Unique Guanine Nucleotide Binding Properties of the Human Placental GTP-Binding Protein G_p[†]

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ABSTRACT: G_p is a major GTP-binding protein of human placenta and platelets [Evans, T., Brown, M. L., Fraser, E. D., & Northup, J. K. (1986) *J. Biol. Chem. 261*, 7052–7059]. High-affinity guanine nucleotide binding is associated with a polypeptide migrating identically with H-ras on SDS–PAGE. We have characterized the interactions of preparations of purified human placental G_p with guanine nucleotides in detergent solution. Equilibrium binding studies with [^{35}S]GTPγS, [^{3}H]Gpp(NH)p, and [^{3}H]GTP identified a single class of sites with a dissociation constant of 10 ± 1, 153 ± 61, and 125 ± 77 nM for the ligands, respectively. These three ligands were mutually competitive with K_i values consistent with the K_d values from direct binding experiments. Competition for the binding of [^{3}H]Gpp(NH)p was used to determine the specificity of the site. K_i values determined from this assay were 14 nM for GTPγS, 143 nM for Gpp(NH)p, 3.3 μM for GDPβS, 69 nM for GTP, and 64 nM for GDP. ATP, ADP, cAMP, cGMP, and NAD^+ had no detectable affinity for this site. While the equilibrium binding data fit well to a single class of sites, association kinetics of these ligands were better fit to two rate constants. Dissociation kinetics, however, were not clearly resolved into two rates. All binding reaction rates were regulated by magnesium ion. In the absence of added magnesium and in the presence of EDTA, no association of the ligands was detected, and the dissociation of bound ligands was facilitated by addition of EDTA. Binding appeared to require only micromolar concentrations of magnesium, and at these concentrations, dissociation of bound ligand was markedly retarded. The binding of [^{3}H]GTP to G_p in the presence of magnesium lead to hydrolysis of the γ-phosphoryl group, with GDP remaining tightly associated with the site. Incubation of G_p with GTP lead to hydrolysis of GTP and the formation of GDP-bound G_p. As opposed to untreated G_p, the GN-binding properties of GDP–G_p were found to be rate-limited by GDP dissociation. Guanine nucleotides stabilized the binding site against thermal inactivation. GTPγS and Gpp(NH)p were most effective, and GDP was partially effective, while aluminum fluoride with or without GDP had no effect. Magnesium was required for the stabilization by guanine nucleotides. These data identify a metal-dependent GTP-specific binding site on G_p and describe a GTP-binding site distinct from that reported for other G-proteins or ras-related GTP-binding proteins.

There is a growing body of evidence for a superfamily of structurally related GTP-binding proteins involved in the regulation of cellular functions (Stryer & Bourne, 1986). This includes the elongation factors (eIF2, EF-TU) regulating protein synthesis (Kaziro, 1978), signal-transducing G-proteins[1] (Gilman, 1987; Casey & Gilman, 1988; Levitzki, 1988), and ras and ras-like proteins (Barbacid, 1987). Members of this superfamily share a highly homologous structure encoding a GTP-specific binding domain and a characteristic GTPase activity (Lochrie & Simon, 1988). For elongation factors and G-proteins, GTPase activity controls the regulatory activity of the GTP-binding protein. Binding of GTP is limited by the dissociation of the hydrolytic product, GDP. Release of GDP is in turn controlled by the interaction of other protein components of the regulatory pathways (TS for EF-TU; activated receptor for signal-transducing G-proteins). All signal-transducing G-proteins which have been isolated share a common structure of α, β, and γ subunits, where the GTP-binding α subunit is unique for each G-protein oligomer (Gilman, 1987). While multiple forms of the β and γ subunits have been identified, the β and γ subunits isolated with distinct G-proteins from a single cell type appear to be identical (Evans et al., 1987; Manning & Gilman, 1983). The release of the GTP-bound α subunit from the G-protein oligomer is thought to initiate the activation of the regulated pathway. Reversal of activation is associated with hydrolysis of GTP and reassociation of the βγ complex with the GDP-bound α.

Isolation strategies similar to those designed for signal-transucing G-proteins first identified an abundant 21-kDa protein from human placenta (Evans et al., 1986), and was named G_p for its placental origin. This protein was subsequently isolated from bovine brain (Waldo et al., 1987), rabbit atria,[2] and human platelet (Polakis et al., 1989a). Modified

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[1] Abbreviations: G-protein, a member of the family of heterotrimeric GTP-binding proteins that serves as an intermediary in transmembrane signaling processes; the family consists of G_s and G_i that regulate adenylyl cyclase activity, G_t or transducin that couples photolyzed rhodopsin to activation of cGMP phosphodiesterase in the vertebrate retinal rods and cones, and G_o, the most abundant G-protein in brain; G_p, predominant GTP-binding protein identified in extracts of human placental membranes with an approximate mass of 21 kDa; DTT, dithiothreitol; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); GDPβS, guanosine 5′-*O*-(2-thiodiphosphate); Gpp(NH)p, guanosine 5′-(β,γ-imidotriphosphate); PAGE, polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; PEI, poly(ethylenimine).

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strategies for purification resulted in the isolation of a number of low molecular weight GTP-binding polypeptides from bovine brain (Kikuchi et al., 1988). The identified members of this set of proteins include the rho (Yamamoto et al., 1988), c-Ki-ras (Yamashita et al., 1988), rap-1 (Pizon et al., 1988), and rab-3 (Matsui et al., 1988) gene products. A similar set of low molecular weight GTP-binding proteins has also been isolated from platelets, including c-ras (Ohmori et al., 1989), ral, and rac-1 (Polakis et al., 1989b) gene products.

Interest in low molecular weight GTP-binding proteins stems largely from the transforming activity of the ras gene products. However, despite considerable cell biological interest and structural information, the biochemical control and the functions for this family of low molecular weight GTP-binding proteins remain obscure. Most of the research along these lines has adopted the model based upon the biochemical regulation of the signal-transducing G-proteins. We wished to determine the extent of biochemical homology between the oligomeric G-proteins and the low molecular weight GTP-binding proteins. As we had isolated the so-called "G_p" protein together with the $\beta\gamma$ subunit complex affiliated with G-proteins both from human placenta (Evans et al., 1986) and from bovine brain membranes (Waldo et al., 1987), but not from human platelet membranes (Evans et al., 1986), it was of interest to determine the similarity of biochemical properties of this small GTP-binding protein as compared with other G-proteins. We report herein on guanine nucleotide binding properties for the isolated human placental protein.

Materials and Methods

Materials

Radiolabeled [^{35}S]GTPγS (1200 Ci/mmol) was obtained from New England Nuclear; [^{3}H]Gpp(NH)p (12 Ci/mmol) and [^{3}H]GTP (19 Ci/mmol) were obtained from ICN Biomedicals, Inc. Lithium salts of GTPγS, Gpp(NH)p, and GDPβS were obtained from Boehringer Mannheim. Sodium salts of GTP, GDP, and GMP were purchased from Sigma Chemical Co. All other materials were of the highest grade commercially available.

Methods

Purification of Human Placental G_p. GTP-binding proteins were isolated from membranes prepared from full-term human placentas as described previously (Evans et al., 1986). Briefly, a microsomal membrane fraction from about 10–12 placentas was extracted in a solution of 1% (w/v) cholate at about 10 mg/mL membrane protein. The solubilized protein supernatant was then subjected to sequential DEAE, Ultrogel AcA-34, heptylamine–Sepharose, and hydroxyapatite chromatography in cholate solution. Migration of G_p was identified by [^{35}S]GTPγS binding (Northup et al., 1982); G_s and G_i were identified by adenylate cyclase activation and pertussis toxin catalyzed ADP-ribosylation, respectively, as described previously (Evans et al., 1986). The fractions of G_p for the present studies were either used as the hydroxyapatite pool or subjected to further chromagraphy on Ultrogel AcA-44 in 20 mM Tris, pH 8.0, 1. mM EDTA, 1 mM DTT, 100 mM NaCl, and 0.8% (w/v) sodium cholate.

Guanine Nucleotide Binding Assays. Guanine nucleotide binding to the isolated G_p was determined by using minor modifications of the nitrocellulose filtration method described by Northup et al. (1982). The standard assay was performed in a volume of 100 μL containing 50 mM Na-HEPES (pH 8.0), 1 mM EDTA, 1 mM DTT, 200 mM NaCl, 2 mM $MgCl_2$, 0.1% Lubrol 12A9, and 1 mg/mL BSA (solution A). G_p was added to this reaction solution at a concentration of 20–200 nM binding sites, and the reaction contained 1 μM radiolabeled guanine nucleotide at a radiospecific activity of 8000–46 000 cpm/pmol. Samples were incubated in silanized glass test tubes at 37 °C for 60 min. Three 25-μL aliquots were removed and diluted with 2 mL of ice-cold TNMg solution (25 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 20 mM $MgCl_2$) followed by rapid filtration through 0.45-μm nitrocellulose filters (Schleicher & Shuell B85 or Millipore HAWP). Filters were washed 5 times with 2 mL of ice-cold TNMg, dried for 20 min with a 250-W infrared lamp (GE250R40/1), and counted with 10 mL of toluene containing 0.5% 2,5-diphenyloxazole in a Trachor 6895 liquid scintillation spectrometer.

As an alternative method to quantitate bound guanine nucleotide, G_p incubated with radiolabeled nucleotides, as described above, was removed from the 37 °C bath, placed on ice, and passed over Sephadex G-25 as described below. Replicate samples of the fractions from this chromatography were counted directly or subjected to nitrocellulose filtration. For [^{35}S]GTPγS, the nitrocellulose filtration value was consistently >90% of the value determined by Sephadex G-25. For [^{3}H]GTP, this value was 30–50% of that determined by Sephadex G-25. Binding values were not corrected for the apparent loss on nitrocellulose filters, and thus are likely to underestimate true maximal binding.

In the analysis of molar binding of guanine nucleotides to G_p, a value of 56 000 Da and protein concentrations determined by Amido Black (Schaffner & Weissmann, 1973) with BSA as a standard were used. The binding data were analyzed by using the programs "LIGAND" of Munson and Rodbard (1980) and the programs "KINETIC" and "EBDA" described by McPherson (1985).

Identification of Bound Nucleotide. The identity of bound guanine nucleotide was determined by resolution of protein-associated ligand from unbound ligand using Sephadex G-25. After incubation of G_p with radiolabeled guanine nucleotide in solution A at 37 °C, the reaction was chilled on ice, and 100 μL of the reaction was applied to 1 mL of Sephadex G-25 in a 7-mm-diameter Bio-Rad Econo column. The column was equilibrated and eluted with solution A, and fractions of 100 μL were collected. Samples of 5 μL were pipetted onto nitrocellulose membranes, dried, and counted with 10 mL of cocktail to determine the resolution profile. Fractions of 75 μL of the bound peak were incubated with 2 mL of absolute ethanol and then dried under a stream of nitrogen in a cold room. Radioactivity was redissolved in 10 μL of 50% ethanol. Nucleotides were separated on Cellulose 300 PEI (Brinkmann) developed with 0.5 M LiCl and 2.0 M formic acid. Unlabeled GTP, GDP, and GMP were added as internal standards and were visualized with a UV lamp (Mineralight UVG-54). Samples of the unbound ligand were also subjected to PEI–cellulose chromatography, but without ethanol precipitation.

Except where indicated, experiments were performed at least 3 times with similar results. The data presented either are representative of quantitatively similar experiments or are the average of values obtained in replicate experiments.

Results

Equilibrium Binding Experiments. Guanine nucleotide binding to G_p was studied by using radiolabeled GTPγS, Gpp(NH)p, and GTP. For each nucleotide, the binding reached apparent equilibrium within 2 h at 37 °C. Figure 1

[2] A. Tamir, H. Tamir, G. L. Peterson, M. R. Tota, M. S. Shimerlik, and J. K. Northup, unpublished observations.

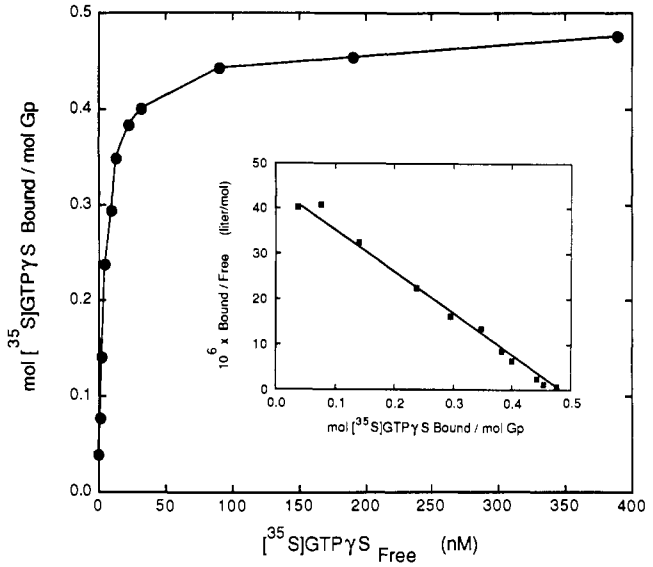

FIGURE 1: Concentration dependence of [^{35}S]GTPγS binding to G_p. Nine samples of 20 nM G_p were incubated with the indicated concentration of GTPγS from 2.5 nM to 0.4 μM (specific activity 46000 cpm/pmol) in a reaction volume of 100 μL at 37 °C. After 60 min, three aliquots of 25 μL were removed from each sample for determination of bound GTPγS as described under Methods. The data are plotted as bound versus free nucleotide, and the curve drawn is the best fit for a single binding site using the program "LIGAND". Constants derived from this fit are K_d = 10 nM and B_{max} = 0.47 mol/mol of G_p (r = −0.99). The inset is the Scatchard plot of these data with the linear regression line (r = −0.99).

Table I: Equilibrium Binding Constants for Guanine Nucleotides[a]

ligand	K_d (nM)	B_{max}[b] (mol/mol of G_p)
[^{35}S]GTPγS (n = 3)	10 ± 1	0.48 ± 0.01
[^{3}H]Gpp(NH)p (n = 4)	153 ± 61	0.47 ± 0.04
[^{3}H]GTP (n = 2)	125 ± 77	0.34 ± 0.01

[a] Saturation isotherms for [^{35}S]GTPγS, [^{3}H]GPP(NH)p, and [^{3}H]-GTP were determined at 37 °C as described in Figures 1 and 2. Scatchard analyses of the binding data were used to derive the reported constants. Values are the mean ± SEM for the indicated number of determinations. [b] Mole fraction of bound was calculated by using an assumed molecular weight of 56000 for human placental G_p.

presents a representative experiment of the binding of GTPγS to G_p at 37 °C. The inset in this figure is the Scatchard (1949) plot of these data, showing a single class of binding sites with a K_d of 10 nM and a B_{max} of 0.47 mol/mol in this experiment. Similarly, the binding of Gpp(NH)p and GTP to G_p displayed a single class of sites, as presented in Figure 2A,B. These two ligands also identified a single class of binding sites with K_d values of 225 and 70 nM at a site density of 0.45 and 0.35 mol/mol for Gpp(NH)p and GTP, respectively, as determined from the data in these experiments. Table I presents a summary of all Scatchard analyses of the binding of GTPγS, Gpp(NH)p, and GTP. In all experiments, these ligands revealed a single class of binding sites with a binding stoichiometry of 0.3–0.5 mol/mol protein. Binding with these ligands also occurred on ice with K_d values similar to those obtained at 37 °C. However, the density of binding sites were reduced to about half of that observed at a more elevated temperature (data not shown).

Figure 3 presents the results of competition experiments for the ligand Gpp(NH)p and various nucleotides. Computer analyses of these curves gave a best fit to a single-site model, indicating that the nucleotides all competed for the same class of binding site. Table II presents the IC_{50} and derived K_i values obtained from the data presented in Figure 3. These values

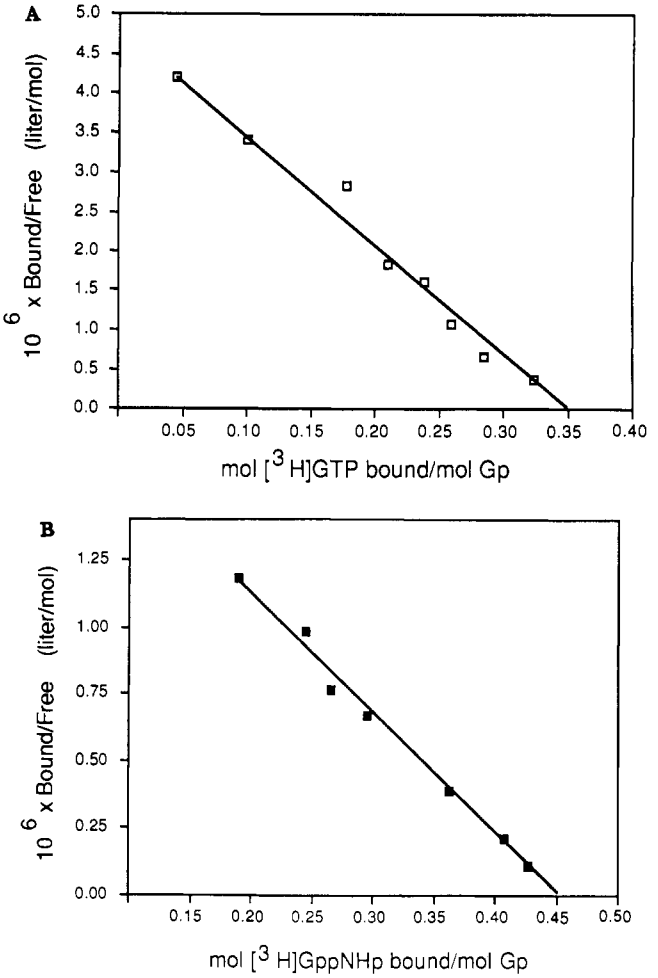

FIGURE 2: Binding of [^{3}H]GTP and [^{3}H]Gpp(NH)p to G_p. The binding of [^{3}H]GTP (A) or [^{3}H]Gpp(NH)p (B) to G_p was determined as described for GTPγS in Figure 1, except the concentration of G_p was 200 nM and the nucleotide concentrations were from 0.02 to 1.0 μM for [^{3}H]GTP (specific activity 8400 cpm/pmol) and 0.2–4.0 μM for [^{3}H]Gpp(NH)p (specific activity 5300 cpm/pmol). Data for both ligands were best fit by a single-site model using the program "LIGAND" with the following constants derived: [^{3}H]GTP, K_d = 70 nM, B_{max} = 0.35 mol/mol of G_p (r = −0.99); Gpp(NH)p, K_d = 225 nM and B_{max} = 0.45 mol/mol of G_p (r = −0.99). The data are presented using the Scatchard (1949) transformation.

Table II: Competition with [^{3}H]Gpp(NH)p by Guanine Nucleotides[a]

guanine nucleotide	IC_{50} (nM)	K_i (nM)
Gpp(NH)p	1080 ± 240	143 ± 6
GTPγS	90 ± 9	14 ± 1
GDPβS	23700 ± 1700	3250 ± 240
GTP	520 ± 63	69 ± 8
GDP	460 ± 54	64 ± 8

[a] Data presented in Figure 3 were analyzed by using the programs "EBDA" and "LIGAND" which derived the values of the concentration of guanine nucleotide displacing 50% of [^{3}H]Gpp(NH)p binding (IC_{50}). K_i values were calculated by using a modified form of the formula of Cheng and Prusoff (1973): $K_i = IC_{50}K_d/L + K_d$. The dissociation constant for [^{3}H]Gpp(NH)p used for this calculation was determined independently: K_d = 153 ± 61 nM (mean ± SEM, n = 4). The concentration of [^{3}H]Gpp(NH)p used was 1 μM. The data presented were also used to generate Hofstee plots; the best fits were linear. Less than 10% displacement was observed with 1 mM samples of the following nucleotides: AMP, ADP, ATP, GMP, cGMP, and cAMP.

are nearly identical with directly determined K_d values for GTPγS, Gpp(NH)p, and GTP (Table I). Similar to the GTP-binding site of other GTP-binding proteins, GTPγS has considerably higher affinity than the physiological ligand, with GDP and Gpp(NH)p nearly equal to GTP in affinity. The

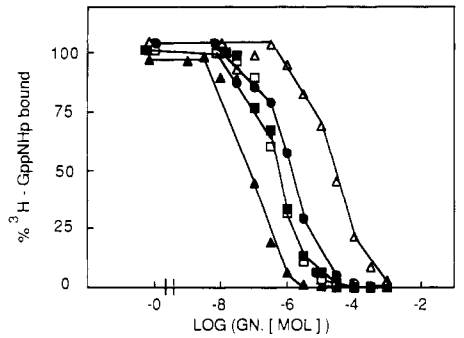

FIGURE 3: Competition for [^{3}H]Gpp(NH)p binding by guanine nucleotides. The binding of 1 μM [^{3}H]Gpp(NH)p to 100 nM G_p was determined in the absence and presence of the indicated concentrations of Gpp(NH)p (●), GTPγS (▲), GDPβS (△), GTP (■), and GDP (□) as described in Figure 1. The data presented are the average of values obtained in three independent experiments, and these have been normalized to the control value for the binding of [^{3}H]GPP(NH)p in the absence of competing ligand which was 0.36 ± 0.036 mol/mol of G_p. The lines are the best-fit curves for a one-site model derived using the program "EBDA". The values derived from these curves are presented in Table II.

GDP analogue GDPβS is a notable exception in that it has considerably reduced affinity for this site compared to the physiological analogue GDP. This preparation of GDPβS was analyzed on PEI–cellulose and tested as an inhibitor of Gpp(NH)p activation of brain adenylate cyclase to rule out degradation (data not shown). Guanosine monophosphate, adenine nucleotides, cyclic nucleotides, and all other tested ligands had no demonstrable affinity for this site, as determined by competition for Gpp(NH)p binding.

Magnesium Dependence. Binding of GTP, Gpp(NH)p, and GTPγS to G_p was dependent on magnesium. In the presence of 1 mM EDTA, there was no detectable binding of these ligands to G_p at 37 °C in 60 min (data not shown). In the absence of added EDTA, however, maximal binding of GTPγS was observed. This was in part due to magnesium contaminating Tris, NaCl, and G_p preparations. In order to determine the magnesium dependence of the binding reaction, EDTA was added at a concentration which half-maximally decreased the binding of GTPγS (10 μM EDTA). Maximal binding of GTPγS was restored when magnesium was added in addition to the 10 μM EDTA. Figure 4 presents the concentration dependence for magnesium restoration of the binding of 1 μM GTPγS to 100 nM G_p. An apparent half-maximal concentration for magnesium of 0.8 μM was calculated from this experiment. Magnesium dependence for Gpp(NH)p- and GTP-binding reactions was similar to that observed for GTPγS (data not shown).

If EDTA was added in excess of magnesium after establishing equilibrium binding, GDP and Gpp(NH)p rapidly dissociated from G_p. The rate of dissociation of GDP from G_p was accelerated from 0.12 to 1.4 min^{-1} (data not shown). It was not possible to determine any effects of magnesium on association rates, as no binding was detected either by Sephadex G-25 or by rapid filtration in the absence of magnesium.

Hydrolysis of GTP by G_p. All characterized G-proteins and ras-like proteins display GTPase activity. Hence, we investigated the GTPase activity of human placental G_p. To assess this activity, we measured the metabolism of [^{3}H]GTP by preparations of G_p. Subsequent to a binding reaction of G_p with [^{3}H]GTP, the reaction was subjected to chromatography on Sephadex G-25 to resolve bound from free nucleotide. Bound ligand was released by ethanol precipitation of the G_p, and both bound and free peaks of the nucleotide were analyzed by PEI–cellulose TLC for ^{3}H-containing nucleotide. Table III presents the results of experiments examining the nucleotide composition of G_p-bound and free nucleotide under various incubation conditions. With all concentrations of G_p examined, from 1 to 4 h of incubation, the bound ligand was about 90% GDP. At initial concentrations of 1 μM GTP and 0.2 μM G_p, in 60 min at 37 °C 39% of the free ligand was identified as GDP. Following 4-h incubation of 2.5 μM G_p with 3 μM GTP at 37 °C, 87% of the free nucleotide was identified as GDP. Thus, it appears that G_p hydrolyzes GTP, and GDP remains associated with the site. For the determination of GTPase activity, similar binding experiments were performed using 4 μm [^{3}H]GTP and 2 μM G_p, and reactions were carried out for 60 min at 37 °C. The relative amounts of guanine nucleotides were determined by using two fractions from each

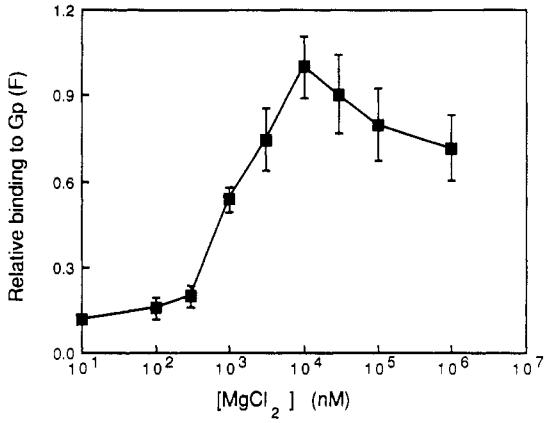

FIGURE 4: Magnesium dependence of GTPγS binding to G_p. The binding of 1 μM [^{35}S]GTPγS to 100 nM G_p was performed in 20 mM Tris-HCl (Bio-Rad electrophoresis grade, pH 8.0) with 10 μM EDTA and the indicated concentrations of magnesium chloride, as described under Methods. The data are presented as the fraction of maximal binding increase in the presence of added magnesium: $F = (B_{Mg} - B_0)/(B_{max} - B_0)$, where B_{Mg} is the binding in the presence of the indicated concentration of magnesium, B_0 is the binding in the absence of added magnesium (0.28 mol/mol of G_p), and B_{max} is the binding in the presence of 10 μM added magnesium (0.64 mol/mol of G_p). The data are mean ± SEM of values obtained in three independent experiments. The apparent half-maximal magnesium concentration obtained in these experiments was approximately 0.8 μM.

Table III: Is G_p a GTPase?[a]

condition	nucleotides (% of total)[b] bound peak GTP	bound peak GDP	free peak GTP	free peak GDP
[^{3}H]GTP			92	1
2.5 μM [^{3}H]GTP (4 h)			93	2
2.5 μM [^{3}H]GTP and 2.5 μM G_p (4 h)	2	93	7	87
1 μM [^{3}H]GTP and 0.2 μM G_p (1 h)	7	87	54	39
2 μM [^{3}H]GTP, 2 μM G_p, and 1 mM ATP (1 h)	4	92	ND[c]	ND

[a] Binding of [^{3}H]GTP to G_p was performed for the indicated times at 37 °C as described under Methods. At the end of the binding reactions, 100-μL aliquots of reaction mixtures were passed over a 1-mL Sephadex G-25 column. Fractions of bound guanine nucleotide or of the free guanine nucleotide were analyzed by using PEI-300 TLC plates as described under Materials and Methods. For determination of GTPase activity, similar binding experiments of 4 μM [^{3}H]GTP to 2 μM G_p were performed for 60 min at 37 °C. Each experiment was performed 3 times. The relative amounts of guanine nucleotides were determined by using two fractions from each experiment representing the unbound guanine nucleotide. The amount of [^{3}H]GDP detected was 70 ± 2%. The hydrolytic rate of GTP was calculated by using the value 0.51 mol/mol of G_p (determined by [^{35}S]GTPγS binding) for correction of G_p concentration. The hydrolysis rate thus obtained was 0.050 ± 0.001 min^{-1} (±SD, n = 6). [b] GMP remained constant in all experiments at 2–6% of total. [c] Not determined.

Table IV: GDP Exchange by GTPγS[a]

incubation of G_p	relative GN bound to G_p	
	[^{35}S]GTPγS	[^{3}H]GDP
60 min at 37 °C	1.000	0.002
60 min on ice	0.054	1.030
48 h on ice	0.910	0.015

[a] G_p (2 μM) was incubated with 2 μM [^{3}H]GTP for 60 min at 37 °C. At the end of this incubation, the reaction mixture was separated by using a Sephadex G-25 column. GDP-bound G_p eluted from this column was used for binding of 1 μM [^{35}S]GTPγS or for dissociation of [^{3}H]GDP in the presence of 100 μM GTPγS. The GTPγS-binding data are presented relative to a control value obtained at 37 °C; this was 0.22 nmol/mL G_p. [^{3}H]GDP dissociation is the fraction of the initial GDP bound G_p; this was 0.13 nmol/mL. All measurements were performed in triplicate, and the SD of measurements were <10% of mean values.

experiment representing the unbound guanine nucleotide. The amount of [^{3}H]GDP detected was 70 ± 2% of total. The hydrolytic rate of GTP was calculated by using the value 0.51 mol/mol of G_p (determined by [^{35}S]GTPγS binding) for correction of G_p concentration. The hydrolysis rate thus obtained was 0.050 ± 0.001 min^{-1} (mean ± SD, n = 6). Because of the low rate of dissocation of GDP (see below), it is likely that this measurement underestimates the k_{cat} and that the turnover for hydrolysis is rate-limited by the dissociation of GDP.

To exclude the possibility that the GTPase activity was contributed by an undetected contaminating nucleotidase, 2 μM G_p was incubated with 2 μM GTP in the presence of 1 mM ATP for 60 min. The bound ligand was still determined to be GDP (see Table III). Thus, the GTP hydrolysis occurred at a guanine nucleotide specific site.

Exchange of Bound GDP. One of the characteristic features of GTP-binding regulatory proteins is that guanine nucleotide binding is limited by the dissociation of tightly bound GDP. Since G_p was found to hydrolyze GTP with GDP remaining associated with the site, we examined the kinetics of GDP dissociation and labeled guanine nucleotide association to G_p. G_p was first incubated with [^{3}H]GTP to label the sites, and dissociation of the resultant bound [^{3}H]GDP was examined. Their was no detected dissociation of bound GDP at 60 min on ice, while [^{3}H]Gpp(NH)p binding was observed for the untreated control under this condition. At 37 °C, GDP was released by incubation with 10 mM EDTA or 100 μM Gpp(NH)p for 60 min (data now shown). This finding indicated that, as for other GTP-binding proteins, release of bound GDP presents an activation energy barrier to the binding of guanine nucleotides. This conjecture was tested by the experiment in Table IV. GDP-bound G_p was prepared as described above, unbound nucleotide was removed by filtration over Sephadex G-25, and then the preparation was incubated with 1 μM [^{35}S]GTPγS to determine nucleotide exchange or with 100 μM GTPγS to determine [^{3}H]GDP dissociation. First, in the presence of 100 μM GTPγS, >99% of the [^{3}H]GDP dissociated in 60 min at 37 °C, while there was no detectable dissociation following 60 min on ice. Concomitant with the dissociation of GDP, [^{35}S]GTPγS bound to G_p at 37 °C in 60 min, while only 5% of the sites bound [^{35}S]GTPγS in 60 min on ice. When incubation on ice was carried out for 48 h, 91% of control [^{35}S]GTPγS binding was obtained, and 85% of the [^{3}H]GDP dissociated. Thus, it appears that G_p bound to GDP displays the GDP release-limiting binding properties hallmark of G-proteins. However, this model was in apparent conflict with data obtained from the kinetics of binding reactions of guanine nucleotides to G_p without preincubation with GTP. In particular, we found significant binding of GTP, Gpp(NH)p, and GTPγS to G_p in 60 min on ice, when GDP dissociation was not detected. About half of the total sites detected at 37 °C were labeled on ice, but with reduced rates of association and dissociation. Hence, without preincubation with GTP, this preparation does not behave as if GDP were bound to all available sites.

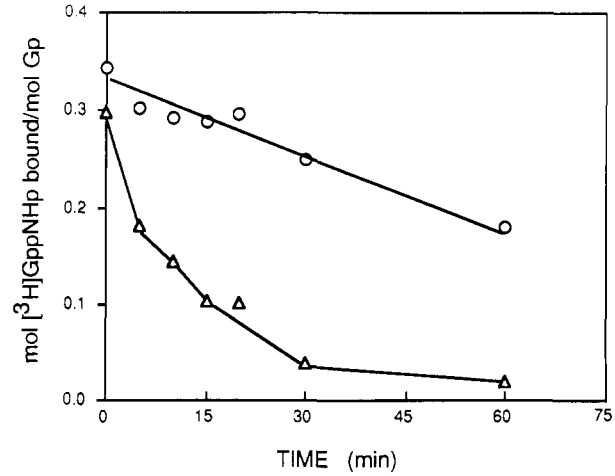

FIGURE 5: Time course of inactivation of G_p at 45 °C. G_p (0.4 μM) was incubated with (○) and without (△) 2 μM [^{3}H]Gpp(NH)p at 45 °C in 520 μL of binding reaction solution. At the indicated times, 50-μL aliquots were removed and held on ice until all time points were accumulated. All samples were then adjusted to 1 μM [^{3}H]Gpp(NH)p by addition of 50 μL of binding reaction solution without (○) or with (△) 2 μM [^{3}H]Gpp(NH)p, and the samples were incubated for 60 min at 37 °C prior to determination of binding, as described under Methods. Rate constants of 0.01 ± 0.001 and 0.04 ± 0.005 min^{-1} were derived by the program "KINETIC" for the presence and absence of Gpp(NH)p, respectively.

Stabilization of G_p by Guanine Nucleotides. As an alternative approach to characterize the interaction of guanine nucleotides, the thermal stability of G_p was examined. In addition to determining apparent affinity, such studies may reveal ligand-induced conformational changes in the protein. Figure 5 presents a representative experiment testing the thermal stability of [^{3}H]Gpp(NH)p-binding activity of G_p at 45 °C. In the absence of guanine nucleotide, this experiment shows a half-life of 17 min for the loss of binding activity at 45 °C. The stability was enhanced dramatically to a half-life of 70 min in the presence of 2 μM Gpp(NH)p and magnesium. A rate constant identical with control (17-min half-life) was observed in the presence of Gpp(NH)p if magnesium was omitted from the incubation at 45 °C (not shown). This indicates that Gpp(NH)p in the presence of magnesium induces a conformation of G_p which is more stable than the protein conformation in the absence of added nucleotide. Because this preparation of G_p appeared to contain a fraction of unoccupied binding sites, we compared the ability of nucleotides to stabilize either untreated G_p or GDP-bound protein (Table V). It is clear from this experiment that GDP association partially stabilizes the binding site as compared with GTPγS. The difference in efficacy between GTPγS and GDP is best seen at 55 °C. At this temperature, 97% of the binding activity was inactivated in 15 min; GDP did not significantly increase the stability, while only 69% inactivation occurred in the presence of GTPγS. In contrast to the signal-transducing G-proteins, addition of 10 μM $AlCl_3$ with 10 mM NaF did not enhance the stability of G_p, even with GDP bound.

Kinetics of Guanine Nucleotide Binding Reactions. In contrast to the equilibrium binding measurements which are consistent with a single population of binding sites, analyses of the kinetics of binding reactions were best fit by two rate

Table V: Stabilization of G_p by Guanine Nucleotides[a]

	relative [^{35}S]GTPγS binding			
	15 min at 45 °C		15 min at 55 °C	
agents	G_p	GDP–G_p	G_p	GDP–G_p
none	0.31	0.39	0.03	0.06
GTPγS	0.97	0.91	0.31	0.16
GDP	0.70	0.64	0.04	0.06
AMF	0.36	0.29	0.04	0.06

[a] Samples of G_p (0.2 μM) and GDP–G_p (prepared by incubation of 2 μM G_p with 2 μM [^{3}H]GTP for 60 min at 37 °C and separation of the free nucleotide over a Sephadex G-25 column) were divided into 50-μL aliquots and incubated for 15 min at 45 °C, or for 15 min at 55 °C in the presence of no addition, 1 μM [^{35}S]GTPγS, 2 μM GDP, and 10 mM NaF + 10 μM $AlCl_3$ (AMF). Binding reactions were then performed for 60 min at 37 °C using 1 μM [^{35}S]GTPγS (33 200 cpm/pmol) in a final reaction volume of 100 μL as described under Methods. Data presented are relative to the control which was 0.56 mol/mol of G_p and 0.42 nmol/mL GDP–G_p. All measurements were performed in triplicate, and the SD of measurements were <10% of mean values.

constants. For GTPγS, GTP, and Gpp(NH)p, about one-third of the available sites bound with a rapid rate (1–4 min^{-1}) at 37 °C, and the remainder bound with constants between 60- and 100-fold lower (data not shown). On ice, the rate constants for GTPγS association were both reduced by a factor of 10 (data not shown). Analysis of the dissociation kinetics for [^{3}H]GDP and [^{3}H]Gpp(NH)p (data not shown) yielded rate constants of 0.12 and 0.14 min^{-1}, respectively. However, these data were equally well-fit by one or two rate constants, and thus, at the resolution of our experiments, we cannot resolve the possibility of two independent binding sites in this preparation.

Discussion

This detailed kinetic and thermodynamic examination of the guanine nucleotide binding properties of the placental G_p protein has revealed many of the features of this "G-protein". All three radiolabeled ligands tested, GTPγS, Gpp(NH)p, and GTP, identified a single class of binding sites with stoichiometry consistent with a single site per molecule of G_p. While the B_{max} values obtained using nitrocellulose filtration were less than 1 mol/mol, these measurements probably underestimate the actual binding capacity somewhat due to dissociation of the bound ligand during filtration. Comparison of the binding amounts determined by Sephadex G-25 exclusion versus nitrocellulose filtration estimates this discrepancy to be about 50% for GTP. Thus, binding stoichiometry was more reliably measured with GTPγS, and these values approached 1 mol/mol with freshly isolated G_p preparations. The mutual competition of GTP, Gpp(NH)p, and GTPγS for the binding of each radiolabeled ligand, and the similarity of K_d values determined directly for each ligand with K_i values obtained in competition for Gpp(NH)p binding argue strongly for a single binding site on the 21-kDa binding polypeptide. Further, the specificity of this site for GTP is well established by the failure of ATP and all other nucleotides tested to compete for Gpp(NH)p binding even at millimolar concentration.

The equilibrium binding constants of G_p for GTP analogues are distinct from those reported for preparations of signal-transducing G-proteins (Neer et al., 1984; Bokoch et al., 1984; Northup et al., 1982; Kelleher et al., 1986). Most notably, the affinity for GDPβS (K_d = 3 μM) is considerably lower than that for GDP (K_d = 100 nM). This is distinct from the signal-transducing G-proteins G_s and G_i for which GDPβS is of higher apparent affinity than GDP or GTP. Also, G_p has a higher affinity for all GTP analogues than has been reported for the signal-transducing G-proteins or ras-like GTP-binding proteins. It is possible that this is due to a distinct GTP-binding domain which differs from that for the signal-transducing G-proteins. Protein sequence which has been derived from human placental G_p is homologous, but distinct from sequences of G-protein α subunits (Polakis et al., 1989a).

The use of [^{3}H]GTP as a binding ligand also revealed two additional features of G_p similar to the G-proteins. Bound GTP was hydrolyzed to GDP which remained bound to the site. The GDP-bound G_p had binding kinetics consistent with a mechanism in which GDP dissociation limits the binding reaction. Our estimate of the GTPase rate at 0.05 min^{-1} most probably represents the overall cycling rate of GDP dissociation, and GTP binding and hydrolysis. Experiments designed to measure both release of bound GDP and association of labeled nucleotide also were consistent with a mechanism of GDP-release limiting the binding reaction rate.

The hydrolysis of GTP to GDP, which only slowly dissociates from G_p, appears to provide an explanation for the complex kinetics of guanine nucleotide dissociation found for all three ligands. The faster rate constant for association would be predicted to represent the bimolecular binding rate for the ligands with an unoccupied site while the slower rate constant is the GDP dissociation rate. Several of our kinetic results are consistent with this explanation. First, the binding reaction observed for G_p with guanine nucleotides on ice for 60 min identified about the same fraction of the sites found to bind with rapid rate constants at 37 °C. Prior binding to GTP prevented the binding reaction on ice, and this correlated with a slow dissociation of GDP from the site at ice temperature.

An alternative explanation for the kinetic results is that the human placental preparations of G_p contain two distinct GTP-binding proteins. This seems unlikely from the equilibrium data, all of which are consistent with a single class of binding sites. However, these data do not exclude two sites with identical equilibrium binding constants, but with different rate constants of binding. Dissociation kinetics did not clearly distinguish two rate constants. The identity of equilibrium constants with different association rate constants would require inversely discrepant dissociation rates to yield identical equilibrium constants. Nevertheless, the dissociation rate constants were not well determined by our experiments, and these data do not rigorously exclude two sites. Our previous studies indicated that all of the GTP-binding activity in placental G_p preparations was contained in the 21-kDa "α" subunit (Evans et al., 1986). This polypeptide did not have a sequenceable amino terminus, and thus this most rigorous criterion for homogeneity cannot be applied. An immunologically indistinguishable protein isolated from human platelets has recently been shown to contain two molecular forms differing in isoelectric point but containing reactivity with antipeptide antisera directed toward sequences obtained from human placental G_p (Polakis et al., 1989b). Two molecular forms of the placental G_p might explain the complex kinetics we have observed.

The data obtained from stabilization of G_p-binding activity against thermal inactivation provide an alternate, indirect assessment of the binding site and conformational states of the protein. The GTP analogues GTPγs and Gpp(NH)p at saturating concentrations both stabilized the site against thermal denaturation. These were more efficacious than GDP in stabilizing the binding site. This suggests that the GTP-bound form of the protein has a conformation that is distinguishable from the GDP-bound form in thermal stability. This is also characteristic of the signal-transducing G-proteins

(Smigel et al., 1982). The two states for G-proteins can be biochemically distinguished as "active" GTP-bound and "basal" GDP-bound with the activated state being more stable to thermal denaturation. The G_p protein differs from signal-transducing G-proteins in that the addition of aluminum and fluoride (Sternweis & Gilman, 1982; Bigay et al., 1987) in the presence of GDP and Mg^{2+} did not enhance the stability of the binding site. This condition both activates and stabilizes the G-protein α chains (Ross & Gilman, 1977; Northup et al., 1982; Smigel et al., 1982). Failure of aluminum fluoride to stabilize G_p was not due to the GDP-binding status of the site, as prior incubation of G_p with GTP to produce GDP–G_p did not affect the outcome. We also could detect no apparent effect of aluminum fluoride on the guanine nucleotide exchange reaction with GTPγS as the binding ligand (data not shown). Thus, it would appear that the GTP-binding site in the G_p protein does not resemble that for G-proteins in its affinity for aluminum fluoride (data not shown). Cholera toxin catalyzed ADP-ribosylation studies in bovine brain membranes suggest that aluminum fluoride does not activate the ARF proteins.[3] It would be of interest to know if this is true also for other low molecular weight GTP-binding proteins, as it has been assumed that the aluminum fluoride complex is a universal GTP-binding protein probe.

Both direct ligand-binding and thermal inactivation studies revealed a divalent metal ion dependence of guanine nucleotide binding. In the presence of EDTA, no stable binding interaction with Gpp(NH)p, GTP, or GTPγS could be observed. Subsequent to binding equilibrium in the presence of magnesium, addition of EDTA dramatically enhanced dissociation rates. Similarly, guanine nucleotide stabilization against thermal inactivation was not observed in the absence of magnesium, even in the presence of 100-fold K_d concentrations of GTPγS. Micromolar concentrations of magnesium were saturating for this dependence, and, thus, it was technically difficult to determine the concentration dependence with precision. With this apparent affinity, it is difficult to imagine that the metal site represents a point of intracellular regulation of guanine nucleotide binding. This metal dependence appears quite similar to the high-affinity metal dependence of G-protein α subunits in regulating guanine nucleotide dissociation. The lower affinity metal effects on G-proteins involve the α and $\beta\gamma$ subunit interactions (Northup et al., 1982, 1983a,b; Higashijima et al., 1987), and these have not been observed for G_p. To date, we have observed no consistent effect of human placental $\beta\gamma$ subunit on human placental G_p guanine nucleotide binding or thermal stability.[4]

The G_p protein presents biochemical features which are similar in many respects to the guanine nucleotide binding properties of G-proteins. The specificity for guanine nucleotides, relatively high affinity of GTPγS as compared with GTP, and rank order affinities of other guanine nucleotides are comparable with G-proteins. However, the low affinity of the GDP analogue GDPβS clearly distinguishes this binding site from the G-proteins. Signal-transducing G-proteins bound with GTP are in an active conformation which is distinguishable from GDP-bound basal conformation in thermal stability. G_p has similarly distinguishable GTP-bound and GDP-bound conformations. The signal-transducing G-proteins are uniformly isolated with GDP bound to the site, while a substantial fraction of the sites in the human placental G_p preparations appear to be empty. For signal-transducing G-proteins, such empty sites would be highly labile (Ferguson et al., 1986), while unoccupied sites on G_p may be less labile. Finally, unlike other G-proteins, the G_p-binding site does not appear to interact with aluminum fluoride as an activating GTP analogue. Thus, we conclude that the GTP-binding site in G_p, while similar, is distinct from that of G-proteins. Partial protein sequence data obtained from the human placental and platelet G_p reveal a sequence (peptide 4: GGEPYTLGLFDTAGQEDYDR) which is homologous, but distinct from sequences contained in the putative GTP-binding domains of all other published GTP-binding proteins (Polakis et al., 1989a). It remains for more complete structural studies to reveal the basis for the unique guanine nucleotide binding properties of the G_p protein.

Registry No. Gpp(NH)p, 34273-04-6; GTPγS, 37589-80-3; GDPβS, 71783-24-9; GTP, 86-01-1; GDP, 146-91-8; GTPase, 9059-32-9; Mg, 7439-95-4.

[3] A. Tamir, manuscript in preparation.
[4] A. Tamir, unpublished observations.

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Rhodopsin-Stimulated Activation-Deactivation Cycle of Transducin: Kinetics of the Intrinsic Fluorescence Response of the α Subunit†

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ABSTRACT: The intrinsic tryptophan fluorescence of the α subunit of transducin (α_T) has been shown to be sensitive to the binding of guanine nucleotides, with the fluorescence being enhanced by as much as 2-fold upon the binding of GTP or nonhydrolyzable GTP analogues [cf. Phillips and Cerione (1988) *J. Biol. Chem. 263*, 15498-15505]. In this work, we have used these fluorescence changes to analyze the kinetics for the activation (GTP binding)-deactivation (GTPase) cycle of transducin in a well-defined reconstituted phospholipid vesicle system containing purified rhodopsin and the α_T and $\beta\gamma_T$ subunits of the retinal GTP-binding protein. Both the rate and the extent of the GTP-induced fluorescence enhancement are dependent on [rhodopsin], while only the rate (and not the extent) of the GTPγS-induced enhancement is dependent on the levels of rhodopsin. Comparisons of the fluorescence enhancements elicited by GTPγS and GTP indicate that the GTPγS-induced enhancements directly reflect the GTPγS-binding event while the GTP-induced enhancements represent a composite of the GTP-binding and GTP hydrolysis events. At high [rhodopsin], the rates for GTP binding and GTPase are sufficiently different such that the GTP-induced enhancement essentially reflects GTP binding. A fluorescence decay, which always follows the GTP-induced enhancement, directly reflects the GTP hydrolytic event. The rate of the fluorescence decay matches the rate of [^{32}P]P_i production due to [γ-^{32}P]GTP hydrolysis, and the decay is immediately reversed by re-challenging with GTP. The GTP-induced fluorescence changes (i.e., the enhancement and ensuing decay) could be fit to a simple model describing the activation-deactivation cycle of transducin. The results of this modeling suggest the following points: (1) the dependency of the activation-deactivation cycle on [rhodopsin] can be described by a simple dose response profile; (2) the rate of the rhodopsin-stimulated activation of multiple α_T(GDP) molecules is dependent on [rhodopsin] and when [α_T] > [rhodopsin], the activation of the total α_T pool may be limited by the rate of dissociation of rhodopsin from the activated α_T(GTP) species; and (3) under conditions of optimal rhodopsin-α_T coupling (i.e., high [rhodopsin]), the cycle is limited by GTP hydrolysis with the rate of P_i release, or any ensuing conformational change, being at least as fast as the hydrolytic event.

Over the past several years, a number of types of receptor-coupled signal transduction systems have been identified and characterized. These include the hormone receptor systems involved in the stimulation and inhibition of adenylyl cyclase activity (Lefkowitz & Caron, 1988), the rhodopsin-mediated stimulation of a visual signal (Stryer et al., 1981; Fung, 1983), and the receptor-mediated regulations of phospholipase C (Smith et al., 1986; Berridge, 1987), phospholipase A_2 (Burch et al., 1986), and ion channels (Hescheler et al., 1987; Logothetis et al., 1987; Yatani et al., 1987). In most of these cases, three membrane-associated proteins are responsible for the change in the levels of a second messenger compound (e.g., cyclic AMP, cyclic GMP, Ca^{2+}, etc.) that is essential for the biological response. These proteins include the cell surface receptor itself, a GTP-binding protein (G protein) which serves as a signal transducer, and the biological effector (enzyme or ion channel) that is directly responsible for altering the levels of a specific second messenger.

The vertebrate vision system offers an especially attractive model for studying the regulatory interactions of a cell surface receptor with a G protein, or the ensuing interactions of the

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