Characterization of Transducin from Bovine Retinal Rod Outer Segments

USE OF MONOCLONAL ANTIBODIES TO PROBE THE STRUCTURE AND FUNCTION OF THE \( \alpha \) SUBUNIT

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A panel of monoclonal antibodies has been developed against the \( T_\alpha \), \( T_\beta \), and \( T_\gamma \) subunits of bovine transducin. Two anti-\( T_\gamma \) antibodies from this panel (TF15 and TF16) and a third one (4A) against frog \( T_\alpha \) (Witt, P. L., Hamm, H. E., and Bownds, M. D. (1984) J. Gen. Physiol. 84, 251-263) were characterized. Each of these monoclonal antibodies recognizes a different region of \( T_\alpha \) and has a specific effect on the function of transducin. The binding of TF15 is reversibly enhanced by treating \( T_\gamma \) with either 1 M guanidinium chloride or, to a smaller extent, by the removal of bound guanine nucleotide. Its epitope is located in a 12-kDa tryptic fragment containing the binding site for the guanine moiety of GTP. Taken together, these results support previous observations that the conformation of \( T_\gamma \) is modulated by the occupancy of the guanine nucleotide binding site. In contrast to TF15, TF16 recognizes only the native form of \( T_\alpha \). Its epitope resides within the central portion of the \( T_\alpha \) molecule. While \( T_\gamma \)-bound TF16 does not inhibit either pertussis toxin-catalyzed ADP-ribosylation, rhodopsin binding, or transducin subunit interaction, it blocks both the light-activated uptake of guanosine 5'-O-(3-thiotriphosphate) (GTP\( \gamma \)S) and the GTP-dependent elution of transducin from photolyzed rhodopsin. These effects are unlikely to be caused by the occupation of the guanine nucleotide binding site by TF16 because this antibody quantitatively precipitates \( T_\alpha \)-GTP\( \gamma \)S. We propose that bound TF16 locks \( T_\alpha \) in a conformation that prevents the entrance of guanine nucleotide and favors \( T_\alpha \)-\( T_\beta \) association. In contrast to TF15, the epitope of 4A was mapped to the amino-terminal region of \( T_\alpha \). This monoclonal antibody blocks pertussis toxin-catalyzed ADP-ribosylation, GTP\( \gamma \)S uptake, and \( T_\alpha \)-\( T_\beta \) association. Moreover, the binding site for 4A becomes inaccessible when transducin binds to photolyzed rhodopsin. These results suggest that the inhibitory effects of 4A are due to a simultaneous steric blockage of both the interaction of \( T_\alpha \) with \( T_\beta \), and their binding to photolyzed rhodopsin. The results obtained from these studies are correlated with the structure and function of \( T_\alpha \).

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* Transducin, a member of the signal-transducing G protein family, plays a pivotal role in converting light into cellular responses in vertebrate retinal rods. Photolyzed rhodopsin catalyzes the exchange of GTP for GDP bound to transducin (Fung and Stryer, 1980). The transducin-GTP complex, in turn, activates a phosphodiesterase, leading to a transient reduction of the intracellular level of cGMP (Fung et al., 1981) and the closure of many cGMP-sensitive channels (Fesenko et al., 1985; Yau and Nakatani, 1985). As a result, the plasma membrane Na⁺ permeability of the rod outer segment decreases and the rod hyperpolarizes (Hagins et al., 1970; Tomita, 1970).

The structure and function of transducin has been the subject of many recent investigations (Fung, 1985; Chabre, 1986; Stryer and Bourne, 1986). It is a peripheral membrane protein consisting of two subunits that exhibit distinct functions (Kuhn, 1980; Fung et al., 1981). The \( T_\beta \) subunit (40 kDa) binds GTP and GDP, and in its GTP-bound form stimulates the phosphodiesterase (Fung et al., 1981). This activation is terminated when bound GTP is hydrolyzed to GDP by a GTPase activity intrinsic to \( T_\alpha \). The \( T_\alpha \) subunit is composed of two tightly associated polypeptides (\( T_\alpha \) 37 kDa, \( T_\gamma \) 8 kDa). It does not participate directly in either phosphodiesterase activation or GTP hydrolysis but is required for the binding of \( T_\gamma \) to photolyzed rhodopsin (Fung, 1985). In this regard, it probably serves to modulate the \( T_\alpha \) activity in the rod outer segment.

Interest in this light-stimulated cGMP cascade is further heightened by the finding that the structure and function of transducin closely resembles that of cellular G proteins of the adenylate cyclase system (Gilman, 1984; Stryer and Bourne, 1986). Similar to the role played by transducin in visual excitation, G proteins transduce the extracellular signals detected by receptors into changes in intracellular messenger concentration. Each G protein is a heterotrimer composed of a GTP-binding \( \alpha \) subunit that interacts with a catalytic moiety and a \( \beta \gamma \) subunit that probably serves as an anchor to the cytoplasmic face of the plasma membrane. Although the G protein \( \gamma \) chain differs from \( T_\gamma \) (Navon et al., 1984; Gierschik et al., 1985), the \( \alpha \) and \( \beta \) polypeptides of G proteins share extensive sequence homologies with \( T_\alpha \) and \( T_\beta \), respectively (reviewed by Stryer and Bourne, 1986). Thus, a better understanding of the structure of transducin is likely to provide new insights into the signal transduction processes that govern hormonal response and cellular development.

Extensive immunological studies on the distribution and similarity between members of the G protein family have been carried out by a number of laboratories (Navon et al., 1984; Gierschik et al., 1985; Pines et al., 1985; Roof et al., 1985; Mumby et al., 1986). We have also successfully used affinity-purified polyclonal antibodies to identify \( T_\gamma \)-producing cDNA clones (Medynek et al., 1985). As an extension of these earlier studies, we have developed monoclonal antibodies to probe the structure and function of the \( \alpha \) subunit of transducin.
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studies, we have developed a panel of monoclonal antibodies which recognize the individual subunits of transducin. In this paper, we describe the characterization of two anti-T$_o$ monoclonal antibodies from this panel and a third one (4A) that has been reported to inhibit the light-activated cGMP pathway in frog rod outer segments (Hamm and Bownds, 1984). The monoclonal antibodies against the T$_o$ and T$_s$ subunits will be the subject of a future study.

**EXPERIMENTAL PROCEDURES**

**Materials**—Frozen bovine retinas were purchased from American Stores Packing Co., Lincoln, NE. Rod outer segment membranes and reconstituted membrane vesicles containing purified rhodopsin were prepared as previously described (Ifong and Hubbell, 1973). The cGMP phosphodiesterase was isolated by a modified procedure of Baehr et al. (1979) as previously described (Fung and Nash, 1983).

Peptides were synthesized by the Merrifield solid-phase method at the Peptide Synthesis Facility, UCLA. Pertussis toxin was purchased from List Biological Laboratories; [35S]GTPyS, [3H]GTP, and carrier-free [3H]NAD were from Amersham Corp.; [3H]NAD was from Du Pont-New England Nuclear; rabbit anti-mouse IgG antibodies were from Coopers Biomedical; Pansorbin was from Behring Diagnostics. Radioactive transducin and protein A were prepared by iodination with chloramine T (Mishell and Shiigi, 1980). The compositions of the buffers were: Buffer A, 10 mM MOPS, pH 7.5, 3 mM MgCl$_2$, 1 mM diithiothreitol, 200 mM NaCl; Buffer B (10 mM sodium phosphate, pH 7.2, 150 mM NaCl); Buffer C (100 mM Tris, pH 8.0, 10 mM thymidine, 0.1 M GTP, 1 mM ATP, 25 mM MgCl$_2$, 1 mM EDTA, 2 mM diithiothreitol).

Preparation of Transducin Samples—Transducin and its subunits were extracted from bovine rod outer segment membranes and purified on aminocyl agarose (Fung, 1983). T$_o$ isolated by this method contained approximately 0.5 mol of bound GDP per mol of T$_o$. This number was obtained by using [3H]GTP during the initial extraction of transducin from the rod outer segment membranes (Fung et al., 1981) and was incorporated into T$_o$, essentially as described (Fung, 1983). Briefly, samples (200 μl) containing purified T$_o$ (0.2 mg), T$_s$ (0.02 mg), and photolyzed rhodopsin (0.02 mg) were incubated with a 20-fold excess of GTPyS overnight on ice under room light illumination. At the end of the incubation period, the reconstituted membranes were removed by centrifugation using a Beckman air-driven centrifuge (5 min. × 2 p.s.). Aliquots of the clear supernatants were used for the competition experiments. To verify the quantitative incorporation of GTPyS, the sample was routinely subjected to cleavage by trypsin (Fung and Nash, 1983), which produced a stable 33-kDa tryptic fragment in the presence of bound GTPyS.

Production of Monoclonal Antibodies—CAF1 mice were each immunized with a total of 400 μg of transducin according to the method of Stahl et al. (1989). Fusion, plating, and subcloning were carried out as described by Harwell et al. (1984) with RPMI 1640 containing glutamine as the medium for cell culture. CAF1 mice were the source of peritoneal feeder cells. Two methods were used to screen antibody-producing hybridomas. In the first method, 50 μl of [3H]-labeled T$_o$-transducin (10,000 cpm, 5 ng) in buffer B containing 1% BSA was added to 50 μl of culture supernatant from each well. After 1 h at 23 °C, the mixture was incubated with 50 μl of rabbit anti-mouse IgG antibodies and then precipitated with immobilized protein A (Pansorbin, Behring Diagnostics). Bound antibodies were eluted from the Pansorbin and analyzed as described below. In the second method, 50 μl of supernatant from each culture well was transfused to polyvinyl 96-well assay plates coated with either T$_o$ or T$_s$. After 1 h at 25 °C, bound monoclonal antibodies were detected by enzyme-linked immunosorbent assay with biotinylated second antibody and avidin-peroxidase (Vector Laboratories). Large amounts of monoclonal antibodies were obtained from the ascites fluid of CAF1 mice according to the protocol of Broder et al. (1984). All were purified by either DEAE-Affit Blue chromatography (Stahl et al., 1982) except for TF16, which was absorbed on protein A-agarose (Bio-Rad) and eluted with 50 μl citrate, pH 3.0. The class chain of each monoclonal antibody was determined by an immunoperoxidase sandwich assay in a commercially available kit (American Qualex).

**Solid-phase Immune Assay**—Antigen-coated polyvinyl 96-well plates were prepared by incubating each well with 50 μl of proteins (200 ng) for 1 h and washing 3 times with 1% BSA to block remaining binding sites on the wells. Monoclonal antibodies (50 μl/well) at various dilutions were added and the plates were incubated at 23 °C for 1 h. They were then washed three times with buffer B containing 0.1% BSA, incubated with 50 μl of rabbit anti-mouse IgG for 1.5 h, and washed again in the same way. Finally, each well was incubated with 50 μl of [3H]-labeled protein A (50,000 cpm, 8–10 ng) for 1 h, washed three times, and counted for radioactivity.

**Immunoprecipitation**—Immunoprecipitation was carried out by adding an aliquot of 1–3 μl of ascites fluid to 50 μl of protein sample in buffer A containing 1% BSA. After 30 min at 23 °C, 20 μl of either Pansorbin or Pansorbin containing bound rabbit anti-mouse IgG was added to precipitate the antibody-antigen complexes, and the mixture was incubated for one additional hour. The bacterial suspension was then centrifuged (5 min, 6000 × g) and washed twice with ice-cold buffer B containing 0.05% Tween 20. Bound antigens were eluted from the Pansorbin by resuspending the pellets in 30 μl of gel electrophoresis sample buffer (Laemmli, 1970) at 70 °C for 10 min. Insoluble bacterial particles were removed by centrifugation, and 25 μl of supernatant was immediately analyzed by SDS-polyacrylamide gel electrophoresis. When radioactive antibodies were used, the gels were subjected to autoradiography by drying and exposing them to Kodak XAR-5 x-ray film and an intensifying screen (Du Pont Lightening Plus). Denatured transducin was prepared by adding SDS to a final concentration of 0.5% to heat for 5 min. Air-driven centrifugation was then used to give a final composition of 1% Nonidet P-40, 1% BSA, and 0.1% SDS, and the samples were directly analyzed by immunoprecipitation as described above.

**Mapping the Antibody Binding Sites**—The binding sites for monoclonal antibodies TF15 and 4A were identified on immunoblots of proteolytic fragments of T$_o$. Cleavage of T$_o$ (0.5 mg/ml) with either trypsin (Fung and Nash, 1983) or Staphylococcus aureus V8 protease was carried out at 23 °C for 2 h at a protease to T$_o$ ratio of 1:40. The reaction was terminated by the addition of either a 10-fold excess (w/v) of denatured trypsin or to the trypsin-containing sample a 100-fold molar excess of diisopropyl fluorophosphate to the V8 protease-containing sample. The proteolytic fragments were then separated by SDS-polyacrylamide gel electrophoresis and analyzed by Western blotting.

**Assays of Transducing Functions**—The binding sites of transducin to photolyzed rhodopsin in synthetic membranes was assayed by a centrifugation method as previously described (Fung, 1983). Briefly, 7 μg of T$_o$ and 8 μg of T$_s$ were incubated with monoclonal antibody (30 μg) for 40 min at 23 °C. Rhodopsin (40 μg) reconstituted into synthetic membrane vesicles was then added, and the mixture was incubated for 5 min at 23 °C under room light illumination. The samples were then centrifuged (5 min at 25 p.s.i) with a Beckman air-driven centrifuge, and the pellets were either analyzed directly or eluted with GTP. To analyze the pellets directly they were washed once with 50 μl of buffer A, solubilized in 30 μl of electrophoresis buffer, precipitated with 100 μl of ascites fluid at 90 °C for 30 min, and the rhodopsin was eluted by SDS-polyacrylamide gel electrophoresis. To elute the transducin bound to the pellets, they were washed once with 50 μl of buffer A, resuspended in 50 μl of buffer A containing 100 μM GTP, pelleted, and 20 μl of the clear supernatant was removed from each sample for analysis.

**GTPase activities and GTPyS binding capacities** of transducin in the presence of added photolyzed rhodopsin in synthetic membranes were measured as reported previously (Fung and Stryer, 1980). The molar ratio of monoclonal antibody to transducin was estimated from their protein concentrations determined by Coomassie Blue binding (Bradford, 1976) and by densitometric analysis of samples separated by SDS-polyacrylamide electrophoresis. The latter method was employed to correct for the presence of non-Ig proteins in the monoclonal antibody preparations.

**ADP-ribosylation of Transducin**—Transducin (10 μg) in 20 μl of buffer C was incubated for 1 h with a 2-fold molar excess of monoclonal antibody followed by the additions of an equal volume of buffer C and 10 μl of buffer C containing 200 μg/ml pertussis toxin and 120 μM (α-32P]NAD (500–800 cpm/ml) at 37 °C for 10 min. The mixture was then analyzed by SDS-polyacrylamide gel electrophoresis. The radioactivity associated with the excised T$_o$ band of the first set was determined by scintillation. The second set was subjected to autora-
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Production and Characterization of Monoclonal Antibodies against Transducin—Murine spleen cells immunized with purified bovine transducin were fused with nonsecreting myeloma cells. The resulting hybridomas were cultured and screened for the production of monoclonal antibody. Of the 76 positive hybridomas identified, 26 were propagated and subcloned by limiting dilution. These cell lines were injected intraperitoneally into mice, 11 of which produced antibody-containing ascites fluid. Analysis of the purified monoclonal antibodies by SDS-polyacrylamide gel electrophoresis showed discrete light and heavy chains, confirming that they were derived from distinct clones (data not shown). Of the 11 monoclonal antibodies characterized, two were specific for Tγ, one was specific for Tα, and six were directed against Tα. Two additional monoclonal antibodies were found to be nonspecific when analyzed on Western blots of rod outer segment proteins and were discarded. Table I summarizes the chain class and subunit specificity of six of these monoclonal antibodies. Despite the use of native transducin as the immunizing antigen, only a small proportion of the monoclonal antibodies recognized native transducin subunits. In this report TF15 and TF16, the two monoclonal antibodies against bovine Tα and 4A, a monoclonal antibody directed against frog Tγ (Witt et al., 1984), were further characterized and used as probes for studying the structure-function relationships of Tα.

Specificity of TF15 and TF16—The specificity of TF15 for Tγ was determined by solid-phase radioimmunoassay. TF15 readily bound to wells coated with Tγ but not to wells coated with Tα. (Fig. 1, left panel). Analysis of the binding by immunoprecipitation indicated that TF15 was not very effective in precipitating nucleotide-depleted Tγ (right panel, lane 1) but readily recognized Tγ that had been pretreated with either 0.5% SDS (lane 2) or 1 M guanidinium chloride (lane 3). The binding of TF15 to guanidinium chloride-treated Tγ was reversible; removing the chaotrope by gel filtration restored Tγ to a form that was poorly accessible to TF15 (lane 4). Thus, TF15 appears to recognize a site on Tγ that is conformation-sensitive.

Since the binding of TF15 to Tγ is apparently sensitive to the reversible unfolding of the polypeptide chain, we surmised that bound guanine nucleotide might change the relative affinity of TF15 for Tγ. To test this hypothesis, GTPγS and GDPγS were incorporated into nucleotide-depleted Tγ, and the samples were used in competition with Tγ immobilized on 96-well polystyrene plates with radioimmunoassay as described under “Experimental Procedures.”

<table>
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<th>Table I</th>
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<td>Monoclonal antibodies against the subunits of transducin</td>
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<td>Assays for chain class and binding specificity are described under “Experimental Procedures.” The specificity of TF16 was determined by immunoprecipitation. The other monoclonal antibodies were assayed by either radioimmunoassay or Western blotting.</td>
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<td>Tγ</td>
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<td>TC06</td>
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FIG. 1. Specificity of TF15. Left panel, 200 ng of either Tγ (●) or Tα, (○) were coated onto polyvinyl wells and incubated with the indicated concentrations of TF15. Radioimmunoassay was then performed using rabbit anti-mouse IgG and 125I-labeled Protein A as described under “Experimental Procedures.” The counts/min are expressed as a percentage of the radioactivity bound at the saturating concentration of TF15. Right panel, TF15 ascites (1 μl) was added to samples containing 10 μg of either native Tγ (lane 1), SDS-treated Tγ (lane 2), Tγ treated with 1 M guanidinium chloride (lane 3), or the sample of lane 3 after removal of guanidinium chloride (lane 4). Pansorbin (20 μl) with bound rabbit anti-mouse IgG was added, and the bound proteins were precipitated, eluted, and analyzed by SDS-polyacrylamide gel electrophoresis as described under “Experimental Procedures.” H and L are, respectively, the heavy and light chains of Ig that were co-eluted from the precipitate.

FIG. 2. Effect of guanine nucleotide on the binding of TF15. Tγ (●), Tγ-GTPγS (■), or Tγ-GDPγS (▲) as shown on the abscissa was preincubated with 50 μl of TF15 ascites (diluted 500-fold). The mixtures were then used as the source of primary antibody and assayed for antibody binding to Tγ immobilized on 96-well polystyrene plates by radioimmunoassay as described under “Experimental Procedures.” mAb, monoclonal antibody.
was used.

Under "Experimental Procedures." An aliquot of 10 pg of each subunit that were co-eluted from the precipitate.

was added to a preformed TF16-Tu complex (data not shown). When Tγ was added, and the bound proteins were precipitated, eluted, and analyzed by SDS-polyacrylamide gel electrophoresis as described under "Experimental Procedures." An aliquot of 10 μg of each subunit was used. H and L are, respectively, the heavy and light chains of Ig that were co-eluted from the precipitate.

Moreover, TF16 readily precipitated the Tγ-Tβ complex under conditions in which the subunits of transducin were associated (lane 2). A similar result was obtained when Tγ was added to a preformed TF16-Tα complex (data not shown).

The Binding Domains of TF15, TF16, and 4A—In previous studies we and others have demonstrated that proteolysis of Tα by trypsin generates four fragments, each containing a distinct functional domain (Fung and Nash, 1983; Hurley et al., 1984; Medynski et al., 1985). Trypsin initially attacks Lys-18 of Tα, to generate a 38-kDa fragment lacking the carboxyl-terminal region. After prolonged digestion, a second cut is made at Arg-310, removing a 5-kDa polypeptide from the carboxyl-terminal region and producing a transient 33-kDa fragment. Finally, the 33-kDa fragment is converted to a 21-kDa fragment and a 12-kDa fragment by a third cleavage at Arg-204. This last cleavage is blocked by the binding of either Gpp(NH)p or GTPγS (Fung and Nash, 1983).

The ability of TF15 to recognize the tryptic fragments of Tα was examined on immunoblots (Fig. 4). The binding site for TF15 was found to be in the 33-kDa fragment generated by tryptic cleavage of the Tα-GTPγS complex (lane 3) and further resolved to the 12-kDa fragment derived from prolonged tryptic digestion of Tα (lane 4), indicating that the TF15 epitope lies somewhere between residues 205 and 310.

We also mapped the epitope of monoclonal antibodies 4A that has previously been reported to react with the Tα subunit of frog transducin (Witt et al., 1984). We have confirmed that 4A cross-reacts with both native and denatured bovine Tα (Fig. 4, lane 1). This antibody, however, did not react with the 33-kDa tryptic fragment of Tα (lane 3). Moreover, the loss of 4A binding was coincident with the removal of the amino-terminal region of Tα by trypsin (data not shown) or by S. aureus V8 protease (lane 2). The latter protease makes a single cut at Asp-22 (Navon and Fung, 1987). To further support our conclusion that the amino-terminal region of Tα contains the 4A epitope, we synthesized an octadecyl peptide of the sequence Cys-Tyr-Glu-Lys-His-Ser-Arg-Glu-Leu-Glu-Lys-Leu-Lys-Leu-Lys-Leu-Lys-Glu-Ala which contains 16 amino acids corresponding to residues 8–23 of Tα. We then tested the ability of this synthetic peptide (8′–23′) to competitively inhibit the binding of 4A to Tα, immobilized on 96-well polystyrene plates by radioimmunoassay as described under "Experimental Procedures." Other peptides which failed to compete for the binding of the antibodies to Tα are Tyr-Gly-Lys-His-Lys-Leu-Glu-Gln-Leu-Arg-Ser-Gly-Ala, Cys-Ala-Ala-Lys-Phe-Glu-Ser-Asn-Phe-Asn-Thr-Gln-Gln-Thr-Aan-Arg-Asn-Asp-Gly-Thr-Asp-Tyr and Asn-Leu-Cys-Asn-Ile-Pro-Ser-Cys-Ala-Leu-Leu-Ser-Ser-Asp-Ile-Thr-Ala-Ser-Val-Asn-Cys-Ala-Lys.

FIG. 4. Binding domains of TF15 and 4A. Left panel, Coomassie Blue staining pattern of uncleaved Tα (lane 1); Tα cleaved with S. aureus protease (lane 2); Tα-GTPγS cleaved with trypsin (lane 3); and Tα, cleaved with trypsin (lane 4). Cleavage conditions and protease inhibitions are described under "Experimental Procedures." Right panel, Western blots of the same samples using TF15 and 4A as the primary antibodies. An aliquot of 10 μg of protein was applied to each well for protein visualization, and a 100-fold smaller amount (100 ng) was used for Western blotting.
ments immobilized on nitrocellulose paper, immunoprecipitation was employed to determine the location of its epitope. As shown in Fig. 6, TF16 readily precipitated Tα (lane 1) and Tα-GTPγS (lane 2), indicating that the bound nucleotide does not interfere with antibody binding. The 33-kDa fragment generated by tryptic cleavage of Tα-GTPγS (lane 4) was also quantitatively precipitated by TF16. Evidently, the TF16 epitope is not located in either the amino- or the carboxy-terminal region of the Tα molecule. Consistent with our previous observations (Fung and Nash, 1983), the 21- and 12-kDa fragments derived from Tα remained tightly associated and were co-precipitated by TF16 (lane 3).

Effect of Anti-Ta Monoclonal Antibodies on the Binding and Hydrolysis of Guanine Nucleotides—Fig. 7 shows the effect of TF15, TF16, and 4A on the GTPase and GTPγS binding activities of transducin. In addition to these anti-Ta antibodies, TF28 which recognized native Tα (Table I) was used as a control. Both TF15 and TF28 had no effect on the ability of transducin to hydrolyze GTP (Fig. 7, lower panel). In contrast, addition of either TF16 or 4A markedly reduced the GTPase activity. The effect was due to an inhibition of GTPγS binding (upper panel) and was concentration-dependent, with more than 80% loss of activity at a stoichiometric amount of antibodies present in the incubation mixture.

Effect of Anti-Ta Monoclonal Antibodies on the Interaction of Transducin with Photolyzed Rhodopsin—Fig. 8, panel A, shows the effect of TF16 on the binding of transducin to photolyzed rhodopsin in reconstituted membranes. Consistent with our previous observations (Fung, 1983), Tα and Tβ, together (lane 2), but not Tα alone (lane 1), readily bound to photolyzed rhodopsin in reconstituted membranes. This binding was not affected by TF16. Moreover, TF16 was quantitatively precipitated together with the membranes (lane 3). This effect was apparently due to the association of TF16 with membrane-bound transducin because the antibody was not found in the membrane fraction of samples lacking transducin (lane 5) or containing only Tα (lane 4). Hence, Tα containing bound TF16 was still able to interact with Tβ, and photolyzed rhodopsin to form a membrane-bound complex. In contrast, a 40% decrease in transducin associated with the membranes, as quantified by scanning densitometry, was observed in the presence of 4A (panel B, lane 2) as compared to the control (lane 1). This result indicates that 4A competitively inhibited the binding of transducin to photolyzed rhodopsin.

The ability of these two types of monoclonal antibodies to recognize the membrane-bound form of transducin was also measured. TF16 was quantitatively precipitated by the preformed transducin-rhodopsin complex (Fig. 8, panel C, lane 1). On the other hand, the binding site for 4A was apparently blocked by the interaction of Tα with photolyzed rhodopsin and Tβ, because it was completely absent in the membrane fraction (lane 2).

The effect of monoclonal antibody on the release of transducin from photolyzed rhodopsin was also tested. GTP was unable to elute transducin from the membranes containing bound TF16 (data not shown). This result was expected since TF16 blocked GTPγS uptake (Fig. 7). In contrast, transducin was readily released from the membranes by GTP in the presence of 4A, TF15, or TF28.

Effect of TF16 and 4A on the Interaction of Tα with Tβ.—TF16 was able to precipitate nearly equal amounts of both subunits (Fig. 3, lane 2 and Fig. 9, left panel, lane 1). By comparison, only 0.2 mol of Tβ was precipitated with each mol of Tα-4A complex (Fig. 9, lane 2), suggesting that the binding of 4A interferes with the association of Tβ. Since Tβ is known to promote pertussis toxin-catalyzed ADP-riboseylation of Tα (Water et al., 1985), this reaction was employed as an additional measure of the effect of 4A on subunit

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**Fig. 6. Binding domain of TF16.** TF16 ascites (1 μl) was added to 15 μg of either Tα (lane 1), Tα-GTPγS (lane 2), Tα cleaved with trypsin (lane 3), or Tα-GTPγS cleaved with trypsin (lane 4). Pansorbin (20 μl) was added, and the bound proteins were precipitated, eluted, and analyzed by SDS-polyacrylamide gel electrophoresis as described under "Experimental Procedures." H and L are, respectively, the heavy and light chains of Ig that were co-eluted from the precipitate. The precipitated proteolytic fragments are indicated by their masses in kilodaltons.

**Fig. 7. Effect of monoclonal antibodies on GTPγS binding and GTPase activities.** Intact transducin (0.25 mg/ml) was incubated at 23 °C for 1 h with TF15 (○), TF28 (■), TF16 (□), and 4A (△) at the monoclonal antibody (mAb) to transducin ratios indicated on the abscissa. An aliquot of 20 μl of the mixture was then removed and used to measure GTPγS binding (upper panel) and GTPase activities (lower panel). GTPγS binding was measured at 23 °C in buffer A containing 10 μM [35S]GTPγS and 0.5 μM photolyzed rhodopsin in reconstituted membranes. Final assay volumes were 30 μl, and the reactions were terminated after 2 min. The GTPase assay was carried out at 23 °C in buffer A containing 10 μM [γ-32P]GTP and 10 μM photolyzed rhodopsin in reconstituted membranes. Final assay volumes were 30 μl, and the reactions were terminated after 25 min. Further details of the assay protocols are given under "Experimental Procedures." Data points represent the results obtained from duplicate samples.
interaction. As shown in Fig. 9, right panels, preincubation of transducin with TF16 (lane 2) and TF28 (lane 4) had no effect on the level of labeling. Under these conditions roughly 0.5 mol of ADP-ribose was incorporated per mol of transducin. Similar preincubation of Tu with 4A, however, resulted in a 96% reduction of radioactivity (lane 3). We further found that 4A quantitatively precipitated Tu containing an inserted ADP-ribose (data not shown), excluding the possibility that 4A inhibits the ADP-riboylation reaction by directing binding to the insertion site at Cys-347 (West et al., 1985).

**DISCUSSION**

The structure and function of the Tu subunit of transducin has been the subject of many recent investigations (Fung, 1985; Chabre, 1985; Stryer and Bourne, 1986). Although the crystal structure of Tu has not yet been determined, much information on the locations of its active sites has been deduced from its amino acid sequence and from the results of proteolysis and chemical modification. The most salient features are presented in a model shown in Fig. 10 and are summarized as follows. 1) The amino acid sequence of Tu contains four regions (A, C, E, G) which exhibit close homology to those of the G proteins, EF-Tu and EF-G, and ras protein (Halliday, 1984; Hurley et al., 1984; Medynski et al., 1985; Tanabe et al., 1985; Yatsunami and Khorana, 1985). In EF-Tu, these four regions are all located in the vicinity of the guanine nucleotide binding site (La Cour et al., 1985; Jurnak, 1986). By analogy, regions A, C, E, and G of Tu are thought to form a binding pocket for the guanine nucleotide. Consistent with this assignment is the specific labeling of the 12-kDa tryptic fragment (containing E and G) by 8-azido-GTP (Ho and Fung, 1984) and a marked reduction in accessibility of Arg-204 (adjacent to C and E) to trypsin cleavage upon the binding of Gpp(NH)p (Fung and Nash, 1983). 2) Tu is a target for ADP-riboylation catalyzed by cholera toxin (Abood et al., 1982; Navon and Fung, 1984) and pertussis toxin (Van Dop et al., 1984; Watkins et al., 1985; West et al., 1985). The insertion site at Cys-347 (Fig. 10) is blocked when transducin is membrane-bound, suggesting that the carboxyl-terminal region of Tu plays a crucial role in rhodopsin binding (Van Dop et al., 1984). This proposal is further supported by the recent finding that another rhodopsin-binding protein, the retinal 48-kDa protein, also contains a sequence which is homologous to that of the carboxyl-terminal region of Tu (Wistow et al., 1986). 3) The amino-terminal region of Tu is thought to participate in subunit interaction because the
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removal of amino acid residues 1-18 by trypsin leads to the dissociation of Tα, and the release of the cleaved transducin from the membranes (Fung and Nash, 1983; Navon and Fung, 1987). This cleavage also blocks the stimulatory effect of Tα on the pertussis toxin-catalyzed ADP-ribosylation of Tα (Watkins et al., 1985). 4) The amino acid sequence between A and C is significantly different among families of GTPase with specific functions of Tu. We found that each antibody recognizes a different region of the Tu polypeptide and exerts a specific effect on transducin. This information is especially useful when integrated with the results from previous studies shown in Fig. 10.

The binding of TF15 to Tα is conformation-sensitive. It readily recognizes both denatured Tα and Tα in the presence of a mild chaotrope (Fig. 1). Modifying Tα, either by iodoination or by amimation using methyl-p-hydroxybenzimidate also converted Tα to a form recognized by TF15 (data not shown). These procedures did not result in denaturation because the modified Tα retained its ability to undergo rhodopsin-catalyzed guanine nucleotide binding. We also found that nucleotide-depleted Tα has a limited ability to compete with denatured Tα for TF15 binding. This effect is reversed by the incorporation of either GDP or GTPyS into the guanine nucleotide-binding site (Fig. 2). The most likely interpretation of this finding is that the epitope for TF15 is partially exposed when the guanine nucleotide binding site is empty. This proposal is consistent with the mapping of the TF15 epitope to the 12-kDa fragment of Tα (Fig. 4) which contains the E and G regions involved in binding the guanine moiety of GTP (Fig. 10). However, we cannot exclude the possibility that the competition for TF15 binding by the nucleotide-depleted samples is due to a small fraction of denatured Tα that reversibly reattaches upon the incorporation of a guanine nucleotide.

In contrast to TF15, 4A readily binds native Tα and inhibits GTP binding and GTPase activities (Fig. 7). Since 4A can quantitatively precipitate Tα-GTPyS (data not shown), it is unlikely that 4A inhibits GTP uptake by simply occupying the guanine nucleotide binding site. We propose that the inhibition exerted by 4A is due to a steric blockage of the transducin-rhodopsin interaction, perhaps by preventing subunit association. This proposal is based on three lines of evidence. First, the epitope for 4A is completely inaccessible when transducin is bound to the reconstituted membranes (Fig. 8). Vice versa, the binding of 4A to Tα blocks the association of transducin with the photolyzed rhodopsin. Second, 4A inhibits the Tα-dependent ADP-ribosylation of Tα and partially blocks the co-precipitation of Tα with Tα (Fig. 9). This inhibitory effect does not involve photolyzed rhodopsin, indicating that the binding of 4A directly perturbs Tα-Tα interaction. Finally, the binding site of 4A was mapped to the amino-terminal region of Tα (Figs. 4 and 5), which has been demonstrated to participate in subunit interaction (Navon and Fung, 1987). Since the epitope of 4A is distinct from the ADP-ribosylation site and the proposed rhodopsin-binding domain in the carboxyl-terminal region (Van Dop et al., 1984; Masters et al., 1986), our observation that 4A blocks ADP-ribosylation and rhodopsin binding raises the possibility that the two termini of Tα are in proximity.

TF16 does not block subunit interaction or the Tα-dependent ADP-ribosylation of Tα by pertussis toxin (Fig. 9). It has no effect on rhodopsin binding and readily recognizes the membrane-bound form of transducin (Fig. 8). These results are consistent with the finding that the TF16 epitope is distant from the two terminal regions involved in the formation of a rhodopsin-transducin complex. We further showed that the TF16 binding domain differs from the guanine nucleotide binding site because this antibody is able to precipitate the Tα-GTPyS complex (Fig. 6). Nonetheless, TF16 does block the entrance of GTP into the guanine nucleotide binding site (Fig. 7) and inhibit the GTP-dependent release of transducin from the reconstituted membranes. The most likely interpretation of our results is that the binding of TF16 locks Tα in a conformation favoring the dissociation of GTP and the association of Tα. This proposal explains both the observed tight binding between the subunits during immunoprecipitation and also the inability of GTP to elute the transducin-TF16 complex from the membranes. It is also consistent with the fact that the binding of TF16 is sensitive to the conformation of Tα. It recognizes only native Tα, most likely to an antigenic determinant composed of different parts of the Tα polypeptide brought together by the folding of the protein structure.

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