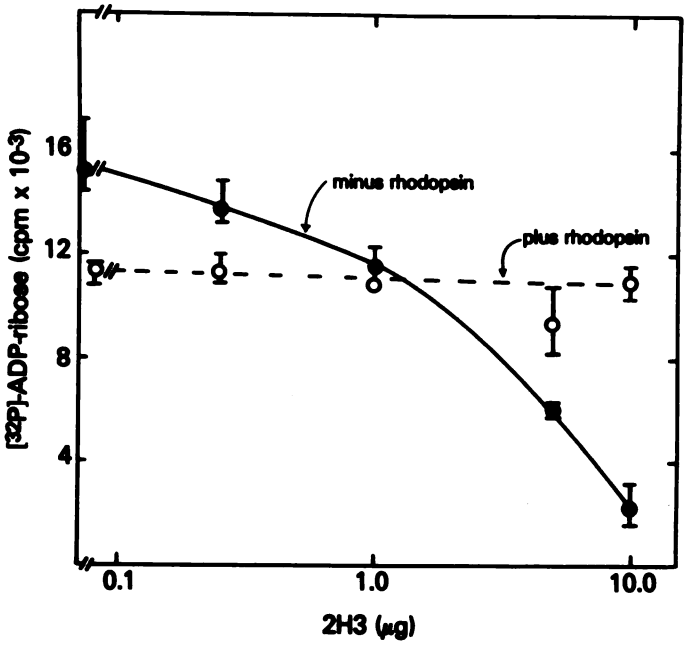

Fig. 2. Effect of 2H3 on pertussis toxin-catalyzed ADP-ribosylation of transducin. G_{t_α} and $G_{t_{\beta\gamma}}$ (3 μg each) were incubated with pertussis toxin (0.25 μg) with (○) or without (●) rhodopsin (1.5 μg) and the indicated amount of 2H3, for 1 hr at 30°, before quantification of [^{32}P]ADP-ribose incorporated into transducin. *Bars*, range of duplicate determinations.

Pertussis toxin-catalyzed ADP-ribosylation of G_{t_α} was decreased ≈20% by the addition of rhodopsin (Fig. 2). When rhodopsin was present, 2H3 had no effect on modification of G_{t_α}. In the absence of rhodopsin, 2H3 inhibited ADP-ribosylation of G_{t_α} in a dose-dependent manner. Inhibition was almost complete with 10 μg of 2H3 (Fig. 2). Fab fragments of 2H3 were similarly effective in inhibiting pertussis toxin-catalyzed modification of G_{t_α} (Fig. 3) but had no effect on toxin-catalyzed ADP-ribosylation in the presence of rhodopsin.

GTPase activity of transducin in the presence of photolyzed

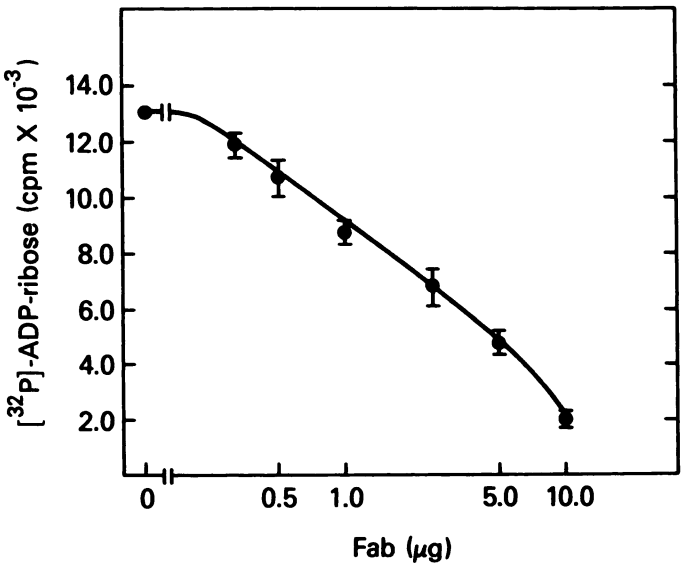

Fig. 3. Effect of Fab fragments of 2H3 on pertussis toxin-catalyzed ADP-ribosylation of transducin. G_{t_α} and $G_{t_{\beta\gamma}}$ (3 μg each) were incubated with pertussis toxin (0.25 μg) and Fab fragments of 2H3 at the indicated concentrations, for 1 hr at 30°, before quantification of [^{32}P]ADP-ribose incorporated into transducin. *Bars*, range of duplicate determinations.

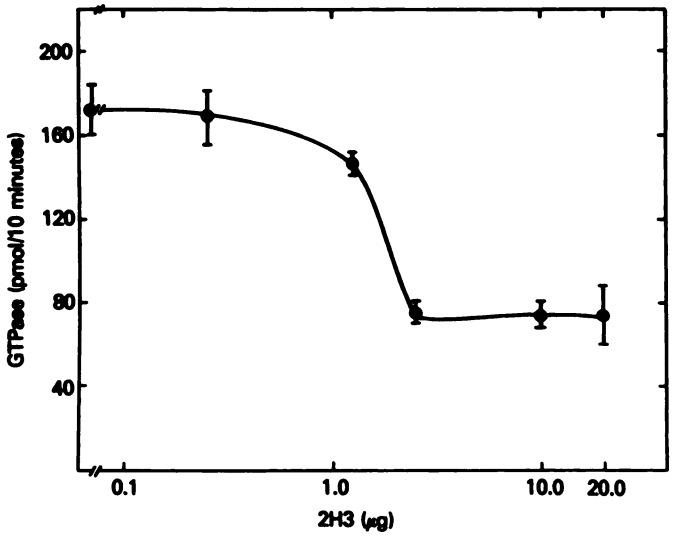

Fig. 4. Effect of 2H3 on GTP hydrolysis in the presence of rhodopsin. G_{t_α} (2.5 μg), $G_{t_{\beta\gamma}}$ (3.0 μg), rhodopsin (1.5 μg), and the indicated amounts of 2H3 were incubated 10 min at 30°. GTPase activity of G_{t_α} in the presence of photolyzed rhodopsin and $G_{t_{\beta\gamma}}$ was >10 times that observed in the absence of receptor and $G_{t_{\beta\gamma}}$. *Bars*, range of duplicate determinations.

rhodopsin was inhibited ≈50% by 2H3 in amounts of 5 μg or more (Fig. 4); similar inhibition was observed with Fab fragments (data not shown). GTPase activity observed in the presence of rhodopsin and antibody was still significantly greater (>10-fold) than the activity of G_{t_α} in the absence of rhodopsin (legend to Fig. 4). To determine whether rhodopsin blocked the accessibility of 2H3 to its epitope, 2H3 was incubated with transducin subunits and the antigen-antibody complex was immunoadsorbed using Protein A-Sepharose (Fig. 5). In the absence of rhodopsin, only β and γ were adsorbed by the Protein A-Sepharose (Fig. 5). A monoclonal antibody against G_{t_α} ($8E_1$), when incubated under identical conditions, bound only G_{t_α} (Fig. 5). In the presence of rhodopsin, none of the

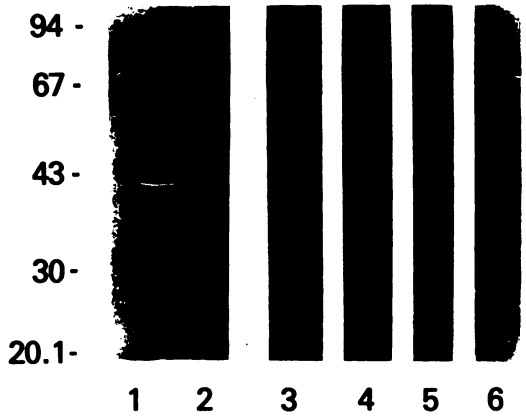

Fig. 5. Immunoadsorption of transducin by monoclonal antibodies against G_t (2H3) and G_{t_α} ($8E_1$). Five micrograms of $8E_1$ (*lanes 3* and *4*) or 2H3 (*lanes 5* and *6*) were incubated for 1 hr at room temperature with (*lanes 3* and *5*) or without (*lanes* 4 and 6) G_{t_α} (2.5 μg) and $G_{t_{\beta\gamma}}$ (2.5 μg) and were adsorbed to Protein A-Sepharose; the material that bound to Protein A-Sepharose was analyzed by SDS-polyacrylamide gel electrophoresis. *Lanes 1* and *2* contain molecular weight standards and transducin, as noted in Fig. 1.

subunits were recovered by immunoadsorption with these antibodies (data not shown).

Discussion

The βγ subunits of the G proteins can influence activity of the α subunits by formation of the inactive αβγ complex and are important for interaction of the α subunit with membrane-associated receptors (1–4). The γ subunit remains tightly associated with the β subunit throughout purification, so that it has not been possible to identify separate functions for the individual subunits. We used a monoclonal antibody, 2H3, against the γ subunit of transducin to investigate possible functions of this protein. Like polyclonal antibodies against G_{t_γ} (14), 2H3 did not react with γ subunits from G proteins other than transducin. This is consistent with previous studies, which demonstrated biochemical, structural, and immunological differences between γ subunits from transducin and from other G proteins (14–16). Monoclonal antibody 2H3 reacted with both native G_{t_γ} and G_{t_γ} that had been denatured in SDS and dithiothreitol for immunoblots.

The present studies demonstrate that monoclonal antibody 2H3 against G_{t_γ} had significant effects on several βγ-dependent activities. Further, it is evident from these studies that the ability of monoclonal antibody 2H3 to interact with G_{t_γ} was significantly affected by the presence of rhodopsin. 2H3 had profound effects on transducin in the absence of rhodopsin and smaller or no effects in the presence of rhodopsin. To determine whether monoclonal antibody 2H3 affected the association of α and βγ subunits, pertussis toxin-catalyzed ADP-ribosylation of G_{t_α} and immunoprecipitation of the αβγ complex were examined. Prior studies have demonstrated clearly that the preferred substrate for pertussis toxin-catalyzed reaction is the αβγ complex (18, 21). In the absence of $G_{t_{\beta\gamma}}$, ADP-ribosylation of G_{t_α} proceeds at a significantly slower rate. As reported here, in the absence of rhodopsin 2H3 completely inhibited pertussis toxin-catalyzed ADP-ribosylation of transducin. These results are consistent with the hypothesis that 2H3 can interfere with the association of βγ with α. Two possible mechanisms could

explain these effects. First, the epitope recognized by 2H3 may be part of the G_α binding site on $G_{t_{\beta\gamma}}$. Alternatively, 2H3 could inhibit $G_{t_{\beta\gamma}}$ association with G_{t_α} by causing a conformational change in $G_{t_{\beta\gamma}}$ that lowers its affinity for G_{t_α}. Thus, in the absence of receptor, monoclonal antibody 2H3 significantly inhibits the association of $G_{t_{\beta\gamma}}$ with G_{t_α}.

The effects of 2H3 on pertussis toxin-catalyzed ADP-ribosylation and on immunoadsorption of G_{t_α} seen in the absence of rhodopsin were not observed in its presence. Under the conditions used in these experiments, rhodopsin and transducin can form a stable complex. After formation of this complex, 2H3 apparently was unable to bind to G_{t_γ}. These results suggest that the epitope recognized by 2H3 is not accessible to the antibody when rhodopsin and transducin are complexed. This result is consistent with the hypothesis that the $\beta\gamma$ complex interacts directly with rhodopsin. The epitope recogized by 2H3 could be part of the binding site for rhodopsin or close enough to the binding site on G_{t_β} to prevent simultaneous binding of rhodopsin and 2H3.

These conclusions are supported in part by studies on rhodopsin-stimulated GTP hydrolysis. $G_{t_{\beta\gamma}}$ is necessary for rhodopsin stimulation of GTP hydrolysis by G_{t_α}. Prior immunological (28) and biochemical (29) studies were consistent with the presence of a $G_{t_{\beta\gamma}}$ binding site on rhodopsin as well as other receptors (30) and with close proximity on G_{t_α} of the $G_{t_{\beta\gamma}}$ binding and rhodopsin binding sites (31). Monoclonal antibodies 2H3 only partially blocked the GTPase activity of G_{t_α} observed in the presence of rhodopsin and $G_{t_{\beta\gamma}}$. Thus, the receptor did not completely prevent antibody binding. The antibody may, thus, be able to disrupt only partially the functional rhodopsin-transducin interaction. It appears, therefore, that the antibody can interact with G_{t_γ} in the presence of rhodopsin; this interaction inhibits the expression of GTPase but does not abolish activity. Rhodopsin can still interact with $G_{t_{\beta\gamma}}$ in the presence of antibody. This interaction may maintain $\alpha\beta\gamma$ and promote ADP-ribosylation.

Essentially no function has been assigned to the γ subunit, in large part because it has not been possible to purify and reconstitute individual β and γ subunits. Our data demonstrate that the γ subunit may be important in the interaction of $\beta\gamma$ with α and receptor. Additional probes of the γ subunit are necessary to help characterize the function of this protein.

Acknowledgments

We wish to thank Carol Kosh for expert secretarial assistance.

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