Smith, G. D., Swenson, D. C., Dodson, G. G., & Reynolds, C. D. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 7093–7097.
States, D. J., Haberkorn, R. A., & Ruben, D. J. (1982) *J. Magn. Reson. 48*, 286–292.
Steiner, D. F., Cunningham, D. D., Spigelman, L., & Aten, B. (1967) *Science 157*, 697–700.
Strickland, E. H., & Mercola, D. A. (1976) *Biochemistry 15*, 3875–3884.
Tager, H., Given, B., Baldwin, D., Mako, M., Markese, J., Rubenstein, A., Olefsky, J., Kobayashi, M., Kolterman, O., & Poucher, R. (1979) *Nature (London) 281*, 122–125.
Tager, H., Thomas, N., Assoian, R., Rubenstein, A., Saekow, M., Olefsky, J., & Kaiser, E. T. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3181–3185.
Ullrich, A., Bell, J. R., Chen, E. Y., Herrera, R., Petrucelli, L. M., Dull, T. J., Gray, A., Coussens, L., Liao, Y. C., Tsubokawa, M., Mason, A., Seeburg, P. H., Grunfeld, C., Rosen, O. M., & Ramachandran, J. (1985) *Nature 313*, 756–761.
Weiss, M. A., Eliason, J. A., & States, D. J. (1984) *Proc. Natl. Acad. Sci. U.S.A.* 6019–6023.
Williamson, K. L., & Williams, R. J. P. (1979) *Biochemistry 18*, 5966–5972.
Wodak, S. J., Alard, P., Delhaise, P., & Renngboog, S. C. (1984) *J. Mol. Biol. 151*, 317–322.
Wollmer, A., Fleischhauer, J., Strassburger, W., Thiele, H., Bradenbury, D., Dodson, G., & Mercola, D. (1979) *Biophys. J. 20*, 233–243.
Wood, S. P., Blundell, T. L., Wollmer, A., Lazarus, N. R., & Neville, R. W. J. (1975) *Eur. J. Biochem. 55*, 531–542.
Wuthrich, K., Wider, G., Wagner, G., & Braun, W. (1983) *J. Magn. Reson. 155*, 311.

Effect of Monoclonal Antibody Binding on α–$\beta\gamma$ Subunit Interactions in the Rod Outer Segment G Protein, G_t[†]

Maria R. Mazzoni and Heidi E. Hamm*
Department of Physiology and Biophysics, University of Illinois, College of Medicine at Chicago, P.O. Box 6998, Chicago, Illinois 60680

Received May 1, 1989; Revised Manuscript Received July 31, 1989

ABSTRACT: The guanyl nucleotide binding regulatory protein of retinal rod outer segments, called G_t, that couples the photon receptor rhodopsin with the light-activated cGMP phosphodiesterase, can be resolved into two functional components, α_t and $\beta\gamma_t$. The effect of monoclonal antibody binding to the α_t subunit of G_t on subunit association has been investigated in the present study. It was previously shown that this monoclonal antibody, mAb 4A, blocks interactions with rhodopsin and its epitope was located within the region Arg^{310}–Phe^{350} at the COOH terminus of the α_t subunit. In this paper, we show that mAb 4A disrupts the G_t complex. G_t migrates in 5–20% linear sucrose density gradients as a monomer, with a sedimentation coefficient of 4.1 ± 0.07 S, while in the presence of mAb 4A, the α_t and $\beta\gamma_t$ subunits show sedimentation coefficients of 7.7 ± 0.2 and 3.7 ± 0.1 S, respectively. The $\beta\gamma_t$ subunit migrates with the same sedimentation rate as pure $\beta\gamma_t$. Nonimmune rabbit IgG does not modify the sedimentation behavior of G_t. The Fab fragment of mAb 4A also dissociates the G_t complex, as suggested by the change of the sedimentation rate of α_t. This effect of mAb 4A on G_t subunit association was also confirmed by immunoprecipitation studies in the presence of detergent. In the presence of detergent, subunit association is not affected, but the formation of G_t oligomers and, therefore, the nonspecific precipitation of $\beta\gamma_t$ subunit are reduced. An anti-peptide immune serum, raised against the last 10 amino acids from the carboxyl terminus of α_t, also immunoprecipitated the α_t subunit, and the $\beta\gamma_t$ subunit was found in the supernatant fraction. Monoclonal antibody 4A also blocks the pertussis toxin mediated ADP-ribosylation of α_t both in holo-G_t and in the purified α_t subunit. These results suggest either that the mAb 4A epitope is close enough to the site of $\beta\gamma_t$ interaction to dissociate binding or that antibody binding causes a conformational change on α_t causing loss of affinity for $\beta\gamma_t$.

A guanine nucleotide-binding regulatory protein, called transducin or G_t,[1] couples light-activated rhodopsin with the cGMP phosphodiesterase [for a review, see Stryer and Bourne (1986), Gilman (1987), Hurley (1987), and Liebman et al. (1987)]. Like all G proteins, G_t is a heterotrimer composed of two distinct subunits: α_t (39 kDa) and $\beta\gamma_t$ (β_t, 36 kDa; γ_t, 8 kDa). The α_t subunit binds GDP and GTP and in its GTP-bound form activates the cGMP phosphodiesterase. The activation is terminated when the bound GTP is hydrolyzed to GDP by an intrinsic GTPase activity (Wheeler & Bitensky, 1977). Although the $\beta\gamma_t$ subunit has not been shown to di-

[†]Supported by USPHS Grant EY-06062, NSF Grant DMB-8804861, and an unrestricted departmental grant from Research to Prevent Blindness. H.E.H. was the recipient of an NSF ROW Research Career Enhancement Award.
*To whom correspondence should be addressed.

[1] Abbreviations: G_t, photoreceptor guanyl nucleotide binding protein; α_t, α subunit of G_t; $\beta\gamma_t$, β and γ subunits of G_t; G_s and G_i, regulatory guanyl nucleotide binding proteins that mediate stimulation and inhibition of adenylate cyclase, respectively; G_k, guanyl nucleotide binding protein mediating muscarinic stimulation of cardiac potassium channels; G_o, guanyl nucleotide binding protein isolated from brain; mAb, monoclonal antibody; ROS, rod outer segment; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); MOPS, 3-(*N*-morpholino)propanesulfonic acid; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; PBS, phosphate-buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; TCA, trichloroacetic acid.

0006-2960/89/0428-9873$01.50/0

rectly participate in either GTP hydrolysis or phosphodiesterase activation, its presence is important for effective binding of α_t to photolyzed rhodopsin and GTP–GDP exchange (Fung, 1983). G_t binds tightly to photolyzed rhodopsin in intact rod outer segment membranes (Kühn, 1980). It also binds selectively to unphotolyzed rhodopsin in rod outer segment membranes (Hamm et al., 1987) and in reconstituted phospholipid vesicles (Fung, 1983).

Several monoclonal antibodies (mAb) have been generated to test the functional roles of G_t in phototransduction (Hamm & Bownds, 1984; Witt et al., 1984). One of these antibodies, mAb 4A, was found to block light-activated GTP–GDP exchange and activation of cGMP phosphodiesterase, whereas other antibodies did not have these effects (Hamm & Bownds, 1984). When the mechanism of action of mAb 4A was examined, this antibody appeared to block a site on G_t that interacts with rhodopsin (Hamm et al., 1987, 1988). Antibody binding to proteolytic fragments of α_t (Deretic & Hamm, 1987) located the major portion of the antigenic site of mAb 4A within the region Arg^{310}–Lys^{329} near the COOH terminus of the α_t subunit. The proteolytic mapping results were confirmed by using competition between synthetic peptides corresponding to α_t sequences and G_t for antibody binding (Hamm et al., 1988). The synthetic peptide studies also showed that the carboxyl-terminal amino acids, Ile^{340}–Phe^{350}, also take part in the mAb 4A epitope, while amino-terminal peptides have no effect (Hamm et al., 1988).

The main purpose of the present study was to investigate the effect of mAb 4A binding on subunit interactions of G_t. The physical properties of the G_t–monoclonal antibody complex were characterized by using sedimentation in sucrose density gradients, immunoprecipitation, and accessibility to pertussis toxin ADP-ribosylation. Sedimentation studies showed that mAb 4A forms a stable complex with the α_t subunit of G_t and dissociates α_t from the $\beta\gamma_t$ subunit. This result was confirmed by using immunoprecipitation studies in the presence of detergent. This suggests either that the mAb 4A epitope is close enough to the site of $\beta\gamma_t$ interaction to dissociate binding or that antibody binding causes a conformational change on α_t causing loss of affinity for $\beta\gamma_t$.

Experimental Procedures

Protein Isolation. Bovine rod outer segment (ROS) membranes were prepared according to Papermaster and Dreyer (1974), with some modifications. Bovine G_t was extracted from ROS membranes with low ionic strength buffer (10 mM Tris–HCl, pH 7.5, 2 mM $MgCl_2$, 1 mM DTT, and 0.1 mM PMSF) in the presence of 0.1 mM GTP. For the preparation of α_t (α_t–GTPγS complex) and $\beta\gamma_t$ subunits, G_t was extracted from ROS membranes with the same low ionic strength buffer in the presence of 0.1 mM GTPγS. The α_t–GTPγS and $\beta\gamma_t$ subunits were further purified and separated by chromatography on Blue-Sepharose CL-6 B (Pharmacia), essentially as described by Kleuss et al. (1987). The α_t–GDP complex was prepared and purified according to Yamazaki et al. (1988). All the purified proteins were stored in 40% glycerol at −20 °C. Protein concentrations were determined by the Coomassie Blue binding method (Bradford, 1976) using γ-globulins as standard. Protein concentration and purity were also quantified by SDS–polyacrylamide gel (12.5%) electrophoresis (Laemmli, 1970) followed by Coomassie Blue staining and densitometric scanning (Ephortec, Joyce Loebl densitometer), using bovine serum albumin (BSA) as standard. Preparations of G_t and its subunits were at least 95% pure.

Generation of Monoclonal Antibody to α_t. Monoclonal antibody 4A was generated and characterized as described by Witt et al. (1984) and by Hamm and Bownds (1984).

Preparation of Fab Fragments. Fab fragments of mAb 4A were prepared by using immobilized papain (Pierce). Briefly, 0.5 mL of a 50% slurry of immobilized papain, prewashed in digestion buffer, was added to 10 mg of mAb 4A in 1.0 mL of digestion buffer (20 mM NaH_2PO_4, 20 mM cysteine hydrochloride, and 10 mM EDTA, pH 7.0), and incubated overnight at 37 °C with shaking. The immobilized papain was removed by centrifugation, and the Fab fragments were purified by chromatography on a protein A–Sepharose 4B column. Fab fragments were found in the PBS wash (0.9% NaCl/10 mM NaH_2PO_4, pH 8.0), while Fc fragments were eluted by 0.1 M citrate buffer, pH 3.0.

Sucrose Density Gradients. Prior to sucrose density gradient centrifugation, the purified proteins, G_t, α_t, or $\beta\gamma_t$ (100 μL), were eluted through a Sephadex G-25 (Sigma) column (0.48 × 6 cm) to remove the glycerol and exchange them into the appropriate gradient buffer. In some experiments, the samples of G_t or its subunits were diluted with equal volumes of gradient buffer containing 1% (w/v) Lubrol PX and then eluted through the column. The protein peaks were concentrated by ultrafiltration (Amicon Centricon). To activate G_t, protein samples (35 μg) were incubated in buffer A with ROS membranes (3 μM rhodopsin) and GTPγS (0.1 mM) for 20 min at 4 °C in light. The ROS membranes were removed by centrifugation at 100000 rpm for 10 min in a Beckman TLA 100.2 rotor at 4 °C. Samples (100–150 μL) containing purified G_t, activated G_t, α_t, or $\beta\gamma_t$ (35–50 μg), and marker proteins were layered on the top of 3.2-mL gradients of 5–20% sucrose prepared in buffer A (10 mM MOPS, 60 mM KCl, 30 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT, and 0.1 mM PMSF, pH 7.5) or buffer B (10 mM MOPS, 60 mM KCl, 30 mM NaCl, 2 mM $MgCl_2$, 0.1 mM DTT, and 0.1 mM PMSF, pH 7.5). Samples containing activated G_t were layered on gradients prepared in buffer A containing 0.1 mM GTPγS. Purified G_t (35 μg) or α_t subunit (30 μg) was incubated for 30 min at room temperature in buffer B in the presence of either mAb 4A, Fab fragments of mAb 4A, or nonimmune rabbit IgG at a molar ratio of antibodies to G_t of 2:1. The marker protein mix consisted of 14 μg of BSA, 24 μg of carbonic anhydrase, and 5 μg of cytochrome *c*. When the sedimentation behavior of G_t was analyzed in the presence of antibodies, catalase (14 μg) was also used as standard. The gradients were centrifuged at 41000 rpm for 15 h in a Beckman SW 50.1 rotor at 4 °C and fractionated into 20–25 fractions. Aliquots of these fractions were subjected to precipitation by acetone (90% final concentration), and the position of the proteins was determined by SDS–polyacrylamide gel (12.5%) electrophoresis, according to Laemmli (1970), followed by Coomassie Blue staining and densitometric scanning. Catalase was localized by assay of enzymatic activity. Sizes of mAb 4A and its Fab fragment were determined by centrifugation on 5–20% sucrose gradients as described above.

Immunoprecipitation of G_t. G_t (10 μg) in 100 μL of 10 mM MOPS, pH 7.5, 2 mM $MgCl_2$, 200 mM NaCl, and 0.1 mM DTT, either in the presence or in the absence of 0.3% Lubrol PX (w/v), was incubated for 1 h at room temperature with mAb 4A or nonimmune rabbit IgG at a molar ratio of antibodies to G_t of 2:1. G_t was also incubated with 15 μL of rabbit immune serum raised against the synthetic decapeptide Lys-Glu-Asn-Leu-Lys-Asp-Cys-Gly-Leu-Phe which corresponds to the last 10 amino acids from the carboxyl terminus of the α_t subunit (Goldsmith et al., 1987). Then 400 μL of 10% (w/v) *Staphylococcus aureus* cell suspension (Bethesda Re-

Table I: Hydrodynamic Properties of α_t, $\beta\gamma_t$, and G_t[a]

	sedimentation coefficient, $s_{20,w}$ (S)
buffer A	
G_t	4.0 ± 0.08 (4)
α_t	3.5 ± 0.3 (3)
$\beta\gamma_t$	3.7 ± 0.2 (3)
G_t + GTPγS / α_t	3.4 (2)
G_t + GTPγS / $\beta\gamma_t$	3.6 (2)
buffer B	
G_t	4.1 ± 0.07 (5)

[a] Purified G_t or subunits were applied to 5–20% linear sucrose density gradients prepared in either buffer A or buffer B and centrifuged as described under Experimental Procedures. Following the centrifugation, each gradient was fractionated, and the sedimentation profiles of G_t, α_t, and $\beta\gamma_t$ were determined by electrophoresis on 12.5% SDS–polyacrylamide gels, Coomassie Blue staining, and densitometric scanning. Sedimentation mobility was defined as follows: mobility = [(total no. of fractions) − (peak fraction)]/(total no. of fractions). The sedimentation coefficients [$s_{20,w}$ in Svedberg units (S)] were determined from the calibration curves, as shown in panel A of Figure 1. The values are the averages ± SE. The numbers in parentheses are the number of experiments.

search Laboratories), prepared as previously described in Hamm et al. (1987), was added and incubated for an additional 1 h. The antigen–antibody–*Staphylococcus aureus* cell complexes were pelleted with a microcentrifuge and washed 3 times in 150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.4, 0.02% NaN_3, and 0.5% Nonidet P-40 (NET buffer) as described (Hamm et al., 1987). The immunoprecipitated proteins were eluted from the *Staphylococcus aureus* cells by resuspending the pellet in electrophoresis sample buffer (Laemmli, 1970) and heating at 95 °C for 5 min. The sample was then centrifuged, and an aliquot of the supernatant was analyzed by SDS–polyacrylamide gel electrophoresis.

The time course of association of G_t with mAb 4A was carried out by incubating G_t with the antibody for various periods of time (0–180 min) under the conditions described above.

ADP-Ribosylation of Purified G_t, α_t–GTPγS, and α_t–GDP. G_t (10 μg), α_t–GTPγS (6 μg), or α_t–GDP (10 μg) was incubated in 50 μL of ADP-ribosylation buffer, containing 100 mM NaCl, 2.5 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, 10 mM thymidine, 20 mM HEPES, pH 7.5, 6 μM [^{32}P]NAD (specific activity 25 Ci/mmol), and 2.5 μg/mL pertussis toxin (preactivated with 20 mM DTT for 10 min at 30 °C), for 30 min at 30 °C.

Results

Sedimentation Properties of G_t, GTPγS-Activated G_t, Free α_t, and $\beta\gamma_t$. The physical properties of G_t, GTPγS-activated G_t, and its subunits were investigated under our experimental conditions before examining the sedimentation behavior of the G_t–antibody complex. Figure 1 shows the sedimentation behavior of G_t in sucrose density gradients, and Table I summarizes the sedimentation coefficients ($s_{20,w}$) of free α_t, free $\beta\gamma_t$, and the associated complex. Under our experimental conditions, standard proteins sedimented in a linear relationship to their $s_{20,w}$ values (panel A, Figure 1). The sedimentation rate of G_t did not change when the sucrose density gradient experiments were carried out either in buffer A (1 mM DTT) or in buffer B (0.1 mM DTT). In fact, similar sedimentation coefficient values were obtained (Table I). This indicates that the formation of G_t oligomers is not facilitated by lower concentrations of DTT. Therefore, sucrose density gradients prepared in buffer B were used to study the physical properties of the G_t–antibody complex because in this low concentration

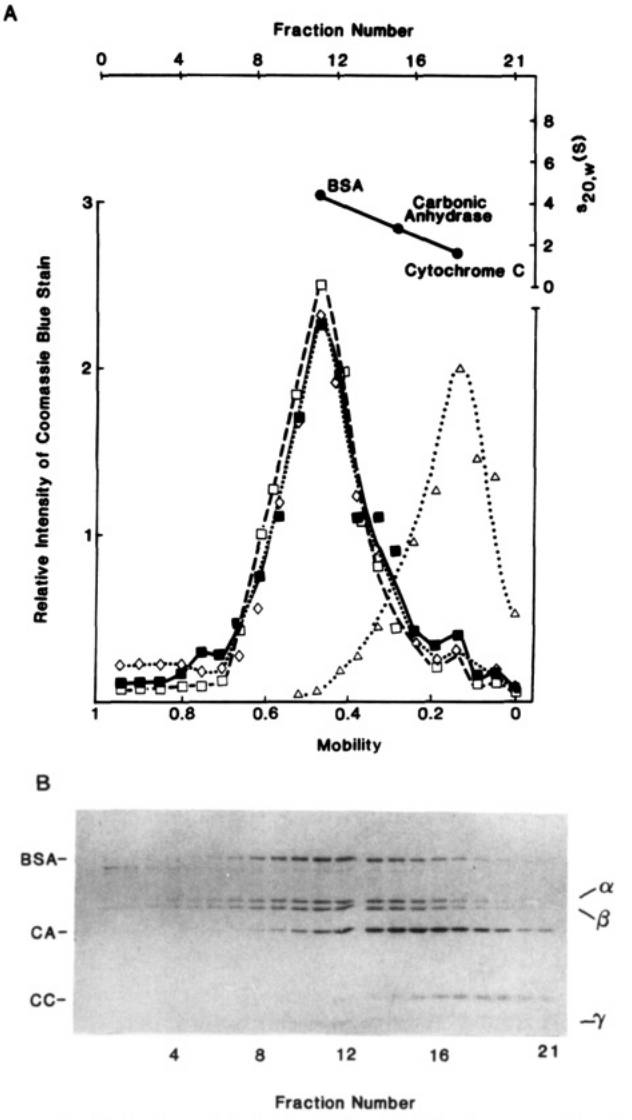

FIGURE 1: Hydrodynamic behavior of G_t. G_t (35 μg) was centrifuged on a 5–20% linear sucrose density gradient prepared in buffer B as described under Experimental Procedures. The gradients were recovered as 155-μL fractions with 1 representing the densest fraction. The results were obtained from one experiment. (A) The sedimentation mobilities of each standard protein and G_t were determined as described in Table I. The profiles of the distribution of α_t (■—■), $\beta\gamma_t$ (◇---◇), BSA (□–□), and cytochrome *c* (△···△) are shown at the bottom, as determined by gel densitometric scanning. (B) Photograph of the Coomassie Blue stained polyacrylamide gel. CA and CC are carbonic anhydrase and cytochrome *c*, respectively.

of reducing agent the disulfide bonds of antibodies were maintained. However, sometimes the formation of G_t oligomers was noted in sucrose gradients prepared in both buffer A or buffer B, as indicated by the presence of α_t and $\beta\gamma_t$ in high-density fractions (relative mobility, 0.8–1.0; data not shown). Therefore, prior to use, samples of G_t were diluted with an equal volume of buffer containing 1% Lubrol PX (w/v) and eluted on a Sephadex G-25 column. Under these conditions, holo-G_t comigrates as a single peak with BSA. A similar sedimentation coefficient for G_t was obtained in both experimental conditions. Holo-G_t as a monomer migrates at a faster rate relative to the individual subunits, α_t, and the $\beta\gamma_t$ complex.

Under physiological conditions, light-activated rhodopsin binds tightly to G_t and promotes its activation by GDP–GTP exchange and subsequent subunit dissociation. This activation was studied in vitro by using a nonhydrolyzable GTP analogue

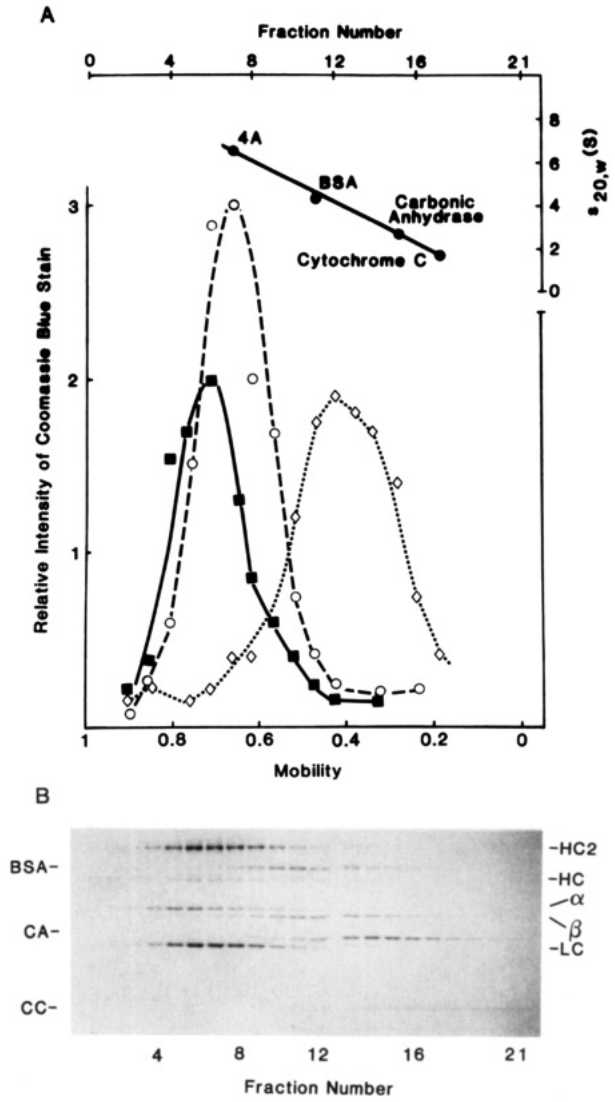

FIGURE 2: Hydrodynamic behavior of G_t in the presence of monoclonal antibody 4A. The sample, containing G_t and mAb 4A (1:2), was layered on the top of a 5–20% linear sucrose density gradient prepared in buffer B, as described under Experimental Procedures. The results were obtained from one experiment. (A) The sedimentation mobilities of each standard protein, α_t, $\beta\gamma_t$, and mAb 4A, were determined as described in Table I. The profiles of distribution of α_t (■—■), $\beta\gamma_t$ (◇---◇), and mAb 4A (○--○) are shown at the bottom, as determined by gel densitometric scanning. (B) Photograph of the Coomassie Blue stained polyacrylamide gel. HC and LC are antibody heavy and light chain, respectively. HC2 is the heavy chain dimer.

(GTPγS). When we incubated G_t with ROS membranes and GTPγS (0.1 mM) before performing the linear sucrose density gradient, we observed that the α_t and $\beta\gamma_t$ subunits display a shift in sedimentation, indicating dissociation of G_t complex. The sedimentation coefficient values of the individual subunits were similar to those obtained from the sucrose density gradient centrifugations either of pure α_t or of $\beta\gamma_t$.

Sedimentation Properties of mAb 4A and Its Effect on G_t. Before performing sucrose density gradient centrifugations of G_t in the presence of mAb 4A, we studied the sedimentation behavior of mAb 4A alone. In this case, a single peak was found in the high-density fractions, and a sedimentation coefficient of 7.2 S was calculated. No other minor peaks were found in fractions of higher density. This suggests that mAb 4A migrates in linear sucrose density gradients as a monomer.

When the sedimentation behavior of G_t was examined in the presence of mAb 4A, a large change in its sedimentation rate was observed (panel B of Figure 2). Monomeric G_t comigrates with BSA in the sucrose density gradient (panel A of Figure 1). However, in the presence of mAb 4A, the $\beta\gamma_t$ subunit migrates as a monomer, with an apparent $s_{20,w}$ value of 3.7 ± 0.2 S (n = 4; panel A of Figure 2), while the α_t peak is found in fractions of much higher density, even higher than mAb 4A alone. This shift in the sedimentation of G_t can only be the result of subunit dissociation by antibody. The main mAb 4A peak (panel A of Figure 2) is found in a lower density fraction, with an apparent $s_{20,w}$ value of 7.1 ± 0.08 S (n = 4), due to an excess of mAb 4A with respect to G_t (2:1) in these experiments. Formation of G_t aggregates facilitated by the presence of mAb 4A cannot explain the observed shift in the sedimentation of α_t, because $\beta\gamma_t$ shows sedimentation behavior similar to the free monomeric subunit (Table I), and the amount of $\beta\gamma_t$ which is found in high-density fractions is low.

Table II: Hydrodynamic Properties of G_t, α_t, and Antibodies[a]

	sedimentation coefficient, $s_{20,w}$ (S)	
IgG + G_t G_t	4.1	(2)
mAb 4A + G_t α_t	7.7 ± 0.2	(4)
mAb 4A + G_t $\beta\gamma_t$	3.7 ± 0.1	(4)
Fab 4A + G_t α_t	5.1	(2)
Fab 4A + G_t $\beta\gamma_t$	3.8	(2)
Fab 4A + α_t	5.1	(2)
Fab 4A	3.7	(2)

[a] The sedimentation coefficients [$s_{20,w}$ in Svedberg units (S)] were estimated from the calibration curves. The values are the averages. The numbers in parentheses are the number of experiments.

As a control experiment, G_t was incubated in the presence of nonimmune rabbit IgG before performing the sucrose density gradient centrifugation. In this case, holo-G_t comigrates with BSA and does not show subunit dissociation.

The binding of a large molecule like an antibody (150 kDa) to the α_t subunit might sterically hinder the association with the $\beta\gamma_t$ subunit. To further investigate the effect of mAb 4A on G_t subunit interactions, Fab fragments of mAb 4A were prepared. Fab fragments still contain the antigen-binding site, but steric hindrance problems should be minimized because of their smaller size (50 kDa). The hydrodynamic behavior of G_t in the presence of Fab fragments of mAb 4A is shown in Figure 3. The α_t subunit migrates in a higher density fraction relative to the $\beta\gamma_t$ subunit (panel A of Figure 3). The $\beta\gamma_t$ subunit shows an $s_{20,w}$ value of 3.8 S (4.2 and 3.4 S in two experiments), while α_t has a greater sedimentation coefficient (5.3 and 5.0 S in two experiments) than that obtained for free α_t subunit. Thus, the α_t peak results from the formation of a complex between α_t and Fab fragment. This is also confirmed by the sucrose density gradient experiments carried out with Fab fragments and purified α_t subunit (Table II). The peak of Fab fragments is found in lower density fractions than the α_t peak, because an excess of Fab mAb 4A was used (panel A of Figure 3). Therefore, binding of Fab fragments of mAb 4A also appears to interfere with the maintenance of subunit association. The sedimentation values of G_t in the presence of nonimmune rabbit IgG, mAb 4A, and Fab fragments of mAb 4A are summarized in Table II.

Immunoprecipitation of G_t. Since sedimentation studies were carried out for 16 h, it was possible that the process of subunit dissociation may have occurred slowly. Another method, immunoprecipitation, was also used to study the effect of mAb 4A on subunit interactions. Figure 4 shows immunoprecipitation results obtained after 2 h of interaction between monoclonal antibody 4A and either G_t or α_t. The antibody clearly causes subunit dissociation, since when mAb 4A was incubated with G_t, no $\beta\gamma_t$ was precipitated (lane 5 of Figure

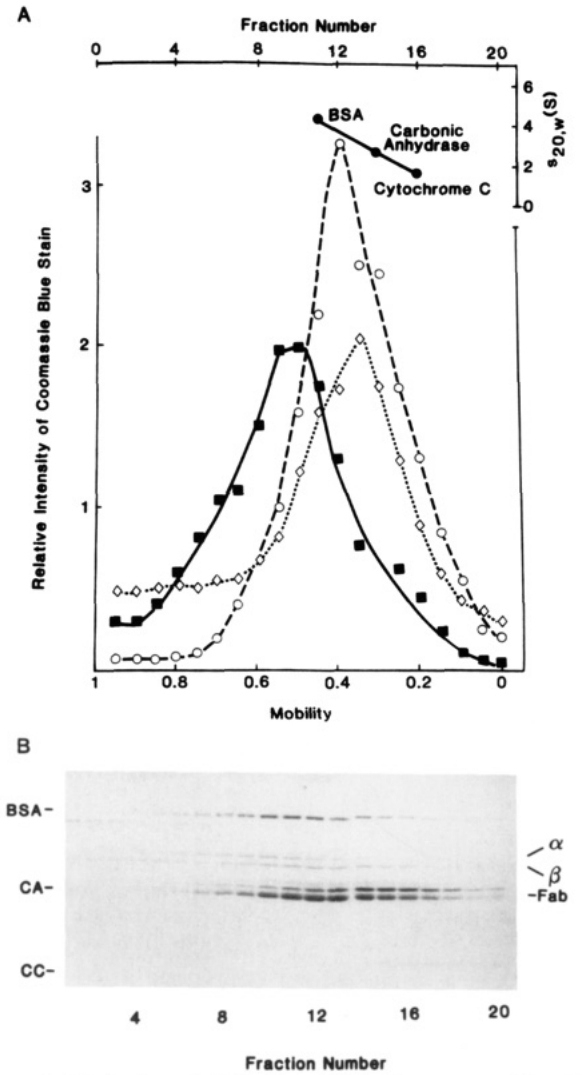

FIGURE 3: Hydrodynamic behavior of G_t in the presence of the Fab fragment of mAb 4A. The sample, containing G_t and Fab 4A (1:5), was layered on the top of a 5–20% linear sucrose density gradient prepared in buffer B as described under Experimental Procedures. The results were obtained from one experiment. (A) The sedimentation mobilities of each standard protein, α_t, $\beta\gamma_t$, and Fab 4A, were determined as described in Table I. The profiles of distribution of α_t (■—■), $\beta\gamma_t$ (◇---◇), and Fab 4A (○—○) are shown at the bottom as determined by gel densitometric scanning. (B) Photograph of the Coomassie Blue stained polyacrylamide gel. Fab represents the heavy and light chain of Fab fragment.

4). The efficiency of immunoprecipitation was not 100%, since 40% of the α_t subunit was found in the supernatant fraction. However, the α_t found in the *Staphylococcus aureus* pellet fraction was approximately 30% (compare lanes 4 and 5 of Figure 4), because some α_t was lost during the pellet washes. A similar efficiency of precipitation was found upon immunoprecipitation of α_t–GTPγS (compare lanes 1 and 2 of Figure 4). There was no nonspecific precipitation in the presence of nonimmune IgG (lane 3 of Figure 4). Hamm et al. (1987) have previously shown that mAb 4A immunoprecipitates both subunits of [^{125}I]iodonaphthyl azide labeled G_t. However, Navon and Fung (1988) reported that mAb 4A immunoprecipitates only the α_t subunit, causing dissociation of the G_t complex. Sucrose density experiments showed that, under the conditions of isolation and storage, G_t can form oligomers and that the use of a detergent in the buffer (0.5% Lubrol PX) can overcome this phenomenon. Therefore, a buffer containing 0.3% Lubrol PX (w/v) was used for the incubation of G_t with antibodies before immunoprecipitation. We have found that

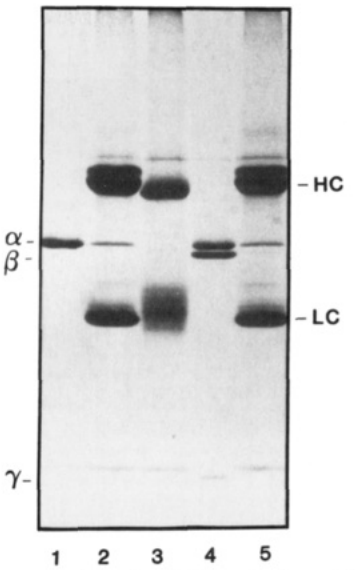

FIGURE 4: Immunoprecipitation of G_t and α_t by mAb 4A. Purified G_t (20 μg) or α_t–GTPγS (10 μg) was incubated in 100 μL of buffer containing 0.3% Lubrol PX in the presence or absence of antibodies for 1 h at room temperature. The molar ratio between antibodies and proteins was 2:1. Then 500 μL of 10% *Staphylococcus aureus* cell suspension was added to precipitate the immunocomplexes as described under Experimental Procedures. Precipitated proteins were separated by 12.5% SDS–polyacrylamide gel electrophoresis, Coomassie Blue stained, and scanned by densitometer. Control: α_t precipitated by 10% TCA (lane 1), α_t immunoprecipitated by mAb 4A (lane 2), and α_t + nonimmune rabbit IgG (lane 3). Control: G_t precipitated by 10% TCA (lane 4), G_t subunit immunoprecipitated by mAb 4A (lane 5).

when G_t was incubated with monoclonal antibody 4A in buffer without any detergent, both α_t and $\beta\gamma_t$ subunits were immunoprecipitated (panel A of Figure 5). Under these conditions, nonspecific precipitation of G_t occurs both in the presence of nonimmune rabbit IgG and in buffer alone (panel A of Figure 5). In the presence of detergent, monoclonal antibody 4A immunoprecipitates only α_t subunit, while the controls, nonimmune rabbit IgG or buffer (panel B of Figure 5), show no precipitation of G_t subunits. Other nonionic detergents like Nonidet P-40 and Triton X-100 or a zwitterionic detergent like CHAPS or a 1% (w/v) solution of BSA have also the same effect (data not shown). To test whether detergent can affect G_t subunit interactions, G_t migration in a 5–20% sucrose density gradient prepared in buffer containing 0.3% Lubrol PX was investigated. G_t migrates in this gradient as a monomer without showing any dissociation of subunits (data not shown). To investigate the time dependence of the antibody effect, we incubated G_t and mAb 4A together for various amounts of time before immunoprecipitation. There was no effect of varying the time of incubation on subunit interactions, suggesting that dissociation of α_t and $\beta\gamma_t$ by mAb 4A was a rapid effect of antibody binding (data not shown).

Since epitope mapping and synthetic peptide competition data (Deretic & Hamm, 1987; Hamm et al., 1988) suggested that mAb 4A binds to the carboxyl terminus of α_t, we investigated the effect of another anti-carboxyl-terminal antibody on subunit association. The antiserum 476 was raised against synthetic peptide α_t341–350 (Goldsmith et al., 1987) and therefore recognizes only the carboxyl-terminal region. This antiserum also immunoprecipitated α_t (panel B of Figure 5), leaving $\beta\gamma_t$ in the supernatant fraction, showing that an antibody directed against the carboxyl terminus of α_t can cause subunit dissociation of G_t.

Effect of mAb 4A on Pertussis Toxin ADP-Ribosylation of G_t and α_t–GDP. Previous results (Deretic & Hamm, 1987)

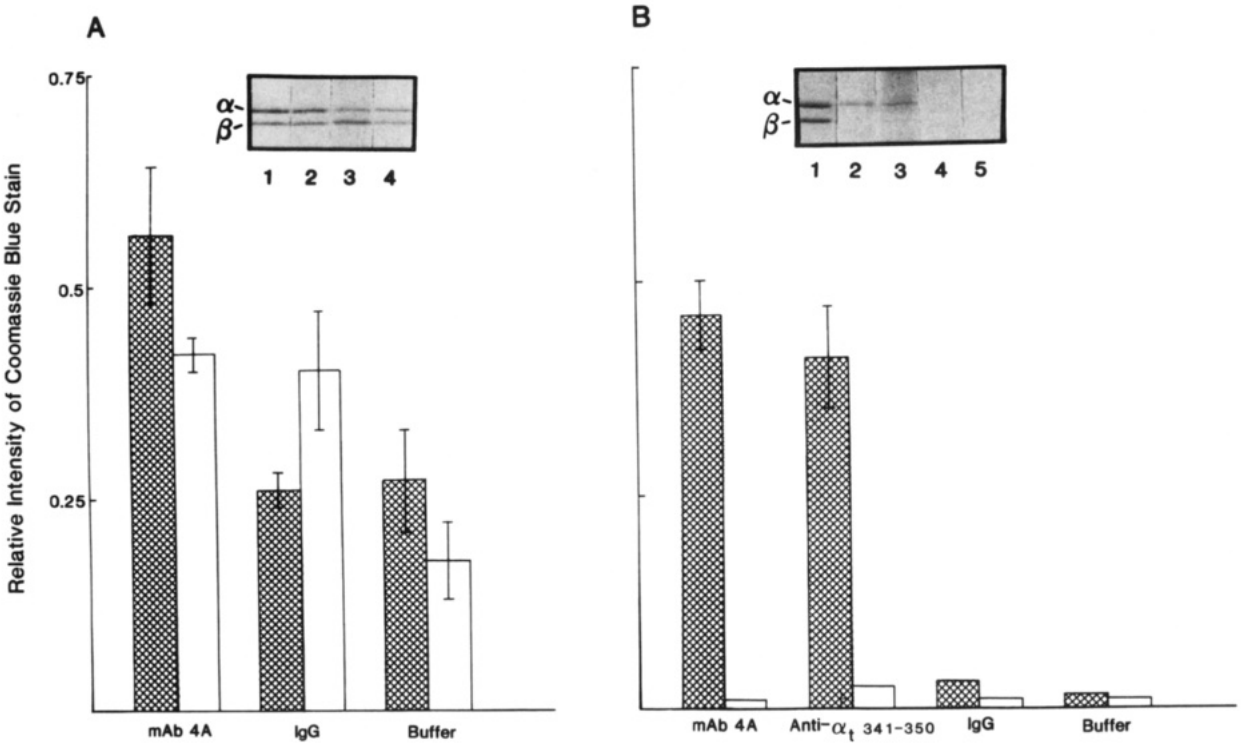

FIGURE 5: Immunoprecipitation of G_t subunits by mAb 4A and anti-peptide antibody (α_t341–350). Purified G_t was incubated either in buffer or in buffer containing 0.3% Lubrol PX with or without antibodies. Immune complexes were precipitated as described under Experimental Procedures. Precipitated proteins were separated by 12.5% SDS–polyacrylamide gel electrophoresis, Coomassie Blue stained, and scanned by densitometer. The relative intensity of Coomassie Blue stain of the α_t (cross-hatched bars) and β_t (open bars) bands is plotted. (A) Immunoprecipitation of G_t subunits in the absence of detergent. The results are the averages of three separate experiments ± SE. Inset: Coomassie Blue stained bands of immunoprecipitated G_t. Control (lane 1); G_t + mAb 4A (lane 2); G_t + IgG (lane 3); G_t + buffer (lane 4). (B) Immunoprecipitation of G_t subunits in the presence of 0.3% Lubrol PX. The results are the average of four separate experiments ± SE. Inset: Coomassie Blue stained bands of immunoprecipitated G_t. Control (lane 1); G_t + mAb 4A (lane 2); G_t + anti-peptide immune serum (lane 3); G_t + IgG (lane 4); G_t + buffer (lane 5).

suggested that mAb 4A blocks the pertussis toxin mediated ADP-ribosylation of G_t. To examine whether this effect is the consequence of antibody-induced subunit dissociation, the [^{32}P]ADP-ribosylation of G_t, α_t–GTPγS, and α_t–GDP was studied in the presence and absence of mAb 4A. The mAb 4A inhibition of G_t ADP-ribosylation was confirmed (lane 2 of Figure 6). Pertussis toxin can also ADP-ribosylate purified α_t subunits, although not as well, since the G_t complex is the preferred substrate for pertussis toxin (Watkins et al., 1985). The purified inactive α_t–GDP is a better substrate than the activated α_t–GTPγS (compare lanes 3 and 4 of Figure 6). This may be because it is this conformation that has a high affinity for $\beta\gamma_t$. Monoclonal antibody 4A also blocks the pertussis toxin ADP-ribosylation of α_t–GDP (lane 5 of Figure 6) and α_t–GTPγS (not shown).

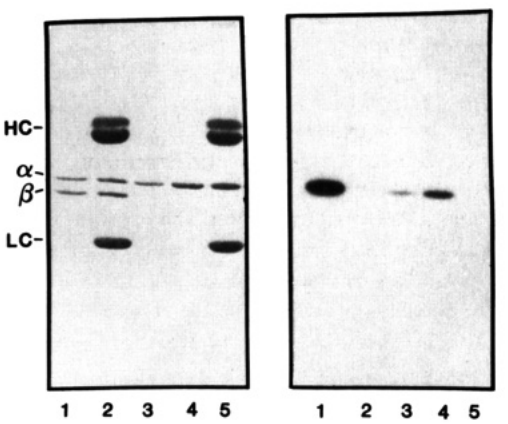

FIGURE 6: Monoclonal antibody 4A blocks pertussis toxin mediated ADP-ribosylation. Purified G_t (10 μg), α_t–GTPγS (6 μg), or α_t–GDP (10 μg) was preincubated for 30 min at room temperature in the presence and absence of mAb 4A (30 μg). Samples were then ADP-ribosylated as described under Experimental Procedures. The reaction was terminated by the addition of electrophoresis sample buffer. The samples were subjected to 12.5% SDS–polyacrylamide gel electrophoresis. The gel was stained with Coomassie Blue (left panel), dried, and subjected to autoradiography (right panel). G_t (lane 1); G_t + mAb 4A (lane 2); α_t–GTPγS (lane 3); α_t–GDP (lane 4); α_t–GDP + mAb 4A (lane 5).

Discussion

Monoclonal antibodies have been useful probes to study the structure and function of GTP-binding proteins. An especially well-studied antibody that recognizes the α subunit and has distinct functional consequences is mAb 4A. The epitope of mAb 4A is of current interest because the antibody blocks light activation of G_t, and it also cross-reacts with and functionally blocks G_s, G_i (Hamm et al., 1989), and G_k (Yatani et al., 1988). Epitope mapping of proteolytic fragments of G_t (Deretic & Hamm, 1987) indicated that Arg^{310}–Lys^{329} is the central part of the mAb 4A epitope. The antibody binds to native α_t with higher affinity than it binds to fragments, suggesting that antibody binding is conformation-dependent; thus, other regions of α_t may also be involved in antibody binding. Competition studies with synthetic peptides corresponding to various regions of α_t (Hamm et al., 1988) showed that peptide Asp^{311}–Val^{328} was the most potent blocker of G_t–mAb 4A interaction. Peptide competition studies also showed that the carboxyl-terminal 11 amino acids, Ile^{340}–Phe^{350}, also take part in the epitope. Synthetic peptides corresponding to the amino-terminal fragment, Asp^{8}–Ala^{23}, do not have any effect on mAb 4A binding in the ELISA assay

(Hamm et al., 1988), suggesting that the amino-terminal region is not a major part of the mAb 4A epitope.

The main purpose of this work was to study the effect of monoclonal antibody 4A on holo-G_t, using another approach under physiological conditions of α_t folding and subunit binding. The sedimentation coefficient which we obtained for G_t is close to those reported for G_o (Sternweis, 1986), G_i (Codina et al., 1984; Huff et al., 1985; Sternweis, 1986), G_s (Codina et al., 1984; Sternweis, 1986), and G_t (Wessling-Resnick & Johnson, 1987). All previous sedimentation studies of G proteins except G_t were carried out in the presence of detergents. Sternweis (1986) showed that G_s, G_i, and G_o aggregated extensively if detergent was not present in the gradient. The α subunits migrated as monomers even in the absence of detergent, while $\beta\gamma$ subunits aggregated very easily, suggesting that the $\beta\gamma$ subunits were responsible for this aggregation of G proteins. Photoreceptor $\beta\gamma_t$ was an exception to this rule, according to Sternweis (1986), and migrated in sucrose density gradients as a monodisperse peak either in the presence or in the absence of detergent. We found, in agreement with Sternweis, that $\beta\gamma_t$ migrated as a monomer but showed a slight concentration-dependent tendency to form oligomers in sucrose density gradients. This was also true for holo-G_t. This tendency to form G_t oligomers was reduced by pretreating the sample with a detergent (0.5% Lubrol PX). The α_t subunit migrated as a single peak, without formation of oligomers and at a slower sedimentation rate than $\beta\gamma_t$. Thus, it is likely that the interaction between the oligomers of G_t occurs through $\beta\gamma_t$ which may be important in anchoring the G_t complex to the ROS membranes.

When monoclonal antibody 4A was incubated with G_t before centrifugation in sucrose density gradients, holo-G_t dissociated and the subunits migrated as an α_t–monoclonal antibody complex and free $\beta\gamma_t$. The Fab fragments of mAb 4A also disrupted the binding between α_t and $\beta\gamma_t$ subunit, suggesting that the effect of the antibody on subunit interactions was not a consequence of bivalency or steric hindrance by the Fc region of the antibody. The effect of mAb 4A on subunit association was also confirmed by immunoprecipitation experiments. Only the α_t subunit was immunoprecipitated by mAb 4A when the experiments were performed in the presence of detergent. We also found that the anti-peptide immune serum, raised against the α_t synthetic peptide Lys^{341}–Phe^{350}, also causes subunit dissociation and immunoprecipitates α_t.

Several possible mechanisms can be postulated for the effect of an antibody binding to a protein antigen resulting in the dissociation of the protein complex.

(1) The carboxyl-terminal region of α_t is involved in the interaction with $\beta\gamma_t$. Recently, it has been suggested on the basis of cross-linking studies that the 5-kDa COOH terminus of α_t directly interacts with the amino-terminal region of the $\beta\gamma_t$ subunit (Hingorani et al., 1988).

(2) The epitope of mAb 4A is completely within the region Arg^{310}–Phe^{350} at the carboxyl terminus of α_t, but the binding of antibody to this region modifies the binding affinity between subunits. That this can occur was shown in the experiments where antiserum against a synthetic peptide corresponding to the carboxyl-terminal region Lys^{341}–Phe^{350} caused subunit dissociation and immunoprecipitated only the α_t subunit. Two distinct mechanisms could be responsible for such an effect. (a) An antibody-induced conformational change in α_t leading to a decreased affinity for $\beta\gamma_t$. The existence of conformational changes in a protein antigen after antibody binding have been clearly described for the neuraminidase–antibody complex (Colman et al., 1987). (b) Steric hindrance by the antibody of $\beta\gamma_t$ binding. It is known that the region of interaction between a protein antigen and an Fab fragment extends over a large area (Amit et al., 1986; Sheriff et al., 1987) and, therefore, some steric interference cannot be excluded.

(3) The region Arg^{310}–Phe^{350} is the center of the antigen–antibody-binding area, but surrounding amino acids, which comprise the $\beta\gamma_t$-binding site, also take part in the formation of the antigen site. Therefore, the interactions between both G_t and rhodopsin and the G_t subunits are directly inhibited by monoclonal antibody 4A. Recently, crystal structure determinations of complexes of monoclonal antibody combining regions (Fab fragments) with their protein antigens have been shown that the epitopes of all protein antigens studied to date are formed from amino acids sequences from different portions of the proteins (Amit et al., 1986; Sheriff et al., 1987; Colman et al., 1987). It was also shown that the surface of the antigen–antibody interaction is rather flat, and the region of complementarity extends over an area of about 750 $Å^2$ (Sheriff et al., 1987). Thus, when the antibodies are elicited by immunization with the native protein, it is likely that these antibodies recognize native conformational, or assembled, determinants. This is certainly true with all monoclonal antibodies raised against native G_t (Witt et al., 1984), which bind to the α_t subunit on Western blots at least 100-fold better than to its tryptic fragments (Deretic & Hamm, 1987). As pointed out by Deretic and Hamm (1987), epitope mapping studies can only identify the most important parts of the epitope of conformational antibodies. To determine other discontiguous segments of the polypeptide chain, alternative approaches must be used, such as synthetic peptide competition with G_t for antibody binding. In this way, another part of the epitope was elucidated, the carboxyl-terminal 11 amino acid segment, Ile^{340}–Phe^{350} (Hamm et al., 1988). This method can positively identify parts of the epitope, but a negative result cannot be used to disprove the participation of a particular region in the epitope, since the binding affinity of a peptide on the periphery of the epitope may not be sufficient to induce competition in the peptide assay. Such a weak interaction could conceivably weaken interaction of another protein binding in that area, however. The determination of the crystal structure of the antigen–Fab complex will be necessary to determine what other segments of the polypeptide chain are also part of the combining region.

To examine these options, it would be useful to know the binding site on α_t for the $\beta\gamma_t$ subunit. The amino-terminal region of α_t has been suggested as the binding domain for $\beta\gamma_t$ (Fung & Nash, 1983; Watkins et al., 1985). Navon and Fung (1987) showed by immunoprecipitation, using an anti-α_t monoclonal antibody, that removal of the amino-terminal region of α_t by *Staphylococcus aureus* V8 protease impairs $\beta\gamma_t$ binding, and the presence of $\beta\gamma_t$ slows down proteolysis. However, as recently pointed out by Neer et al. (1988), it is not yet clear whether the amino-terminal region of the α subunit interacts with $\beta\gamma$ subunit directly or whether its presence maintains some conformation of the α subunit that is required for heterotrimer formation. Another reason to question whether the amino terminus of α is the only site of $\beta\gamma$ interaction is that one might expect a conserved sequence in various α subunits that interact with the same $\beta\gamma$ subunits, and there is very litte conservation of sequences in the amino-terminal region (Jones & Reed, 1987).

There is some evidence that other portions of the α subunit take part in the $\beta\gamma$ interaction site. As mentioned above, Hingorani et al. (1988) studying the chemically cross-linked products of G_t subunits, have shown that the 5-kDa COOH-

terminus fragment of α_t directly interacts with β_t. Further, Kahn and Gilman (1984) have shown that cholera toxin mediated ADP-ribosylation of Arg^{201} of the α_s subunit changes its affinity for $\beta\gamma$, suggesting that this region might also take part in the $\beta\gamma$-binding site.

One possibility we feel is excluded by all the information is the one put forth by Navon and Fung (1988), that the amino terminus is the only mAb 4A epitope and the consequences of mAb 4A binding result only from G_t subunit dissociation. Several observations are inconsistent with this interpretation. (1) Binding of mAb 4A to holo-G_t, α_t, and neighbors of α_t. Yatani et al. (1988) showed that the antibody binds equally well to the α_t subunit alone and to α_t when $\beta\gamma_t$ is present. If mAb 4A recognized the $\beta\gamma_t$-binding site, the presence of $\beta\gamma_t$ should block mAb 4A access. By contrast, binding of rhodopsin and mAb 4A are mutually exclusive. Navon and Fung (1988) showed that mAb 4A does not bind to α_t when it is bound to rhodopsin, suggesting that the mAb 4A epitope is covered by rhodopsin. Conversely, in the presence of mAb 4A, binding to rhodopsin is blocked (Hamm et al., 1987). (2) Pertussis toxin ADP-ribosylation of Cys^{347} of α_t is blocked by mAb 4A both in the presence (Figure 6; Hamm et al., 1987) and in the absence (Figure 6) of the $\beta\gamma_t$ subunit. Thus, the antibody effect cannot be simply to block the known enhancement of ADP-ribosylation by $\beta\gamma_t$ (Watkins et al., 1985).

We have shown that an anti-peptide antibody that recognizes the carboxyl-terminal 10 amino acids of α_t can cause subunit dissociation (Figure 5). This could be the consequence of conformational modification of α_t upon antibody binding to the carboxyl-terminal region decreasing its affinity for $\beta\gamma_t$. Alternatively, the amino and carboxyl termini of α_t could be spatially close. In fact, three-dimensional models of the α_t molecule based on its structural homology to other GTP-binding proteins (Deretic & Hamm, 1987; Hingorani & Ho, 1987) reflect this and suggest that these regions are brought relatively close together by the native folding of the protein.

Because of this functional association between the amino and carboxyl termini, it has been experimentally difficult to untangle the effects involved in receptor binding and subunit interactions in terms of structural correlates on the carboxyl and amino termini of the molecule. Further structural studies clarifying this interaction may yield important insights into the dynamics of the molecule during its activation sequence, which is at the heart of the high amplification of the visual signal.

Acknowledgments

We thank Dr. Justine Malinski for purification of α_t-GDP and helpful discussions and Dusanka Deretic for helpful discussions and advice. We are grateful to Dr. Svetlana Salov for technical assistance. We acknowledge the gift of anti-peptide antiserum against $\alpha_t 341-350$ from Dr. A. M. Spiegel.

References

Amit, A. G., Mariuzza, R. A., Phillips, S. E. V., & Poljak, R. J. (1986) *Science 233*, 747–753.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Codina, J., Hildebrandt, J. D., Sekura, R. D., Birnbaumer, M., Bryan, J., Manclark, C. R., Iyengar, R., & Birnbaumer, L. (1984) *J. Biol. Chem. 259*, 5871–5886.

Colman, P. M., Laver, W. G., Varghese, J. N., Baker, A. T., Tulloch, P. A., Air, G. M., & Webster, R. G. (1987) *Nature 236*, 358–363.

Deretic, D., & Hamm, H. E. (1987) *J. Biol. Chem. 262*, 10831–10847.

Fung, B. K.-K. (1983) *J. Biol. Chem. 258*, 10495–10502.

Fung, B. K.-K., & Nash, C. R. (1983) *J. Biol. Chem. 258*, 10503–10510.

Gilman, A. G. (1987) *Annu. Rev. Biochem. 56*, 615–649.

Goldsmith, P., Gierschik, P., Milligan, G., Unson, C., Vinitsky, R., Malech, H. L., & Spiegel, A. M. (1987) *J. Biol. Chem. 262*, 14683–14688.

Hamm, H. E., & Bownds, M. (1984) *J. Gen. Physiol. 84*, 265–280.

Hamm, H. E., & Deretic, D., Hofmann, K. P., Schleicher, A., & Kohl, B. (1987) *J. Biol. Chem. 262*, 10831–10838.

Hamm, H. E., Deretic, D., Arendt, A., Hargrave, P. A., Koening, B., & Hofmann, K. P. (1988) *Science 241*, 832–835.

Hamm, H. E., Deretic, D., Mazzoni, M. R., Moore, C. A., Takahashi, J. S., & Rasenick, M. M. (1989) *J. Biol. Chem. 264*, 11475–11482.

Hingorani, V. N., & Ho, Y.-K. (1987) *FEBS Lett. 220*, 15–22.

Hingorani, V. N., Tobias, D. T., Henderson, J. T., & Ho, Y.-K. (1988) *J. Biol. Chem. 263*, 6916–6926.

Huff, R. M., Axton, J. M., & Neer, E. J. (1985) *J. Biol. Chem. 260*, 10864–10871.

Hurley, J. B. (1987) *Annu. Rev. Physiol. 49*, 793–812.

Jones, D. T., & Reed, R. R. (1987) *J. Biol. Chem. 262*, 14241–14249.

Kahn, R. A., & Gilman, A. G. (1984) *J. Biol. Chem. 259*, 6235–6240.

Kleuss, C., Pallat, M., Brendel, S., Rosenthal, W., & Schultz, G. (1987) *J. Chromatogr. 407*, 281–289.

Kühn, H. (1980) *Nature 283*, 587–589.

Laemmli, U. K. (1970) *Nature 227*, 680–685.

Liebman, P. A., Parker, K. R., & Dratz, E. A. (1987) *Annu. Rev. Physiol. 49*, 765–791.

Navon, S. E., & Fung, B. K.-K. (1987) *J. Biol. Chem. 262*, 15746–15751.

Navon, S. E., & Fung, B. K.-K. (1988) *J. Biol. Chem. 263*, 489–496.

Neer, E. J., Pulsifer, L., & Wolf, L. G. (1988) *J. Biol. Chem. 263*, 8996–9000.

Papermaster, D. S., & Dreyer, W. J. (1974) *Biochemistry 13*, 2438–2444.

Sheriff, S., Silverton, E. W., Padlan, E. A., Cohen, G. H., Smith-Gill, S. J., Finzel, B. C., & Davies, D. R. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8075–8079.

Sternweis, P. C. (1986) *J. Biol. Chem. 261*, 631–637.

Stryer, L., & Bourne, H. R. (1986) *Annu. Rev. Cell Biol. 2*, 319–419.

Watkins, P. A., Burns, D. L., Kanaho, Y., Liu, T.-Y., Hewelett, H. L., & Moss, J. (1985) *J. Biol. Chem. 260*, 13478–13482.

Wessling-Resnick, M., & Johnson, G. L. (1987) *Biochemistry 26*, 4316–4323.

Wheeler, G. L., & Bitensky, M. W. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4238–4242.

Witt, P. L., Hamm, H. E., & Bownds, M. D. (1984) *J. Gen. Physiol. 84*, 251–263.

Yamazaki, A., Tatsumi, M., & Bitensky, M. W. (1988) *Methods Enzymol. 159*, 702–710.

Yatani, A., Hamm, H., Codina, J., Mazzoni, M. R. Birnbaumer, L., & Brown A. M. (1988) *Science 241*, 828–831.