Antibodies against a retinal guanine nucleotide-binding protein cross-react with a single plasma membrane protein in non-retinal tissues

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Received 7 May 1984

Antisera (AS/1-AS/6) to purified bovine retinal transducin, a guanine nucleotide-binding protein, were produced in 6 rabbits. Immunoblots showed that the antisera varied in their reactivity with the subunits of transducin; AS/1 reacted strongly with all 3 subunits, while the others reacted with only the β and/or γ subunits. Only AS/1 specifically immunoprecipitated the α subunit radiolabeled with non-covalently bound guanine nucleotides. Immunostaining of plasma membrane proteins from non-retinal tissues with AS-1 revealed a single protein (approx. 35 kDa), most likely representing the β subunit of the guanine nucleotide-binding proteins (G_s and G_i) associated with adenylate cyclase. Cerebral cortex showed the highest content of this protein. Antisera against transducin provide a highly specific and sensitive probe for quantitation of the β subunit of G_s and G_i.

Guanine nucleotide-binding protein *Adenylate cyclase* *Retina* *Rod outer segment membrane*

1. INTRODUCTION

Transducin (TD) is a guanine nucleotide-binding protein (G-protein) located in the retinal rod outer segment (ROS) disc membranes. TD couples light activation of rhodopsin to increased cGMP phosphodiesterase activity [1], thus serving as a key intermediate in the visual transduction process [2]. Recent evidence suggests a close functional [1] and structural [3] relationship between TD and the G-proteins associated with the adenylate cyclase complex, i.e., G_s and G_i. We here describe the production and initial characterization of antisera raised against purified TD which cross-react with a single plasma membrane protein in non-retinal tissues.

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Abbreviations: G_s, G_i, the stimulatory and inhibitory G-proteins, respectively, of adenylate cyclase; ELISA, enzyme-linked immunosorbent assay; SDS–PAGE, SDS-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; Tw, Tween 20; staph A, staphylococcal protein A; GTP-γ-S, guanosine 5′-(3-*O*-thio)triphosphate; GppNHp, guanosine 5′-(β,γ-imido)triphosphate

2. MATERIALS AND METHODS

2.1. *Membrane preparations and TD purifications*

Fresh bovine eyes, liver, kidney cortex, and cerebral cortex were obtained from a local slaughterhouse and transported on ice to the laboratory. Liver, kidney, and cerebral cortex were homogenized in buffer [10 mM Tris–HCl (pH 7.5), 0.25 M sucrose], spun at low speed to remove unbroken cells, and then at 10000 × *g* for 10 min to collect crude plasma membranes in the pellet. These were resuspended and washed twice in buffer and then frozen and stored in liquid nitrogen. The retinas were dissected from fresh eyes, and

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00145793/84/$3.00

ROS membranes prepared [4]. TD was purified from ROS membranes essentially as in [5] with the following modifications: elutions with isotonic and hypotonic buffers were each performed 6 times. ROS membranes from 80 retinas were then eluted with 1 ml of hypotonic buffer containing 40 μM GppNHp (or other guanine nucleotide as specified). The supernate after centrifugation at 48000 × g for 10 min was collected, and spun again at 48000 × g for 1 h to remove residual particulate material.

2.2. *Immunization*

Six New Zealand White rabbits were injected intradermally with purified TD, initially 100 μg/animal in complete Freund's adjuvant, and 2 weeks later, 50 μg in incomplete Freund's. Animals were bled before immunization and 2 weeks after the booster injection, and heat-inactivated sera were collected.

2.3. *ELISA*

Polyvinyl chloride microtiter plates (96 well) were coated with purified TD (overnight incubation at 4°C with 50 μl/well of 50 μg/ml solution). The plates were washed twice with PBS + 0.05% Tw, incubated with 50 μl indicated dilutions of antisera for 1 h at 37°C, washed 3 times with PBS-Tw, and then incubated with 100 μl/well of a 1:1000 dilution of goat anti-rabbit immunoglobulin (conjugated with horseradish peroxidase) for 1 h at 37°C. After 3 further washes with PBS-Tw, enzyme substrate, 100 μl of 1 mg/ml *O*-phenyldiamine and 4 μl of 3% H_2O_2/ml of 0.1 M citrate buffer (pH 4.5) was added and color developed at room temperature for 30 min. After adding 50 μl/well of half-concentrated sulfuric acid, absorbance was read in an ELISA reader at 480 nM.

2.4. *Immunoblots*

ROS membranes and purified TD were subjected to SDS–PAGE on 10 and 15% acrylamide gels [6]. Transfer to nitrocellulose paper was performed with constant current (20 mA) for 12 h in a Biorad transblot apparatus [7]. After transfer, the paper was incubated first in 10 mM Tris–HCl (pH 7.5), 500 mM NaCl with 3% gelatin, and then with the same buffer containing 1% gelatin and 1:250 dilution of antiserum for 4 h at room temperature. After rinsing, the paper was incubated with second antibody (1 μg/ml peroxidase conjugated goat anti-rabbit IgG) for 2 h at room temperature. Papers were then stained in 8.3 mM Tris–HCl (pH 7.5), 415 mM NaCl, 20% methanol, 0.015% H_2O_2, and 0.5 mg/ml 4-chloro-1-naphthol for 10 min at room temperature.

2.5. *Immunoprecipitation of radiolabeled TD*

TD was purified as above but radiolabeled guanine nucleotides (54 nM GTP-[γ-^{35}S] (925 Ci/mmol) or [^{3}H]GppNHp (1.3 Ci/mmol) were used for elution. The radiolabeled TD was gel filtered over a PD-10 column to remove free nucleotide. Radiolabeled TD (about 10000 cpm) was incubated with antiserum diluted in 100 μl of 1% bovine serum albumin/PBS for 4 h at 4°C. Antigen–antibody complexes were precipitated with 20 mg formalin-fixed staph A/assay volume in a total of 300 μl, after 10 min incubation at 4°C. After centrifugation at 3000 × g for 10 min, supernates were collected and counted by liquid scintillation spectrometry.

2.6. *Protein*

Protein was assayed as in [8] using bovine IgG as standard.

Nonradioactive guanine nucleotides were obtained from Boehringer. GTP-[γ-^{35}S] was from New England Nuclear, and [^{3}H]GppNHp from Amersham. Reagents for SDS–PAGE and immunoblotting were from Biorad, except for goat anti-rabbit IgG which was from Kirkegaard and Perry. Prestained M_r markers and formalin-fixed staph A were from BRL. PD-10 was from Pharmacia. Goat anti-rabbit IgG for ELISA was from New England Nuclear. Protein assay reagents were from Biorad.

3. RESULTS AND DISCUSSION

TD purified (>90% as judged by Coomassie blue-stained SDS–polyacrylamide gels) according to [5] was used to immunize rabbits and to coat plates for ELISA. All 6 animals developed antibodies to purified TD within 2 weeks of booster injection (see fig.1). Half-maximal titers varied from about 1/300 (AS/6) to 1/10000 (AS/1, AS/5). Preimmune serum from each animal gave a negligible response (<10%) in the ELISA.

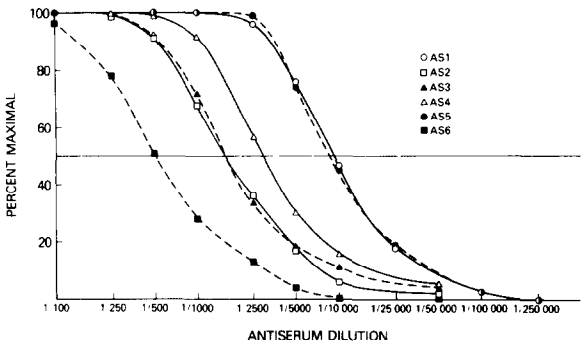

Fig.1. ELISA of rabbit antisera (AS/1–AS/6) raised against purified bovine retinal transducin. ELISA was performed as described in section 2 with indicated dilutions of each antiserum. Results are expressed on the ordinate as a percent of the maximal absorbance (1.7 units at 480 nM) obtained. Controls run with either no transducin or with transducin plus preimmune serum gave readings less than 10% of maximal.

TD is a multisubunit protein [2,5]. The ELISA presumably measures the ability of each antiserum to recognize any or all of the subunits. We therefore separated the TD subunits by SDS–PAGE, transferred them to nitrocellulose paper, and performed immunostaining to define the specificity of the antisera. The α and β subunits of TD are resolved on a 10% acrylamide gel but the γ subunit runs with the dye front. The γ subunit can be resolved from the dye front on a 15% gel. Immunostaining of purified TD transferred from a 10% gel showed that AS/1, AS/2, AS/3 and AS/5 differed in their specificity for the TD subunits. AS/1 reacted strongly with both α and β subunits; AS/3 and AS/5 reacted predominantly with β, and AS/2 showed faint reactivity with both α and β (fig.2A). Varying reactivity of the antisera with material at the dye front (presumably the γ subunit) was also evident on 10% gels (not shown). No other stained bands were seen. On immunostaining of purified TD transferred from 15% gels, it was evident that AS/1 also reacted strongly with the γ subunit, AS/5 and AS/2 reacted less strongly, and AS/3 showed faint reactivity (fig.2B). After SDS–PAGE of whole ROS membranes and transfer to nitrocellulose paper, immunostaining with AS/1 revealed only bands corresponding to the 3 subunits of TD (see lane 1 in fig.4B); immunostaining

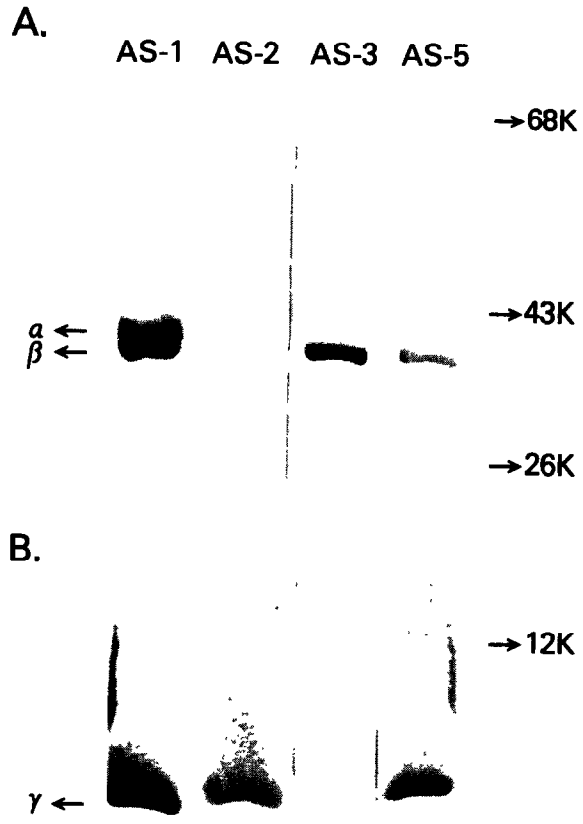

Fig.2. Immunostaining of nitrocellulose blots of purified subunits of transducin. Purified transducin was separated into α, β, and γ subunits by SDS–PAGE [(A) 10% acrylamide, (B) 15% acrylamide]. The separated subunits were transferred to nitrocellulose paper as described in section 2. Immunostaining with antisera (AS/1, AS/2, AS/3, and AS/5) was then performed (see section 2). The arrows to the left indicate the positions of the transducin subunits and those to the right, the positions of molecular mass standards (bovine serum albumin, 68 kDa, ovalbumin, 43 kDa, chymotrypsinogen, 26 kDa, and cytochrome *c*, 12 kDa) on Coomassie blue-stained gels.

of material eluted from ROS membranes by hypotonic buffer without guanine nucleotides (containing predominantly the subunits of cGMP phosphodiesterase [5]) revealed no bands (not shown). Immunostaining of TD subunits with preimmune sera also failed to show any bands.

The α subunit of TD contains a site for noncovalent binding of guanine nucleotides [1,2]. When nonhydrolyzable analogs of GTP such as GppNHp or GTP-[γ-S] are used to elute TD, the α subunit dissociates from β and γ and retains bound guanine nucleotide [2]. We took advantage of this property by using GTP-[γ-^{35}S] or [^{3}H]GppNHp to elute TD. After gel filtration to remove unbound nucleotide, we tested the ability of the antisera to bind to the specifically labeled α subunit of TD (separation of α from β-γ subunits of TD as in [2] confirmed that the labeled guanine nucleotide was exclusively associated with the alpha subunit). Only AS/1 was able to immunoprecipitate the labeled α subunit (fig.3). Similar results were obtained with [^{3}H]GppNHp as the label (not shown). The inability of the other antisera to immunoprecipitate labeled α subunit is not surprising in view of the specificity seen on immunostaining (fig.2A). The finding that the half-maximal titer of AS-1 defined by immunoprecipitation of labeled α is substantially lower than that defined by ELISA is explained in part by additional determinants on the β and γ subunits available for reaction with AS-1 in the ELISA, and may also relate to the instability of the radioligand, i.e., guanine nucleotide noncovalently bound to α subunit, in the immunoprecipitation assay. Nonetheless, the ability of AS/1 to immunoprecipitate TD α labeled in a biologically relevant way provides further evidence for the specificity of this antiserum.

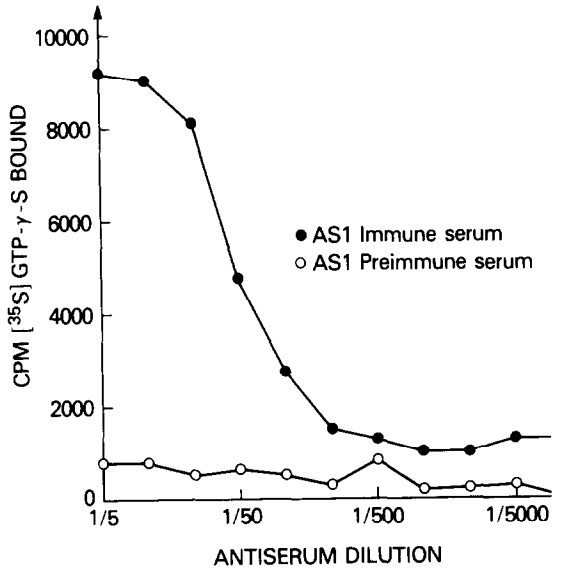

Fig.3. Specific immunoprecipitation of transducin radiolabeled with non-covalently bound guanine nucleotide. Transducin was eluted from ROS membranes with GTP-[γ-^{35}S], and unbound nucleotide removed by gel filtration as described in section 2. Radiolabeled transducin was incubated with indicated dilutions of immune and preimmune serum, and antibody bound transducin precipitated with staph A as indicated in section 2. The results on the ordinate are expressed as total cpm GTP-[γ-^{35}S] in the pellet.

Given the structural homologies between TD and the adenylate cyclase associated G_s and G_i [3,10], it was obviously of interest to test whether these antisera cross-reacted with the adenylate cyclase G-proteins. Since G_s and G_i are found in plasma membranes from virtually all tissues, albeit in extremely low amounts [3], we tested the ability of the antisera to recognize plasma membrane proteins in bovine liver, kidney cortex, and cerebral cortex. Immunostaining with AS/1 of membrane proteins blotted from 10% SDS gels showed a single peptide band of about 35 kDa in each tissue (fig.4A,B, lanes 2–4). AS/3, which recognizes TD β but not α, also recognized this protein (not shown). Several points, in addition to the apparent M_r, strongly suggest that this protein is the β subunit of G_s and G_i. The β subunits of G_s, G_i, and TD have been shown by peptide mapping to be virtually identical [3], suggesting that antibodies to TD β might well cross-react with G_s and G_i β. The ability of AS/3 to recognize the protein excludes the possibility that it is the α subunit of either G_s or G_i. If, in fact, the protein recognized with TD antisera is the β subunit, the particularly heavy band seen in cerebral cortex suggests that this tissue is relatively rich in G_s and/or G_i. Definitive identification of this protein will require testing the cross-reactivity of these antisera with the purified resolved subunits of G_s and G_i.

These findings demonstrate that we have succeeded in producing antisera, several in relatively high titer, specific for TD. Although all 6 animals were injected exclusively with purified holoprotein, immunostaining reveals that the antisera differ quantitatively in their ability to recognize the 3 TD subunits. AS/1 reacts strongly with all 3, AS/2 almost exclusively with γ, AS/3 almost exclusively with β, and AS/5 mainly with γ. These antisera, therefore, may prove useful in helping to define

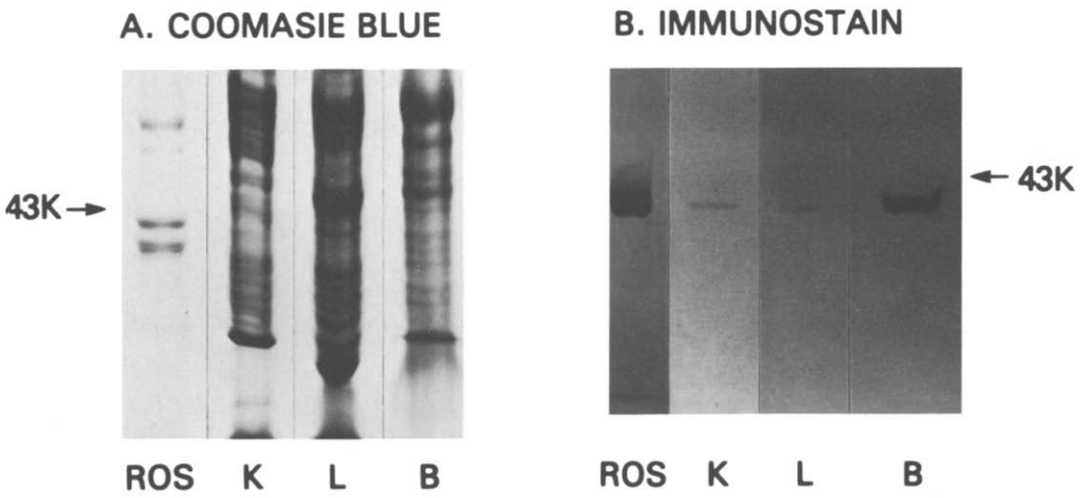

Fig.4. Protein staining (A) and immunostaining (B) of SDS–PAGE separated proteins from bovine ROS (lane 1), kidney cortex (K, lane 2), liver (L, lane 3), and cerebral cortex (B, lane 4) plasma membranes. 100 μg ROS membranes and 300 μg of each of the other membranes were loaded onto 10% gels and SDS–PAGE performed. One gel was stained with Coomassie blue (A), and the other (B) blotted and immunostained with AS/1, as described in section 2. The arrow indicates the position of the 43-kDa-marker, ovalbumin.

the functions of individual subunits of TD. They may also be useful in developing radioimmunoassays for the holoprotein or its subunits, in immunocytochemical studies of the retina, and in screening expression vector (e.g., GT11 [9]) gene libraries for the TD subunit genes. Perhaps most importantly, these antisera may prove useful in studies of the adenylate cyclase associated G-proteins, G_s and G_i. Because of the difficulty in purifying substantial quantities of these proteins, antisera against them have not been readily produced. AS/1 reacts in a highly specific and sensitive manner with a single plasma membrane protein from several bovine tissues. If, as is likely, this protein is the β subunit of G_s and G_i, these antisera provide a unique tool for quantitation and possible immunoaffinity purification of G_s and G_i.

ACKNOWLEDGEMENTS

We are grateful to Mark Griffo, Johns Hopkins University, for assistance in purifying TD. P.G. was supported in part by a grant (Gi 138/1-1) from the Deutsche Forschungsgemeinschaft.

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