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Structural and Functional Characterization of Guanyl Nucleotide-Binding Proteins Using Monoclonal Antibodies to the α-Subunit of Transducin

JANE L. HALPERN, SU-CHEN TSAI, RONALD ADAMIK, YASUNORI KANAHO, EVA BEKESI, HSIANG-FU KUNG, JOEL MOSS, and MARTHA VAUGHAN

Laboratory of Cellular Metabolism, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892 (J.L.H., S.-C.T., R.A., Y.K., J.M., M.V.) and Hoffmann-La Roche, Inc., Department of Molecular Oncology, Roche Research Center, Nutley, New Jersey 07110 (E.B., H.-F.K.)

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SUMMARY

Transducin, the GTP-binding protein of the retinal light-sensitive phosphodiesterase system, and G_s and G_i, regulatory proteins of the hormone-sensitive adenylate cyclase, are members of a family of guanyl nucleotide-binding proteins termed G proteins that are important in signal transduction. To probe relationships within this family of G proteins, monoclonal antibodies were prepared against the α-subunit of bovine transducin (T_α). Three of four monoclonal antibodies were specific for T_α and did not cross-react with other G proteins. One, MAB1, cross-reacted strongly with the α-subunit of G_i ($G_{i\alpha}$) purified from rabbit liver and, to a lesser extent, with the α-subunit of G_o ($G_{o\alpha}$) purified from bovine brain and the proto-oncogene product H-ras p21. All four monoclonal antibodies recognized epitopes on a 23-kDa tryptic peptide fragment of T_α which is derived from the N-proximal region. The three monoclonal antibodies that recognized only T_α inhibited rhodopsin-stimulated GTP binding and hydrolysis by transducin, whereas MAB1 had no significant effect in these assays. These studies demonstrate that, within the 23-kDa tryptic peptide of T_α, there is a domain(s) unique to T_α that is involved in GTP binding and hydrolysis and another domain which is highly conserved in T_α and to a lesser extent in other G proteins. Prior studies have identified regions involved in nucleotide binding and hydrolysis that are homologous in all G proteins. The observations reported here are consistent with the conclusion that the G proteins may have in addition unique regions involved in these functions.

The guanyl nucleotide-binding proteins referred to as G proteins are important in a number of membrane signal-transducing systems, including the hormone-sensitive adenylate cyclase system and the retinal light-sensitive phosphodiesterase (1–3). The activity of adenylate cyclase is regulated by two G proteins, G_s, and G_i, which are coupled to stimulatory and inhibitory receptors, respectively (1, 3). In the rod outer segments, the photon receptor rhodopsin is coupled to its effector through the G protein transducin (2). A fourth G protein, G_o, has been purified from bovine brain (4); it can interact with muscarinic receptors but its effector is unknown (5). The G proteins are heterotrimers with α-, β-, and γ-subunits (1, 6–8). The respective subunits in different G proteins have in common numerous structural and functional characteristics. The α-subunit binds guanyl nucleotides and possesses an intrinsic GTPase activity (6, 8–11). T_α and $G_{i\alpha}$ have similar proteolytic peptide maps (12), and partial amino acid sequencing of T_α and $G_{o\alpha}$ has revealed regions of homology (13). T_α also has regions of homology with two other families of GTP-binding proteins, the bacterial elongation factors and the yeast and mammalian ras gene products (14–17). Based on peptide mapping (12) and immunological analysis, (18, 19) it appears that the β-subunits are very similar, if not identical, in different G proteins.

The G proteins are activated by interaction with agonist-occupied receptor or photolyzed rhodopsin (1–3, 6, 10, 11, 20). The βγ complex is necessary for effective interaction which promotes guanyl nucleotide exchange on the α-subunit, thereby accelerating GTP binding and hydrolysis (11, 21). $G_{i\alpha}$ and $G_{\beta\gamma}$ can replace the corresponding subunits of transducin in reconstituting light-stimulated GTPase activity with purified rhodopsin (11, 22), thus demonstrating their functional homology. In contrast, $G_{i\alpha}$ and T_α did not couple efficiently with the β-adrenergic receptor that mediates adenylate cyclase activation (22), consistent with an expected specificity in the G protein-receptor interaction.

To probe further relationships within the family of G pro-

ABBREVIATIONS: G_s and G_i, respectively, stimulatory and inhibitory GTP-binding proteins of adenylate cyclase: $G_{s\alpha}$ and $G_{i\alpha}$, respectively, α-subunits of G_s and G_i; $G_{o\alpha}$, α-subunit of GTP-binding protein of unknown function; $G_{\beta\gamma}$, β, and γ subunits of G_s, G_i, and G_o; T_α and $T_{\beta\gamma}$, respectively, α- and βγ-subunits of transducin; ELISA, enzyme-linked immunosorbent assay; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetate; TBS, 20 mM Tris/500 mM NaCl, pH 7.5; TTBS, TBS containing 0.05% Tween-20.

teins, monoclonal antibodies were prepared against T_α. Their cross-reactivity with other G proteins and effects on function are described here.

Experimental Procedures

Materials. GTP and TPCK-trypsin were purchased from Sigma, Gpp(NH)p was from Boehringer Mannheim, [γ-^{32}]GTP (16.9 Ci/mol) was from New England Nuclear, and [^{3}H]Gpp(NH)p (7.7 Ci/mmol) was from Amersham.

Monoclonal antibodies. Female BALB/c mice were injected subcutaneously with 100 μg of purified transducin emulsified with an equal volume of Freund's complete adjuvant. Three booster injections (each 100 μg of transducin in incomplete Freund's adjuvant) were administered at monthly intervals. About 2 weeks after the last injection, mice were injected intraperitoneally on four successive days with 100 μg of transducin in normal saline. Spleens were then removed and cells were fused with Sp2/O-Ag14 myeloma cells using 30% polyethylene glycol (Hana Biologics, Berkeley, CA) following standard procedures (23). Hybrid cells were grown in selective medium containing aminopterin. Production of antibodies to transducin was assessed with an ELISA (described below) using horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad). Approximately 800 clones from two separate fusions were tested. Positive hybridoma lines were then screened for production of antibodies to T_α. Four lines which remained stable were selected and subcloned. These clones provided the monoclonal antibodies described here. Hybridoma clones were grown to stationary phase in HB101 serum-free medium (Hana Biologics). Immunoglobulins purified from concentrated (10-fold) spent medium by Protein A-Sepharose (Pharmacia) chromatography (24) were > 90% pure as determined by SDS-PAGE. Each of the monoclonal antibodies was an IgG; subtypes determined using a mouse immunoglobulin subtype identification kit (Boehringer Mannheim) are reported in Table 1.

ELISA on microtiter dishes. Vinyl microtiter dishes (96-well, Falcon) were coated with purified transducin, (0.5 μg) or transducin subunits (0.25 μg) in 2.7 mM KCl/1.5 mM KH_2PO_4/150 mM NaCl/8 mM Na_2HPO_4 (buffer A), 50 μl/well, for 1 hr at room temperature followed by incubation with bovine serum albumin, 5 mg/ml, in buffer A for 1–5 hr. Wells were rinsed and 50 μl of culture supernatant or purified IgG was added. After 3 hr at room temperature, wells were then washed three times with buffer A containing 2 mM EDTA, 0.05% Triton X-100, and 5% horse serum (buffer B) and three additional times with buffer A before incubation for 1 hr with 50 μl of horseradish peroxidase-conjugated goat anti-mouse IgG diluted 1:2000 in buffer A containing 1 mg/ml of bovine serum albumin. Wells were then washed three times with buffer B and three times with buffer A followed by incubation with 100 μl/well of peroxidase substrate (0.04% phenylene diamine and 0.012% H_2O_2 in 25 mM citrate buffer, pH 5.0) for 15–30 min before reading the absorbance at 495 nm with an ELISA plate reader (Flow Laboratories).

Western blots. Proteins separated by SDS-PAGE were transferred electrophoretically to nitrocellulose paper in a Bio-Rad Trans-blot apparatus at a constant voltage (150 V) for 8 hr in 25 mM Tris/192 mM glycine buffer containing 20% methanol (25). After transfer, blots were incubated for 1 hr in TBS containing 3% gelatin, and rinsed twice with TTBS. Blots were then incubated with monoclonal antibody (culture supernatant or purified IgG) for 2–12 hr. After two 5-min washes with TTBS, blots were incubated for 2 hr with horseradish peroxidase-conjugated goat anti-mouse IgG diluted 1:2000 in TTBS containing 1% gelatin, rinsed twice with TTBS and once with TBS, and incubated with peroxidase substrate (0.04% 4-chloro-1-napthol/0.005% H_2O_2/15% methanol in TBS) at 4° for 30–60 min. Blots were rinsed in distilled water and reaction product was quantified using a laser densitometer (LKB).

ELISA on nitrocellulose. Using a Minifold II apparatus (Schleicher and Schuell), samples of protein were applied to nitrocellulose paper which was then treated as described for Western blots.

Assay of GTPase activity. Transducin (or transducin subunits) reconstituted with rhodopsin (1.5 μg) was incubated with purified monoclonal antibody for 45 min at 4° before assay of GTP hydrolysis at 30° for 10 min as described (11).

Gpp(NH)p binding. Rhodopsin (1.5 μg), T_α (2.5 μg) and $T_{\beta\gamma}$ (3.0 μg) were incubated with 10 μg of control IgG or purified monoclonal antibody in a total volume of 75 μl for 45 minutes at 4°C. Reaction mixture (25 μl) was added to the samples to give final concentrations of 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EDTA and 1 μM (^{3}H)Gpp(NH)p (~7×10^5 cpm). After incubation at 30°C for 10 minutes, samples were filtered through 0.45 μm type HA Millipore filters which were then washed four times with 2.5 ml of reaction mixture without Gpp(NH)p and transferred to vials for counting.

Purification of proteins. Transducin was purified by the procedure of Kühn (26). T_α was separated from $T_{\beta\gamma}$ using Blue Sepharose CL-6B (27). Rhodopsin was purified under dim red light as described by Hong and Hubbell (28) except that membranes were not lyophilized. $G_{i\alpha}$ and $G_{\beta\gamma}$ were prepared from rabbit liver membranes by the method of Sternweis *et al.* (29) with modifications as described (30). $G_{o\alpha}$ was purified from bovine brain (4). The p21 ras protein was produced in *Escherichia coli* and purified as described earlier (31).

Protein. Protein concentrations were determined by the Coomassie blue binding method (Bio-Rad) using γ-globulin as the standard.

Results

Each of the four monoclonal antibodies against T_α reacted also with holotransducin but not with $T_{\beta\gamma}$ (Table 1). Similarly, when the subunits of transducin were separated by gel electrophoresis, each antibody reacted only with T_α on Western blots (data not shown). Initial experiments revealed that MAB1 cross-reacted with certain other G proteins, whereas the other monoclonal antibodies did not. After SDS-PAGE and transfer to nitrocellulose paper of $G_{i\alpha}$ and T_α, the reaction with MAB1 on a Western blot was quantified by densitometric scanning. The reaction of MAB1 with $G_{i\alpha}$ was very similar to that with T_α (Fig. 1). MAB1 did not react with $G_{s\alpha}$ (data not shown). The reaction of MAB1 with $G_{o\alpha}$ was < 3% of that with T_α (Fig. 2). Reactivity of MAB1 with the proto-oncogene product H-ras p21 was proportional to the amount of p21, although it was < 2% of that with T_α (Fig. 2).

To begin to map the binding sites for the monoclonal antibodies, T_α was proteolyzed with trypsin and resulting peptide fragments were resolved by SDS-PAGE. Trypsinization in the presence of Gpp(NH)p yielded a stable 32-kDa peptide; in the absence of Gpp(NH)p, the largest peptide was 23 kDa (Fig. 3). Other workers have shown that the 32-kDa peptide results from removal of 2-kDa N-terminal and 5-kDa C-terminal fragments from T_α; the 23-kDa product is the N-terminal portion of the 32-kDa peptide (32). On Western blots, MAB4 reacted with

TABLE 1

Reaction of monoclonal antibodies with transducin (T) and subunits in an ELISA

Samples of culture supernatant from the indicated hybridoma clone were incubated with antigen for 3 hr at 25°. Absorbance of wells coated with $T_{\beta\gamma}$ was not different from that of wells without antigen (i.e., coated with albumin) or wells with T or T_α when first antibody was omitted.

Hybridoma clone	Isotype	Antigen (A_{495nm})		
		T	T_α	$T_{\beta\gamma}$
MAB1	IgG_{2b}	0.63	0.64	0.08
MAB2	IgG_{2a}	0.50	0.32	0.08
MAB3	IgG_{2a}	0.57	0.55	0.07
MAB4	IgG_1	0.61	0.58	0.05

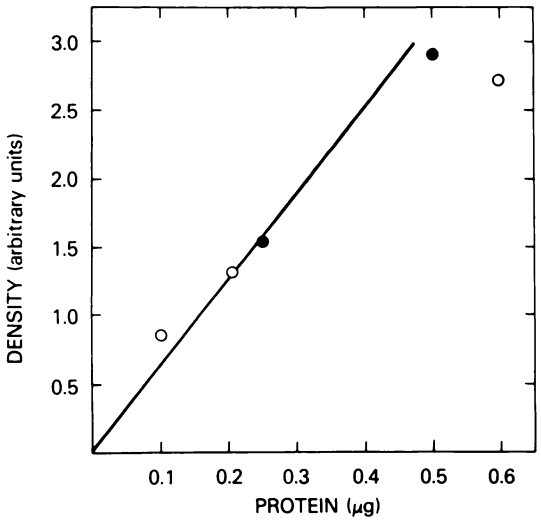

Fig. 1. Reaction of MAB1 with T_α and $G_{i\alpha}$. Samples (0.1–1.0 μg) of T_α (●) or $G_{i\alpha}$ (○) were subjected to electrophoresis on a 12% SDS-polyacrylamide gel and transferred electrophoretically to nitrocellulose paper, after which they were reacted with MAB1. Reaction products were quantified by densitometric scanning. The reaction of MAB1 with different concentrations of each protein was tested on at least four separate Western blots with similar results.

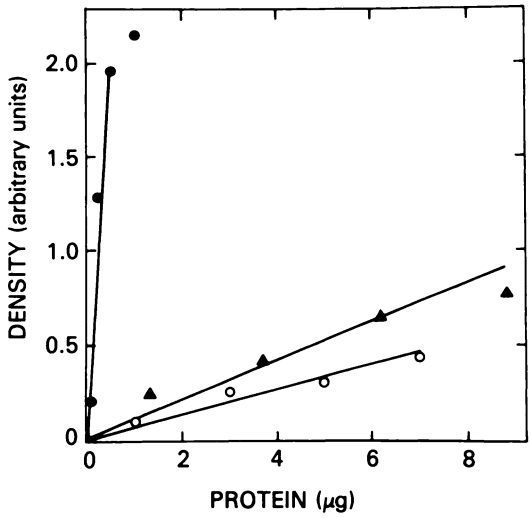

Fig. 2. Reaction of MAB1 with T_α, $G_{o\alpha}$, and H-ras p21. The indicated amounts of T_α (●), $G_{o\alpha}$ (▲), and H-ras p21 (○) were applied to nitrocellulose paper by vaccum filtration and reacted with MAB1. Products were quantified by densitometric scanning. There was no detectable reaction with 8 μg of bovine serum albumin. Similar results were obtained when these proteins were reacted with MAB1 on Western blots.

the 23-kDa as well as the 32-kDa peptide (Fig. 3). The other three monoclonal antibodies also reacted specifically with the 32-kDa peptide (data not shown) and the 23-kDa peptide (Fig. 4).

Effects of the antibodies on T_α function were investigated by assaying GTP hydrolysis and Gpp(NH)p binding to purified transducin subunits reconstituted with rhodopsin in phospholipid vesicles (Fig. 5). With equimolar concentrations of T_α and antibody, MAB2, MAB3, and MAB4 inhibited GTP hydrolysis 50–80%; MAB1 consistently stimulated GTP hydrolysis slightly. Effects on Gpp(NH)p binding were similar to those on GTPase activity. MAB2, MAB3, and MAB4 inhibited Gpp(NH)p binding 60–80%, whereas MAB1 had no significant effect. In the experiment shown in Fig. 6, MAB4 at a molar

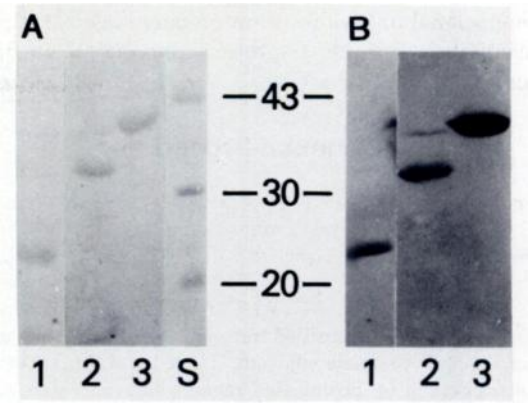

Fig. 3. Reaction of MAB4 with tryptic peptides from T_α or T_α-Gpp(NH)p. T_α-Gpp(NH)p was prepared by incubating purified T_α with 0.1 mM Gpp(NH)p in the presence of rod outer segments (50 μg) that had been depleted of transducin. The mixture was incubated at 4° for 1 hr, then centrifuged to remove membranes (31). T_α or T_α-Gpp(NH)p was incubated with 0.0004% trypsin for 45 min at 30°. The reaction was stopped by the addition of 10% trichloroacetic acid. Intact T_α and trypsinized T_α or T_α-Gpp(NH)p (5 μg each) were subjected to SDS-PAGE and transferred to nitrocellulose sheets. Replicate sheets were either stained for protein with amido black (A) or reacted with MAB4 (B). *Lane 1*, T_α plus trypsin, *lane 2*, T_α-Gpp(NH)p plus trypsin, *lane 3*, intact T_α. *S*, low molecular protein standards.

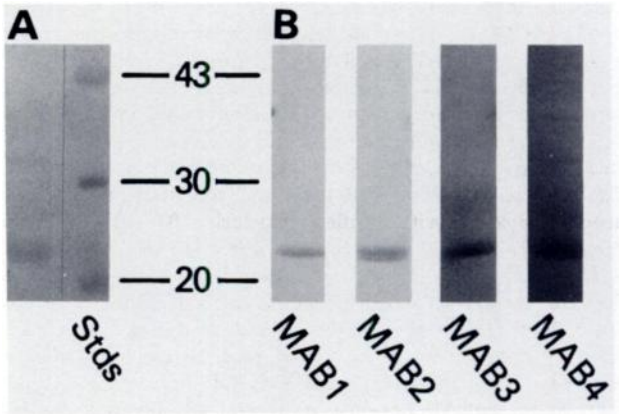

Fig. 4. Reaction of monoclonal antibodies with the 23-kDa tryptic peptide of T_α. T_α (5 μg) was digested with trypsin using the conditions described in Fig. 3. The samples were subjected to electrophoresis and transferred to nitrocellulose paper. Replicate sheets were stained for protein (A) or reacted with the indicated monoclonal antibody (B). *Stds*, low molecular weight protein standards.

concentration one-fourth that of T_α inhibited GTPase activity 60%, and inhibition was 95% with equimolar MAB4.

Discussion

Of the four monoclonal antibodies against T_α, one recognized an epitope shared with certain other guanyl nucleotide-binding proteins, the others an epitope(s) apparently unique to T_α. The 23-kDa tryptic peptide recognized by all four monoclonal antibodies contains regions of sequence homology with $G_{o\alpha}$, as well as regions common to two other guanyl nucleotide-binding protein families, the bacterial elongation factors and the ras gene products (13–17). Two regions of sequence homology in the 23-kDa tryptic peptide, termed A and C, respectively, by Halliday (14), include amino acids 31–49 and amino acids 80–93. Because these sequences have been conserved in guanyl

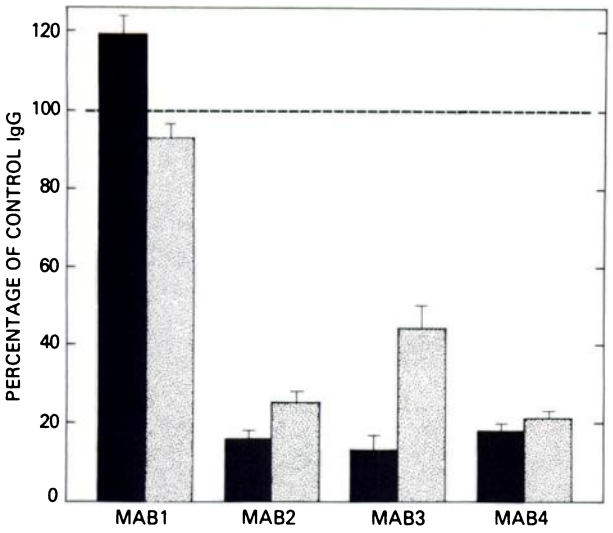

Fig. 5. Effect of monoclonal antibodies on GTPase activity and Gpp(NH)p binding by T_α. T_α, $T_{\beta\gamma}$, and rhodopsin were reconstituted and incubated with 10 μg of the designated antibody for 45 min at 4°. GTPase activity or Gpp(NH)p binding was then assayed for 10 min at 30°. Activity is expressed as a percentage of that in the presence of 10 μg of control IgG. *Bars* indicate the range of duplicate determinations. ^{3}H-Gpp(NH)p binding experiments were repeated twice and GTPase experiments were repeated three times with similar results.

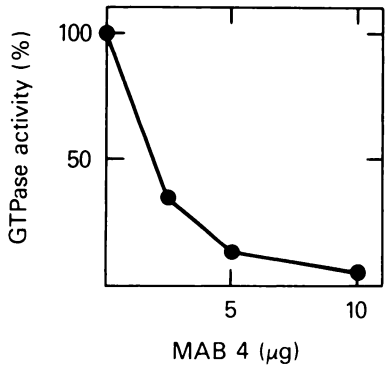

Fig. 6. Inhibition of GTPase activity by MAB4. The indicated amounts of MAB4 were incubated with rhodopsin, T_α, and $T_{\beta\gamma}$ for 45 min at 4° before assay of GTPase activity. The range of duplicate determinations was within the *symbol* for each point. This experiment was repeated twice with similar results.

nucleotide-binding proteins, it has been proposed that these regions are part of the GTP-binding site.

Three of the four monoclonal antibodies against T_α that recognized determinants in the 23-kDa tryptic peptide did not cross-react with other G proteins. Similarly, Gierschik *et al.* (19) found that only one of eight different antisera raised against T_α in rabbits recognized other α-subunits. We also have prepared antisera against transducin and T_α which do not cross-react with the α subunits of G_o, G_i, or G_s.[1] These data suggest that the immunological homology among α-subunits is confined to limited regions of the molecule. As highly conserved proteins are often poor antigens it may be difficult to obtain antibodies to such regions of G proteins. All three monoclonal antibodies that recognized only T_α inhibited GTP binding and hydrolysis. Thus, it appears that there is a region(s) in the 23-kDa tryptic peptide that is not common among G proteins but is necessary for the GTPase activity of transducin. Since guanyl nucleotide binding and GTPase activity depend on the formation of a complex including T_α, $T_{\beta\gamma}$, and rhodopsin (21), antibody binding at any of the sites involved in these interactions could effectively inhibit these activities.

It is unclear whether the epitope conserved in several G proteins, which is recognized by MAB1, is in the regions of sequence homology designated A and C. Several points argue against this, however. First, MAB1 reacted very poorly with $G_{o\alpha}$ and H-ras p21, although T_α and $G_{o\alpha}$ are identical in sequence from amino acid 30–51, and T_α and H-ras p21 are highly homologous in this region (15–17). Second, MAB1 did not recognize $G_{s\alpha}$. If regions A and C are involved in GTP binding, it is reasonable to assume that they would be conserved in $G_{s\alpha}$. Finally, interaction of MAB1 with T_α did not significantly affect either GTP hydrolysis or Gpp(NH)p binding. This lack of effect was not due to the inability of the monoclonal antibody to interact with T_α in solution, since it could effectively immunoprecipitate T_α (data not shown). These results are consistent with the proposal that the epitope recognized by MAB1 is not contained in one of the two regions common to several families of guanyl nucleotide-binding proteins but represents a distinct site not related to GTP binding or hydrolysis. From the strong reaction of MAB1 with $G_{i\alpha}$, it may be inferred that these two proteins are highly homologous at this epitope. The role of this conserved epitope in the function of $G_{i\alpha}$ and T_α remains to be determined.

[1] Unpublished data.

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Send reprint requests to: Dr. Jane L. Halpern, Laboratory of Cellular Metabolism, Building 10, Room 5N307, National Institutes of Health, Bethesda, MD 20892.