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Biochemistry

Antisera of designed specificity for subunits of guanine nucleotide-binding regulatory proteins

(G proteins/transducin/adenylate cyclase/immunoblots)

SUSANNE M. MUMBY, RICHARD A. KAHN, DAVID R. MANNING*, AND ALFRED G. GILMAN

Department of Pharmacology, Southwestern Graduate School, University of Texas Health Science Center, Dallas, TX 75235

Contributed by Alfred G. Gilman, September 9, 1985

ABSTRACT **Antisera were raised against purified subunits of regulatory GTP-binding proteins (G proteins) and against synthetic peptides that correspond to defined regions of G proteins. Peptide antisera were generated that recognized all α or all β subunits from G_s, G_i, G_o, and transducin; others recognized only $G_{s\alpha}$ or $G_{o\alpha}$. Such cross-reaction or complete specificity for a given α subunit was not obtained when purified subunits were injected. Peptide antisera were used to identify G protein subunits in selected tissue membrane preparations by immunoblots.**

Members of a family of GTP-binding regulatory proteins (G proteins) transduce signals from membrane-bound receptors to intracellular effectors. The family includes G_s and G_i, which are responsible for stimulation and inhibition, respectively, of adenylate cyclase (1, 2). Transducin (T), localized in the disc membranes of retinal rod outer segments, couples activation of rhodopsin by light to increased cyclic GMP phosphodiesterase activity (3). G_o, found originally in bovine brain, is a fourth member of the family (4, 5). While its function has not yet been established, structural and biochemical characteristics of G_o justify its designation as a distinct G protein (4–8).

Purified G proteins have similar physical properties. They are heterotrimers composed of α, β, and γ subunits. The α subunits bind and hydrolyze GTP and can be ADP-ribosylated by NAD with cholera toxin or pertussis toxin. The α subunits are distinct and vary in size from 39 to 52 kDa [T and G_o, 39; G_i, 41; G_s (two forms), 45 and 52]. The 36-kDa β subunit is a component of all G proteins. Under certain conditions, the β subunit of G_s, G_i, and G_o (but not T) can be resolved into a doublet with various amounts of 36- and 35-kDa polypeptides by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (4, 9). Although the relationship of the two proteins is not clear, β subunits from G_s, G_i, and T are indistinguishable by peptide mapping and amino acid analysis (10). The γ subunit ($\approx$8 kDa) of the G proteins is presumed to be similar; the amino acid sequence of T_γ has been determined (11, 12). Little is known about the function of this subunit.

There is a critical need for specific high-affinity antibodies to individual subunits of G proteins. However, only modest quantities of proteins such as G_s are available, and certain subunits are not very antigenic when used as immunogens in rabbits. Some progress has been made by using T as antigen. Injection of holoprotein gave rise to antisera with various reactivities with the different subunits (8, 13, 14). Pines *et al.* (14) produced six antisera against the purified α subunit of T but found no cross-reactivity with α subunits of other G proteins. We have purified α and $\beta\gamma$ subunits of G_o and T for injection into rabbits and for amino acid sequencing (6). A cDNA clone corresponding to $G_{s\alpha}$ has also been isolated (15). Peptides have been synthesized according to sequence information and have then been used as immunogens as reviewed by Lerner (16). We have been able to generate antisera of desired specificity by the use of peptides that correspond to either common or unique G protein amino acid sequences.

MATERIALS AND METHODS

Membrane, Protein, and Peptide Preparations. Bovine tissues were obtained from a slaughterhouse. Brain membranes were prepared as previously described (4). The preparation of membranes from adrenal cortex and medulla was essentially the same as that described by Schneider *et al.* (17), with further purification by sucrose gradient centrifugation (18). Sarcolemmal vesicles were isolated from heart by the published procedure of Slaughter *et al.* (19). Retinal rod outer segment disc membranes were prepared as described by Papermaster and Dreyer (20). Tracheal smooth muscle membranes were prepared from muscle dissected from fresh tissue, frozen in liquid nitrogen, and stored at −80°C. Frozen tissue (25 g) was rapidly thawed in 100 ml of 10% (wt/wt) sucrose in buffer A (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid/2 mM $MgCl_2$/1 mM EDTA, pH 8.0). All subsequent procedures were at 0–4°C. The tissue was coarsely chopped with scissors and macerated with a Potter–Elvehjem tissue grinder. The tissue was further disrupted by homogenization with a Brinkmann Polytron (30 sec; setting 7). Twenty-milliliter aliquots of the homogenate were loaded on 20-ml cushions of 40% (wt/wt) sucrose in buffer A and were centrifuged at 25,000 rpm in a Beckman SW-28 rotor for 2 hr. The interfacial material was collected and diluted with 3 vol of cold water and sedimented at 35,000 rpm for 1 hr in a Beckman 35 rotor. The pellets were resuspended in a total volume of 1.3 ml in 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 8. Aliquots of membrane preparations from each tissue were rapidly frozen and stored at −80°C.

G proteins were purified by established procedures (4, 9, 21, 22). T (for injection) was purified by a modification of the procedure described by Fung (23). Guanosine 5'-[γ-thio]triphosphate was used instead of guanosine 5'-[β,γ-imido]triphosphate, and heptylamine-Sepharose was used in place of hexylagarose to resolve subunits. T subunits were eluted with a linear gradient of 0.3–0.85% sodium cholate.

Peptides for this study were synthesized commercially by Peninsula Laboratories (San Carlos, CA). The G_β peptide

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Abbreviations: G proteins, guanine nucleotide-binding regulatory proteins; G_s and G_i, the G proteins that mediate stimulation and inhibition, respectively, of adenylate cyclase; G_o, a related G protein of unknown function; T, transducin, the major G protein of the retinal rod outer segment.

*Present address: Department of Pharmacology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104.

was synthesized according to the partial amino acid sequence determined for tryptic fragments (24) of G_β protein purified from bovine brain, bovine retina, and rabbit liver. The fragments were purified electrophoretically and sequenced by automated Edman degradation. Partial sequences for $G_{o\alpha}$ and T_α and for a cDNA clone corresponding to $G_{s\alpha}$ have been published (6, 15). The peptides were synthesized with a cysteine residue at the amino terminus to facilitate cross-linking to keyhole limpet hemocyanin (Sigma) with *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester as described by Green *et al.* (25).

Immunization. The method and schedule for immunization of rabbits with peptides coupled to hemocyanin were the same as those described by Green *et al.* (25). Exceptions were rabbits injected with the $G_{s\alpha}$ peptide-hemocyanin conjugate; they received larger amounts of antigen (15). Rabbits immunized with purified G proteins received 15–20 intradermal injections (1 ml total; 25–100 μg of protein) in the back. The first injection was made with complete Freund's adjuvant and was followed by a boost 2 weeks later with half as much antigen with incomplete adjuvant.

ELISA. Peptide or protein (50 μl; 10 μg/ml in 100 mM $NaHCO_3$, pH 9.6) was incubated in wells of Immulon 2 plates (Dynatech, Alexandria, VA) overnight at 4°C. Plates were then rinsed four times with 0.05% Tween-20 in 0.9% NaCl. Primary antisera were diluted with a solution of 0.1% gelatin (Sigma), 0.05% Tween 20, and 0.02% NaN_3 in phosphate-buffered saline. Serum solutions (50 μl) were incubated in the coated wells for 2 hr at room temperature. The plates were rinsed as before and then incubated for 2 hr with 50 μl of alkaline phosphatase conjugated to protein A diluted 1:1000 (Zymed Laboratories, Burlingame, CA). The plates were rinsed again before addition of 50 μl of substrate, *p*-nitrophenyl phosphate (Sigma), at 1 mg/ml in 0.2 M 2-amino-2-methyl-1,3-propanediol and 1 mM $MgCl_2$, pH 10.3. The reaction was stopped after 15–30 min by the addition of 12.5 μl of 1 M NaOH. The absorbance at 405 nm was determined with a Dynatech plate scanner.

Immunoblots. Conditions for gel electrophoresis, transfer of proteins to nitrocellulose, and processing of blots have been described previously (15). Samples were pretreated with *N*-ethylmaleimide for electrophoresis as described by Sternweis and Robishaw (4).

RESULTS

The peptides synthesized for production of antisera are shown in Table 1, as are the preparations of G protein subunits used as immunogens. The $G_{\alpha,common}$ peptide sequence, found in both $G_{o\alpha}$ and T_α (6), was chosen with the hope that antisera generated against it would also recognize other G proteins. The same sequence was subsequently found in $G_{i\alpha}$ (unpublished observation) and, with the substitution of arginine for lysine at the carboxyl terminus, in $G_{s\alpha}$ (15). The $G_{s\alpha}$ peptide sequence was derived from a cDNA clone that codes for $G_{s\alpha}$ (15). During the early stages of DNA sequencing, the identities of two amino acid residues were not certain, and two peptides were synthesized. One peptide contained lysine residues in the sixth and eighth positions and the other arginines. The peptide designated $G_{s\alpha}$ was later determined to be correct; the other peptide was designated $G_{s\alpha,Arg}$. The G_β peptide sequence was derived from partial amino acid sequence obtained from tryptic fragments of β subunits purified from bovine brain (G_o, G_i), bovine retina (T), and rabbit liver (G_i). Cleavage of each β subunit resulted in the production of a 27-kDa fragment with the amino-terminal sequence shown in Table 1.

Each peptide was cross-linked to keyhole limpet hemocyanin and injected into two or four rabbits. All rabbits developed a titer to peptide and to hemocyanin as determined by ELISA. Results of assays for specificity of reaction with peptides for representative antisera are shown in Fig. 1. Rabbits injected with $G_{\alpha,common}$, $G_{o\alpha}$, or G_β peptides reacted with the injected peptide only (Fig. 1 *A*, *D*, and *E*). Antiserum C-519 reacted with the injected $G_{s\alpha}$ peptide and about half as well with the related $G_{s\alpha,Arg}$ peptide (Fig. 1*B*). Antiserum A-572 reacted equally well with the $G_{s\alpha,Arg}$ and the $G_{s\alpha}$ peptide (Fig. 1*C*).

Table 1. Antigens and antisera

Antigens		Antisera	
Desig-nation	Amino acid sequence*	Titer to G protein(s)†	Serum code number‡
	Synthetic peptide antigens		
$G_{\alpha,common}$	CGAGESGKSTIVKQMK	4/4	A-569
$G_{s\alpha}$	CKQLQKDKQVYRATHR	1/4	C-519
$G_{s\alpha,Arg}$	CKQLQRDRQVYRATHR	1/2	A-572
$G_{o\alpha}$	CNLKEDGISAAKDVK	2/2	U-46
G_β	CEGNVRVSRELAGHTGY	2/2	U-49
	Bovine protein antigens		
$G_{o\alpha}$ ($G_{i\alpha}$)§		2/2	S-214
G_β		2/2	S-217
T_α		2/2	U-42
T_β		2/2	U-45

*Amino acid sequence is given in the one-letter code. For each peptide the amino-terminal cysteine was used to couple peptide to hemocyanin and is not part of the G protein sequence.
†Numbers for antisera reactions refer to the number of rabbits with antibody to G protein/number of rabbits injected.
‡Serum code number refers to antiserum obtained from the rabbit with the best titer to G protein.
§The preparation of $G_{o\alpha}$ was contaminated with approximately 10% $G_{i\alpha}$.

Antisera were assayed for the ability to recognize G protein subunits by immunoblotting. Nearly quantitative transfer of microgram quantities of resolved α and β subunits was achieved as determined by staining of the nitrocellulose and the polyacrylamide gel after transfer (not shown). It was difficult to estimate the extent of transfer of the γ subunit. Immunoblots failed to reveal any reaction in the region corresponding to γ. Antiserum A-569 from a rabbit injected with the $G_{\alpha,common}$ peptide recognized the α subunits of G_s, G_i, G_o, and T but did not recognize any of the β subunits (Fig. 2*B*). It is not known why the extent of reaction of the $G_{\alpha,common}$ peptide antiserum was variable for the different α subunits. The $G_{s\alpha}$ protein preparation used for these experiments was contaminated with $G_{i\alpha}$ (Fig. 2*A*), which accounts for the band at 41 kDa in the $G_{s\alpha}$ lane of Fig. 2*B*. Detection of the 52-kDa form of the $G_{s\alpha}$ subunit was evident on longer exposure (not shown). The $G_{i\alpha}$ preparation contained an unidentified protein migrating at 47 kDa (Fig. 2*A*), which was recognized by the $G_{\alpha,common}$ peptide antiserum A-569 (Fig. 2*B*).

Both the $G_{s\alpha}$ and the $G_{s\alpha,Arg}$ peptide antisera recognized the two forms of $G_{s\alpha}$ (Fig. 2 *C* and *D*) and did not react with other subunits. As shown in Fig. 2*E*, antiserum U-46, from a rabbit injected with the $G_{o\alpha}$ peptide, recognized the α subunit of G_o but none of the other G proteins. Reactivity with antisera made by injection of protein subunits was not as specific, presumably because of unavoidable contamination of protein antigens and/or cross-reactivity. Thus, serum from the rabbit injected with a preparation of $G_{o\alpha}$ protein (which contained approximately 10% $G_{i\alpha}$) reacted with $G_{o\alpha}$, $G_{i\alpha}$, and T_α (Fig. 2*F*), while serum from a rabbit injected with T_α also reacted weakly with $G_{i\alpha}$ (Fig. 2*G*). Weak cross-reaction between T_α and $G_{i\alpha}$ has been previously observed for other T antisera as well (8, 13, 14).

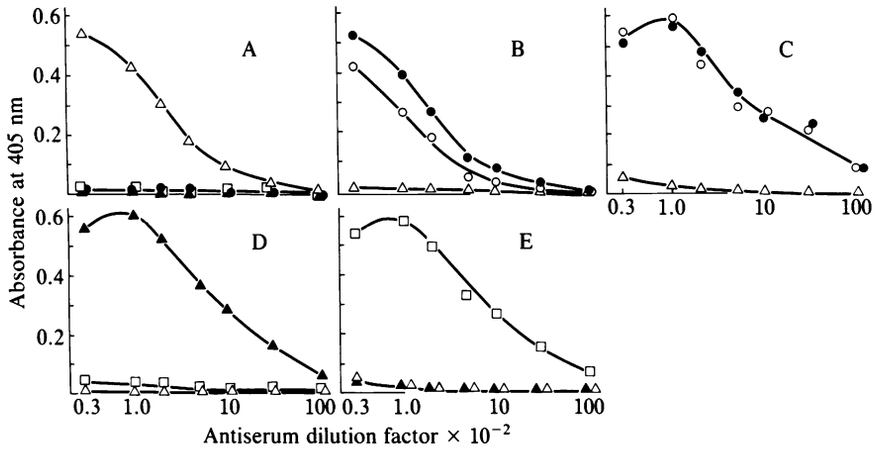

FIG. 1. Peptide specificity of antisera determined by ELISA. △, $G_{\alpha,common}$; ▲, $G_{o\alpha}$; ○, $G_{s\alpha,Arg}$; ●, $G_{s\alpha}$; □, G_{β}. Each panel represents the results obtained with a single antiserum from a rabbit injected with a peptide coupled to hemocyanin. (*A*) Serum A-569, peptide $G_{\alpha,common}$; (*B*) serum C-519, peptide $G_{s\alpha}$; (*C*) serum A-572, peptide $G_{s\alpha,Arg}$; (*D*) serum U-46, peptide $G_{o\alpha}$; (*E*) serum U-49, peptide G_{β}.

All three of the β antisera reacted with the β subunits of G_s, G_i, G_o, and T (Fig. 2 *H*, *I*, and *J*). G_s, G_i, and G_o β subunits transfer as resolved 35/36-kDa doublets from the polyacrylamide gel to nitrocellulose (data not shown). Shorter exposures of film to immunoblots shown in Fig. 2, and other experiments not shown, indicate that antisera U-49 and U-45 react exclusively with the 36-kDa form of the β subunit. Only a slight reaction of serum S-217 has ever been observed with the 35-kDa protein, although the $G_{o\beta}$ used as antigen was composed of both 35- and 36-kDa proteins.

The peptide antisera were used to identify G protein subunits in membrane preparations from bovine brain, adrenal gland, heart, retinal rod outer segments, and tracheal smooth muscle by immunoblotting. The $G_{\alpha,common}$ peptide antiserum A-569 reacted with proteins of approximately 40 kDa in each membrane preparation, corresponding to $G_{i\alpha}$, $G_{o\alpha}$, and/or T_{α} (Fig. 3*A*). Overexposure of the blot resulted in the ability to detect the 45- and 52-kDa $G_{s\alpha}$ subunits (not shown). Both forms of $G_{s\alpha}$ were detected in each membrane preparation with antiserum A-572 (Fig. 3*B*). $G_{o\alpha}$ was found in all of the membrane preparations, but the amount detected varied widely (Fig. 3*C*). Smaller quantities of brain and heart membrane proteins were loaded on this gel because these tissues contain relatively large amounts of $G_{o\alpha}$ (Fig. 3*C*, lanes 1 and 4) (4, 5, 26). Another protein, most prominent in the adrenal cortex and the tracheal smooth muscle membrane preparations, migrates at about 70 kDa and is recognized by the affinity-purified $G_{o\alpha}$ peptide antiserum (Fig. 3*C*, lanes 3 and 6). The β subunit is detected in all the membrane preparations by the G_{β} peptide antiserum U-49 (Fig. 3*D*). Use of preimmune sera with the membrane protein blots resulted in essentially blank autoradiograms (not shown).

The specificity and sensitivity of the peptide antisera are demonstrated by the experiment shown in Fig. 4. Immunoblots with serum U-42 (anti-T_{α}) indicate that 5–10% of the rod outer segment disc protein consists of T_{α} (Fig. 4*A*). In view of this high concentration of T_{α}, the reaction of the $G_{o\alpha}$ peptide antiserum (U-46) with a retinal protein migrating with an apparent molecular mass of 39 kDa (Fig. 3*C*, lane 5) raised the possibility of cross-reaction of the U-46 antiserum with T_{α}. However, blots of increasing amounts of $G_{o\alpha}$, T_{α}, and rod outer segment disc membranes indicate that the

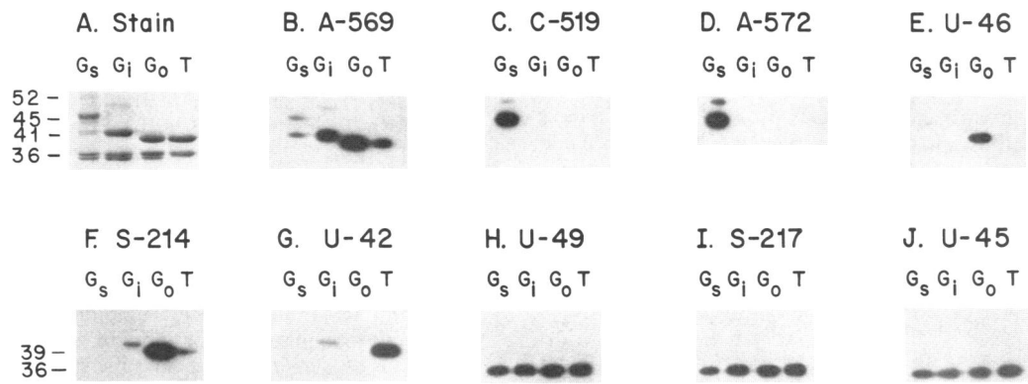

FIG. 2. Specificity of antisera to G protein subunits assessed by immunoblotting. Purified G proteins were resolved on sodium dodecyl sulfate/polyacrylamide gels and either stained with Coomassie brilliant blue (*A*) or transferred to nitrocellulose (*B–J*). One microgram of each protein was loaded for staining and 0.1 μg was loaded for blotting. The blots were processed with the indicated antisera and with ^{125}I-labeled goat antibodies to rabbit IgG (2×10^5 cpm/ml for each blot, except *C* and *D*, which required 1×10^6 cpm/ml). The antibody solutions contained 0.2% Nonidet P-40. Each blot was exposed to film for 16 hr, except for *I* and *J*, which were exposed for 30 hr. Only the portions of the blots corresponding to the α and β subunits are shown; no other bands were observed. (*A*) Protein stain; (*B*) antiserum A-569 ($G_{\alpha,common}$ peptide antigen), 1:1000 dilution; (*C*) antiserum C-519 ($G_{s\alpha}$ peptide antigen), 1:200 dilution; (*D*) antiserum A-572 ($G_{s\alpha,Arg}$ peptide antigen), 1:1000 dilution; (*E*) antiserum U-46 ($G_{o\alpha}$ peptide antigen), 1:1000 dilution; (*F*) antiserum S-214 ($G_{o\alpha}$ protein antigen), 1:2000 dilution; (*G*) antiserum U-42 (T_{α} protein antigen), 1:5000 dilution; (*H*) antiserum U-49 (G_{β} peptide antigen), 1:10,000 dilution; (*I*) antiserum S-217 ($G_{\beta\gamma}$ protein antigen from bovine brain), 1:2000 dilution; (*J*) antiserum U-45 ($T_{\beta\gamma}$ protein antigen from bovine retina), 1:400 dilution. The numbers to the left of *A* and *F* indicate the apparent molecular masses (kDa) of G protein standards.

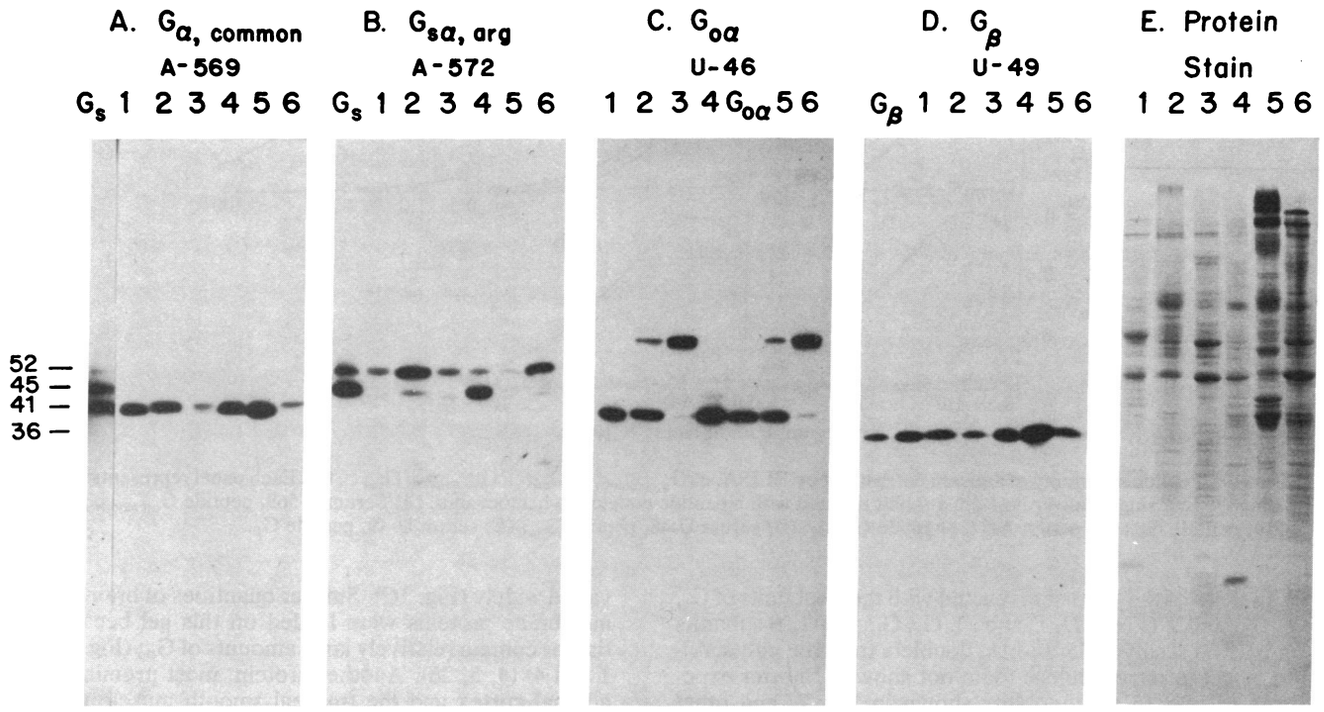

FIG. 3. Immunoblots of tissue membrane proteins resolved by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. Sample buffer was used to solubilize membrane preparations for electrophoresis. Proteins were either stained with Coomassie brilliant blue (*E*) or transferred to nitrocellulose (*A–D*). The letters at the top of the lanes refer to G protein standards ($G_{s\alpha}$ = 100 ng, $G_{o\alpha}$ = 20 ng, G_{β} = 40 ng) and numbers refer to membrane preparations: 1, 30 μg from brain except 5 μg in *C*; 2, 30 μg from adrenal medulla; 3, 30 μg from adrenal cortex; 4, 10 μg from cardiac sarcolemma; 5, 30 μg from rod outer segment discs; 6, 30 μg from tracheal smooth muscle. The blots were processed with the indicated primary antisera (diluted 1:200 except U-49, which was diluted 1:5000) in buffer containing 0.2% Nonidet P-40 and with 1×10^6 cpm/ml of ^{125}I-labeled goat antibodies to rabbit IgG in buffer containing 2% Nonidet P-40 and 0.2% sodium dodecyl sulfate. Serum U-46 was affinity purified with $G_{o\alpha}$ peptide coupled to cyanogen bromide-activated Sepharose. Each blot was exposed to film for 3 hr except the G_s standard lane of *A* and all of *B*, which were exposed for 15 hr. Numbers to the left of *A* indicate apparent molecular masses (kDa) based on mobility of G protein standards.

reaction was indeed with low concentrations of $G_{o\alpha}$ (about 0.02% of the preparation) (Fig. 4*B*). It is possible that G_o is a trace component of discs or that the disc membranes were contaminated with membranes that contain G_o.

DISCUSSION

We have generated a number of antisera that react with G proteins by injecting rabbits with either purified G protein subunits or synthetic peptides (coupled to hemocyanin) derived from amino acid sequences of G proteins. We have found the peptide antisera to be particularly useful. Their great advantage has been the ability to manipulate specificity by the choice of peptides with a common or unique sequence. The $G_{\alpha,common}$ peptide, synthesized according to a region of identical amino acid sequence found originally in $G_{o\alpha}$ and T_{α}, allows production of antisera that recognize $G_{s\alpha}$, $G_{i\alpha}$, $G_{o\alpha}$, and T_{α}. Such complete cross-reactivity has not been achieved with antisera derived by injection of $G_{o\alpha}$ or T_{α} proteins, as shown in Fig. 2 and as described by others (8, 13, 14). Peptides were also effective in the production of antisera specific for $G_{s\alpha}$; such antisera have not been obtained previously. Each antiserum recognizes both the 52-kDa and the 45-kDa forms of $G_{s\alpha}$. Wide variation in the ratios of the 52- and 45-kDa $G_{s\alpha}$ subunits was observed among the various tissues tested by immunoblotting (Fig. 4*B*). The exact relationship of the two forms of this α subunit remains unknown.

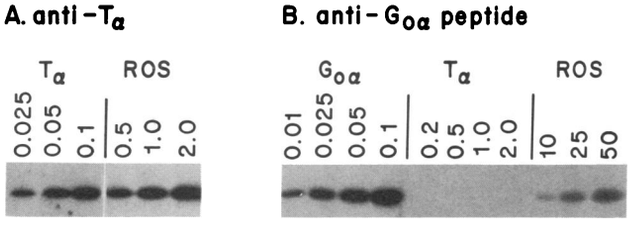

FIG. 4. Detection of T_{α} and $G_{o\alpha}$ in a rod outer segment (ROS) disc preparation from bovine retina. T_{α}, $G_{o\alpha}$, and ROS disc proteins were resolved by polyacrylamide gel electrophoresis and transferred to nitrocellulose. The amount of protein loaded is denoted in μg above each autoradiographic image. One blot (*A*) was incubated with anti-T_{α} serum U-42 at a 1:5000 dilution in buffer containing 0.02% Nonidet P-40 and the other (*B*) with anti-$G_{o\alpha}$ peptide serum U-46 at 1:400 dilution (affinity purified). Both blots were processed with ^{125}I-labeled goat antibody to rabbit IgG at 1×10^6 cpm/ml. Exposures for autoradiography were 2 hr for *A* and 3 hr for *B*.

The $G_{o\alpha}$ peptide antiserum (U-46) recognized only $G_{o\alpha}$ and not other known G proteins (Fig. 2*E*; Fig. 4). The function of G_o has not yet been determined. Use of the $G_{o\alpha}$ peptide antiserum in immunoblots of membrane preparations and immunocytochemical studies should help to define the location of G_o and may suggest possible functions. The identity of the 70-kDa protein that reacts with the $G_{o\alpha}$ peptide antiserum is not known (Fig. 3*C*). It is intriguing that the protein is more prevalent in the adrenal cortex and tracheal smooth muscle membranes, which are strikingly deficient in $G_{o\alpha}$. However, the extent of homology of the 70-kDa protein with $G_{o\alpha}$ may be limited since the protein reacts with neither the $G_{\alpha,common}$ peptide antiserum (Fig. 3*A*) nor the $G_{o\alpha}$ protein antiserum S-214 (data not shown).

In contrast to the α subunits of G proteins, the β subunits are difficult to distinguish. Knowledge of the similarity of β subunits is extended further by amino acid sequence data and

reactivities of antisera reported here. Antisera generated against $G_{o\beta}$ protein, T_β protein, and G_β peptide all reacted with the β subunits of G_s, G_i, G_o, and T. Cross-reactivity between β subunits has been described previously for T antisera by Gierschik *et al.* (13) and Roof *et al.* (8). Our T_β protein antiserum and β peptide antiserum reacted with the 36-kDa β subunits and not the 35-kDa protein purified with G_s, G_i, and G_o. Such specificity was also observed for a T antiserum described by Roof *et al.* (8). These data strongly suggest that the 35-kDa protein is not simply a proteolytic product of the 36-kDa β subunit. However, the relationship between these polypeptides remains unknown.

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