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G PROTEINS: TRANSDUCERS OF RECEPTOR-GENERATED SIGNALS

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PERSPECTIVES AND SUMMARY

To be considered in this discussion is a family of guanine nucleotide–binding proteins (G proteins) that serve as membrane-bound transducers of chemically and physically coded information. Knowledge of this family, particularly that acquired over the past 10 years, permits a rather restrictive definition of the characteristics of its closest members. I present such a definition here in the interest of generalization and describe these characteristics in more detail throughout this review. However, we must remain alert for deviations from the "rules" that may be practiced by as yet undiscovered members of the immediate family or by other related proteins.

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The G proteins function as intermediaries in transmembrane signaling pathways that consist of three proteins: receptors, G proteins, and effectors. The receptors that participate in such reactions are legion and include those for a large array of biogenic amine, protein, and polypeptide hormones; autacoids; and neurotransmitters. Best characterized of these receptors are those for β-adrenergic agonists (e.g. epinephrine and the more selective agent isoproterenol) and antagonists. Rhodopsin, too, is a G protein–linked receptor, as, apparently, are those for various odorants. The number of effector molecules known to be controlled by G proteins is more modest: interactions of adenylyl cyclase and a retinal cyclic GMP–specific phosphodiesterase with G proteins are rather well understood. Regulation of the activity of a phosphoinositide phosphodiesterase (phospholipase C) and the function of ion channels by G proteins is strongly suspected, but the details remain unknown.

The G proteins are heterotrimers, with subunits designated α, β, and γ in order of decreasing mass. The α subunits clearly differ among the members of the family and, at least for the moment, define the individual. Common β and γ subunits are probably shared among some α subunits to form the specific oligomers.

The functions of G proteins are regulated cyclically by association of GTP with the α subunit, hydrolysis of GTP to GDP and P_i, and dissociation of GDP. Binding of GTP is closely linked with "activation" of the G protein and consequent regulation of the activity of the appropriate effector. Hydrolysis of GTP initiates deactivation. Dissociation of GDP appears to be rate limiting (or, more precisely, occurs as a result of the rate-limiting process), and this step is accelerated by interaction between G protein and receptor. There is considerable (but not conclusive) evidence that a cycle of dissociation and association of G protein subunits is superimposed on this regulatory GTPase cycle.

G proteins share other unique or unusual characteristics. For example, they are activated by fluoride plus aluminum—the actual ligand probably being AlF_4^-. Also distinctive is that the α subunits of individual G proteins are substrates for ADP-ribosylation catalyzed by bacterial toxins. Best characterized are the reactions carried out by toxins elaborated by *Vibrio cholerae* and *Bordetella pertussis*.

Other recent and related reviews include those by Schramm & Selinger (1), Smigel et al (2), Gilman (3), Levitzki (4), and Stryer (5).

HISTORY

Although space does not permit an extensive historical introduction, I will mention a few of the most important observations, particularly of the 1970s, that serve as background for this review.

The involvement of a G protein in transmembrane signaling was first suggested by the requirement for GTP for hormonal activation of adenylyl cyclase (6). Although knowledge of the biochemistry of the enzyme permitted only speculation on the significance of the phenomenon, this fundamental observation by Rodbell, Birnbaumer, and their colleagues set the stage. Perhaps more confusing than illuminating was the simultaneous finding that GTP interfered with detection of hormone (glucagon) binding to receptors responsible for regulation of adenylyl cyclase activity (7). Maguire et al subsequently found that the effect of guanine nucleotides on receptor binding was specific for agonists and that their affinity for the receptor was reduced (8); although the interpretation was still less than obvious, specificity for agonists lent a strong aura of relevance to function. Crucial, then, were the observations of Cassel & Selinger (9–11), who first assayed catecholamine-stimulated GTPase activity in turkey erythrocyte membranes. These experiments were technically demanding, but their quantitation and interpretation have proven to be essentially correct. Thus, G protein–linked systems are activated on binding of GTP; hydrolysis of GTP initiates or is responsible for deactivation; dissociation of GDP is linked with the rate-limiting step and is controlled by receptor. The latter fact is explained by the negative heterotropic binding interaction between receptor and guanine nucleotide, which must be reciprocal. Schramm's demonstrations that components of the adenylyl cyclase system could be mixed and exchanged by cell fusion (12) presaged their reconstitution in vitro (13–16). The assays that evolved permitted purification of G proteins that are associated with the enzyme (17). The capacity of certain bacterial toxins to ADP-ribosylate specific G proteins was discovered first for cholera toxin (18–20) and subsequently for pertussis toxin (21, 22) and proved to be extraordinarily useful. In the meantime, affinity chromatographic techniques greatly facilitated purification of labile and low abundance molecules such as the β-adrenergic receptor (23) and adenylyl cyclase (24). Identification of analogous systems in the retina (25) and realization of the fact that the basic rules had been worked out before with elongation factor Tu (26) bring us to the present.

INDIVIDUAL G PROTEINS: FUNCTIONS AND MOLECULAR ENTITIES

A chicken and egg problem presents itself in attempts to describe the functions and structure of individual G proteins, since, at the moment, there exist proteins (and "deduced proteins") whose functions are unknown and functions to which a specific molecule has yet to be assigned. I start with a description of those functions that have been implicated as part of the repertoire. Confusion will hopefully be minimized by reference to Table 1.

Table 1 Properties of G protein subunits

Subunit	M_r (kd)	Toxin[a]	Receptor[b]	Effector[c]	Specific peptide[d]
$G_{s\alpha}$	46[e]	C-Arg 201[f]			GEEDPQAARSNSDG
			$\beta>>\alpha$,Rho	AC(+)	KQLQKDKQVYRATHR[g]
$G_{s\alpha}$	44.5	C-Arg 187[f]			TPEPGEDPRVTRAKY[g]
$G_{i\alpha 1}$	40.4	P-Cys 351[f]	M,α,Rho>β	AC(−) Others?	
$G_{i\alpha 2}$	40.5	P-Cys 352[h]	—	—	SKFEDLNKRKDT[i]
$G_{o\alpha}$	39[j]	P-Cys 331[f]	M,α,Rho	—	NLKEDGISAAKDVK
$G_{t\alpha 1}$	40	P-Cys 347 C-Arg 174	Rho>$\alpha>>\beta$	PDE	SDLERLVTPGYVPT[k]
$G_{t\alpha 2}$	40.4	P-Cys 351[h] C-Arg 178[h]	—	PDE (?)	LDRITAPDYLPN[k]
G_{β}[l]	37.4				EGNVRVSRELAGHTGY
G_{γ}[l]	8.4				

[a] C = Cholera, P = Pertussis
[b] β = β adrenergic, α = α adrenergic, Rho = rhodopsin, M = muscarinic cholinergic
[c] AC = adenylyl cyclase, PDE = retinal cyclic GMP phosphodiesterase
[d] Peptides utilized successfully for production of specific antisera. Antisera that recognize $G_{s\alpha}$, $G_{i\alpha}$, $G_{o\alpha}$, and $G_{t\alpha}$ have been produced with two peptides, GAGESGKSTIVKQM and HMFDVGGQRDERRK.
[e] Apparent M_r by SDS-PAGE, ~52 kd
[f] Deduced site of ADP-ribosylation. Full $G_{o\alpha}$ sequence not known; residue number refers to Figure 1.
[g] For both forms of $G_{s\alpha}$
[h] Assumed substrate for ADP-ribosylation at this site
[i] Synthesized from sequence of $G_{i\alpha 2}$; $G_{i\alpha 1}$ has amino terminal CQ instead of SK. Antiserum recognizes α_{41}.
[j] Full sequence not available; M_r by SDS-PAGE
[k] Specific for one $G_{t\alpha}$ versus the other
[l] Values for $G_{t\beta\gamma}$

Criteria for Involvement of a G Protein

We now fully appreciate the significance of the early experiments that indicated a role for G proteins in transmembrane signaling reactions, and many of them continue to be repeated frequently in the exploration of other systems. It has become possible to define criteria for involvement of a G protein when a new situation is approached, and it will be useful to list these before consideration of proven or potential functions that are regulated by G proteins.

1. An appropriate ligand for the receptor of interest and GTP are both required to initiate the response in question.
2. The response can be provoked independently of receptor by inclusion of nonhydrolyzable analogues of GTP (GTPγS or Gpp[NH]p) or F^- plus Al^{3+}. It has been possible to introduce nucleotide analogues into intact cells by injection or perfusion (27, 28) or after permeabilization (29). F^- and Al^{3+} have occasionally proven useful with intact cell preparations (30, 31).
3. There is a negative heterotropic interaction between the binding of guanine

nucleotide to a G protein and the binding of agonist to a G protein–linked receptor.

4. Cholera toxin and/or pertussis toxin have characteristic effects on the functions of known G proteins, and they can be utilized with either intact cells or purified components.
5. Certain mutants, particularly of the murine S49 lymphoma, have been extraordinarily useful in the definition of some G protein–regulated functions. It is hoped that novel mutants, deficient in the activities of various G proteins, can be developed.
6. Antibodies with differing reactivities for individual G proteins have recently become available.
7. Purification and reconstitution of individual components of a pathway is the ultimate criterion. This has been achieved with the adenylyl cyclase complex and the retinal phosphodiesterase system.

Functions Regulated by G Proteins

ACTIVATION OF ADENYLYL CYCLASE G_s, named as the stimulatory regulator of adenylyl cyclase, is required for observation of significant levels of adenylyl cyclase activity under physiological conditions (15). The protein was recognized as a discrete entity following its partial resolution from adenylyl cyclase by affinity chromatography on GTP-Sepharose (16) and its functional reconstitution into plasma membranes prepared from an S49 cell mutant (cyc^-) that has subsequently been proven to be devoid of $G_{s\alpha}$ (13–15). The latter assay permitted purification of the protein from several sources (17, 32–35). Mechanisms of regulation of adenylyl cyclase activity and structural properties of G_s are discussed below.

INHIBITION OF ADENYLYL CYCLASE GTP is also required for receptor-mediated inhibition of adenylyl cyclase. Although a considerable amount of indirect evidence suggested the existence of a distinct G protein to account for this fact, isolation of the species was facilitated greatly by the fruits of an independent line of investigation—studies on the mechanism of action of a toxin from *B. pertussis*. This toxin had been found to abolish hormonal inhibition of adenylyl cyclase and, in some cases, to potentiate stimulation of the enzyme (36, 37). These effects appeared to result from ADP-ribosylation of a 41-kd membrane protein, a reaction catalyzed by the toxin (21, 22). Purification of the substrate for pertussis toxin revealed a guanine nucleotide–binding protein with an obvious resemblance to G_s and G_t (38–41). It was possible to inactivate receptor-mediated inhibition of adenylyl cyclase by treatment of platelet membranes with toxin and, subsequently, to restore hormonal inhibition by reconstitution of membranes with the purified toxin

substrate (42, 43). This protein (M_r of α subunit = 41,000) has thus been termed G_i (i = inhibitory for adenylyl cyclase).

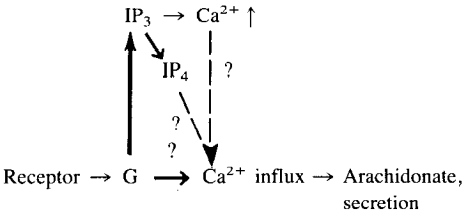
STIMULATION OF RETINAL CYCLIC GMP PHOSPHODIESTERASE Light activates a cyclic GMP–specific phosphodiesterase in retinal rod outer segments (44, 45). The observation of a light-activated GTPase activity in the retina (46) and a guanine nucleotide requirement for activation of the phosphodiesterase (47, 48) led to purification of transducin (G_t), another member of the G protein family (25, 49, 50). It thus became clear that the flow of information was from light to rhodopsin, G_t, and the phosphodiesterase in sequence. The concentration of cyclic GMP in retinal rods is a crucial determinant of visual excitation (see 5 for review).

STIMULATION OF PHOSPHOINOSITIDE HYDROLYSIS Many hormones mobilize intracellular stores of Ca^{2+} by virtue of their ability to stimulate the phosphodiesteratic cleavage of phosphatidylinositol-4,5-diphosphate (PIP_2) to inositol-1,4,5-triphosphate (IP_3) (51). The relevant phosphodiesterase (phospholipase C) is influenced by guanine nucleotides. Thus, GTPγS or Gpp(NH)p (but not GDP, ATP, or ATPγS) stimulate the hydrolysis of polyphosphoinositides by neutrophil membranes (52), and GTP, Gpp(NH)p, or GTPγS is largely required for stimulation of inositol phosphate synthesis by blowfly salivary gland membranes in response to 5-hydroxytryptamine (53). Similar results have been obtained with plasma membrane preparations from rat hepatocytes (54) and human polymorphonuclear leukocytes (55, 56). In these latter two systems the combination of hormone and guanine nucleotide lowered the concentration of Ca^{2+} required to support enzymatic activity to the physiological range. In further support of the notion that a G protein controls phosphoinositide phosphodiesterase activity are the observations that the affinities of agonists for several receptors that stimulate IP_3 synthesis are reduced in the presence of guanine nucleotides (e.g. 57). F^- and Al^{3+} are also able to stimulate the hydrolysis of PIP_2 (31). Beyond this generally consistent set of observations, the situation becomes less clear cut.

There are clearly discrepant observations on the effects of pertussis toxin on receptor-stimulated synthesis of IP_3. For example, the toxin blocks this effect in polymorphonuclear leukocytes (58), in membranes derived therefrom (55), and in mast cells (59). However, it fails to alter the response in hepatocytes (54), cardiac myocytes (60), astrocytoma cells (60), and fibroblasts (61). These observations suggest participation by different G proteins in different cell types or differential modification of the same G protein. G_i and G_o (see below) are the predominant substrates for pertussis toxin. If G_i or G_o is involved in the response of leukocytes and mast cells, it should be possible to stimulate IP_3 synthesis by addition of the activated (GTPγS-bound) G protein

to appropriately prepared membranes; this result has not been reported. The conclusion is that a G protein is likely involved, but its identity is unknown.

Introduction of nonhydrolyzable guanine nucleotide analogues into permeabilized mast cells permits exocytotic secretion in response to addition of extracellular Ca^{2+} (29). Thus, it has been suggested that a G protein may regulate plasma membrane Ca^{2+} channels. However, it is difficult to decide if this is a relatively direct or an indirect response. Treatment of mast cells with pertussis toxin blocks the array of effects that are seen in response to compound 48/80 (a polymeric releaser of histamine), including breakdown of PIP_2, accumulation of inositol polyphosphates, ^{45}Ca influx, generation of arachidonate, and histamine secretion (59). Similar observations have been made with neutrophils (30, 62, 63). The mast cell inositol response to compound 48/80 is not dependent on extracellular calcium, and the Ca^{2+} ionophore A23187 fails to stimulate PIP_2 breakdown. These facts and the observation of guanine nucleotide–mediated IP_3 synthesis in membrane preparations indicate that Ca^{2+} influx does not explain pertussis toxin–sensitive PIP_2 breakdown in neutrophils and mast cells. Arachidonate release and histamine secretion, on the other hand, are largely dependent on extracellular Ca^{2+} and can be evoked by A23187. It is possible that inositol polyphosphates other than IP_3 (e.g. inositol, 1,2,3,4-tetrakisphosphate [IP_4]) may facilitate influx of extracellular Ca^{2+} (64). IP_4 appears to arise by phosphorylation of IP_3. Thus, the scheme might be ordered as follows:



Activation of phosphoinositide breakdown may account for other receptor-mediated effects that likely involve G proteins. Interaction of agonists with a subset of muscarinic receptors of 1321N1 astrocytoma cells (65, 66) or with α_1-adrenergic receptors of rat ventricular myocytes (67) leads to attenuation of cyclic AMP accumulation by stimulation of cyclic nucleotide phosphodiesterase activity. This response is not sensitive to pertussis toxin, is accompanied by hydrolysis of PIP_2, and (at least in the case of astrocytoma cells) is dependent on extracellular Ca^{2+}. It seems most reasonable for the present to assume that the effect on the cyclic nucleotide phosphodiesterase is mediated indirectly by Ca^{2+}.

A pertussis toxin substrate also appears to be involved in reduction of

intracellular Ca^{2+} concentrations (68). Somatostatin inhibits K^+-induced prolactin secretion by GH_4C_1 cells and lowers intracellular [Ca^{2+}]. These effects are not dependent on cyclic AMP. Pertussis toxin blocks this effect of somatostatin. It is possible that a G protein may be negatively linked to generation of inositol polyphosphates (see 69).

REGULATION OF ION CHANNELS A few recent reports lend more credence to the exciting possibility that G proteins may exert direct control over the function of ion channels. Pfaffinger et al (27) measured ionic currents in single atrial cells with a whole-cell voltage-clamp technique that permits equilibration of the cytoplasm with a solution of choice. Muscarinic agonists activate an inward rectifying K^+ channel in this preparation in a cyclic nucleotide–independent manner. Observation of the response to acetylcholine required perfusion of the cells with a GTP-containing solution (ATP was also present) and was blocked by prior treatment of cells with pertussis toxin. Breitwieser & Szabo (28) found that this channel could also be activated irreversibly by exposure to acetylcholine after intracellular injection of Gpp(NH)p. This effect was not overcome by addition of isoproterenol, which should stimulate cyclic AMP accumulation. These experiments appear to rule out cyclic nucleotides as mediators of the response. They do not prove direct interaction between G protein and channel. In particular, channel regulation by G protein–mediated alteration of the activity of a protein kinase or a phosphoprotein phosphatase remain as possibilities. Heart is known to contain both G_i and G_o (70); both are ADP-ribosylated by pertussis toxin; either can interact with muscarinic cholinergic receptors in reconstituted systems (71, 72). G_i or G_o has also been suggested as a mediator of neurotransmitter-induced inhibition of voltage-dependent Ca^{2+} channels in chick dorsal root ganglion cells (73).

Mg^{2+} uptake by S49 lymphoma cells is inhibited by β-adrenergic agonists. This response, which is not mediated by cyclic AMP, is absent in cyc^- or UNC S49 cell mutants (which lack $G_{s\alpha}$ or have an altered $G_{s\alpha}$ that cannot interact with receptor, respectively) (74). The implication is that the response requires the β-adrenergic receptor and G_s but not cyclic AMP and, therefore, perhaps not the only effector with which G_s is known to interact, adenylyl cyclase. Beyond these facts, the mechanism of this interesting effect is unknown.

Molecular Entities and Structure

A detailed view of certain aspects of the structures of G proteins is beginning to emerge, thanks in particular to cDNA cloning and sequencing and to the solution of the crystal structure of a related guanine nucleotide–binding protein, the bacterial elongation factor Tu (EF-Tu) (75). Molecular cloning

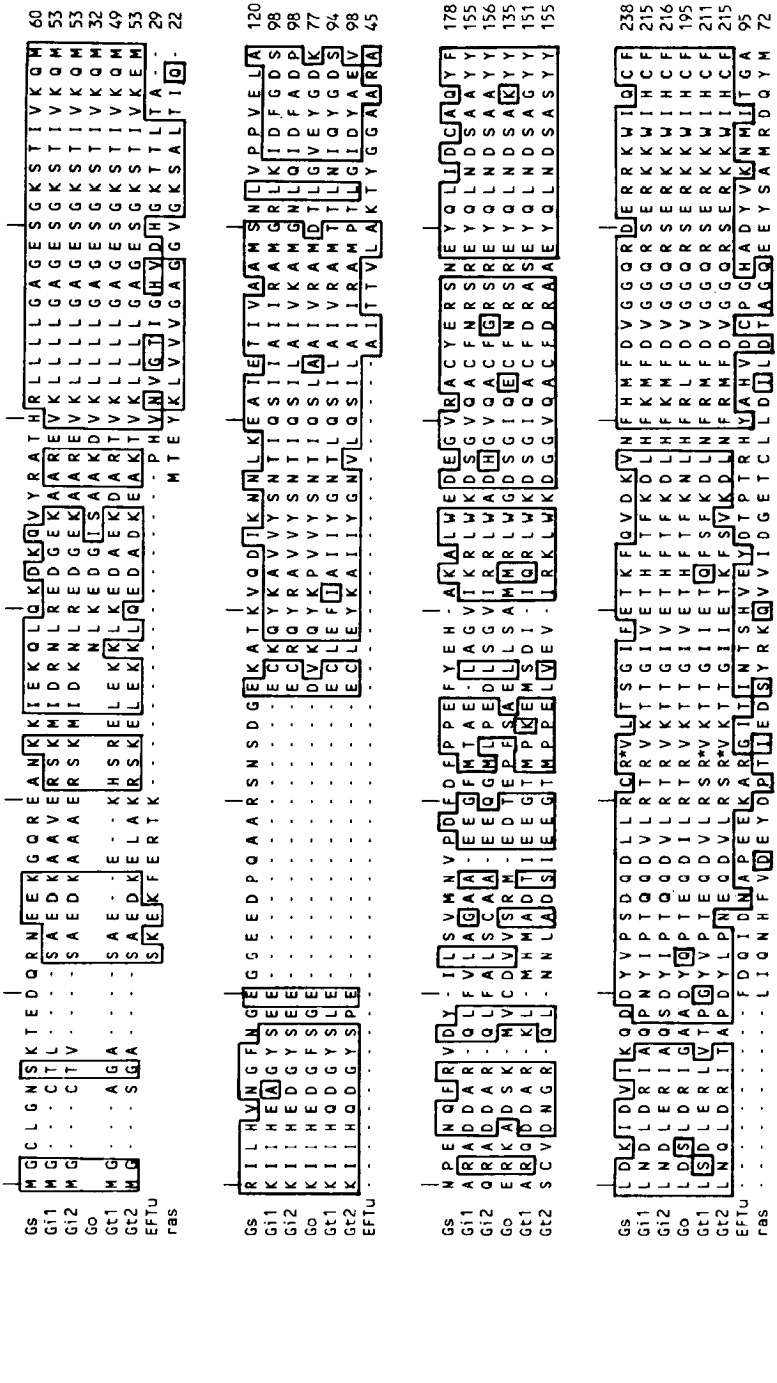
has revealed the primary structures of nearly all of the G proteins that have been purified and has led to an appreciation of at least two additional entities (referred to below as $G_{i\alpha 2}$ and $G_{t\alpha 2}$). To date, however, this approach has not resulted in the discovery of a myriad of novel structures.

$G_{s\alpha}$ G_s, first defined functionally by its ability to activate adenylyl cyclase, was found on purification to be a mixture of two oligomers with differing α subunits (apparent M_r on SDS-polyacrylamide gels, 52,000 and 45,000) and indistinguishable β and γ subunits (17, 32, 76). The relative concentration of the two forms of $G_{s\alpha}$ varies among cells and tissues; functional differences are not yet appreciated.

cDNAs corresponding to $G_{s\alpha}$ have been cloned from bovine brain (77, 78), bovine adrenal (79), and rat brain (80) (Figure 1). The first cDNA was obtained by hybridization with an oligonucleotide probe based on protein sequence obtained from a highly conserved region of $G_{o\alpha}$ and $G_{t\alpha}$ (81). It was identified as $G_{s\alpha}$ by immunoblotting with antibodies generated to peptides synthesized according to sequence deduced from the cDNA (77). This identification was confirmed by failure to find mRNA in the cyc$^-$ ($G_{s\alpha}$-deficient) S49 cell mutant that would hybridize with the cDNA clone (77) and by expression of the cDNA (79, 82). The amino acid sequences that are revealed by the bovine and rat cDNAs differ in only three residues.

The first cDNA for $G_{s\alpha}$ that was isolated encodes a protein of 394 residues and, therefore, an apparent M_r of 46,000. However, upon transient expression in COS-m6 cells, this cDNA was found to direct the synthesis of the 52-kd form of $G_{s\alpha}$ (79). The same result was obtained by expression in *Escherichia coli,* using prokaryotic expression vectors containing either the tac or T7 promoters (M. Graziano, unpublished observation). The implication is that the M_r of the larger form of $G_{s\alpha}$ is actually 46,000 and that its electrophoretic behavior in SDS is anomalous. An alternative cDNA for $G_{s\alpha}$ has also been detected (82). It differs from the first in only 46 contiguous nucleotides, resulting in the alteration of two and the deletion of 14 amino acid residues (residues 73–86 in the larger form). It appears to encode a 44.5-kd protein and directs the synthesis of the 45-kd form of $G_{s\alpha}$ in COS-m6 cells or *E. coli*. Messenger RNA corresponding to each cDNA is detectable by S1 nuclease analysis. In view of their otherwise identical sequence, the two mRNAs are presumed to arise from a single gene for $G_{s\alpha}$ by alternative splicing of internal exons.

$G_{i\alpha}$ First visualized as a 41-kd substrate for ADP-ribosylation by pertussis toxin, oligomeric G_i was purified from rabbit liver (38, 41) and human erythrocytes (35, 39) by techniques nearly identical to those developed for G_s. Although the α subunit of G_i was clearly distinguishable from those of G_s and



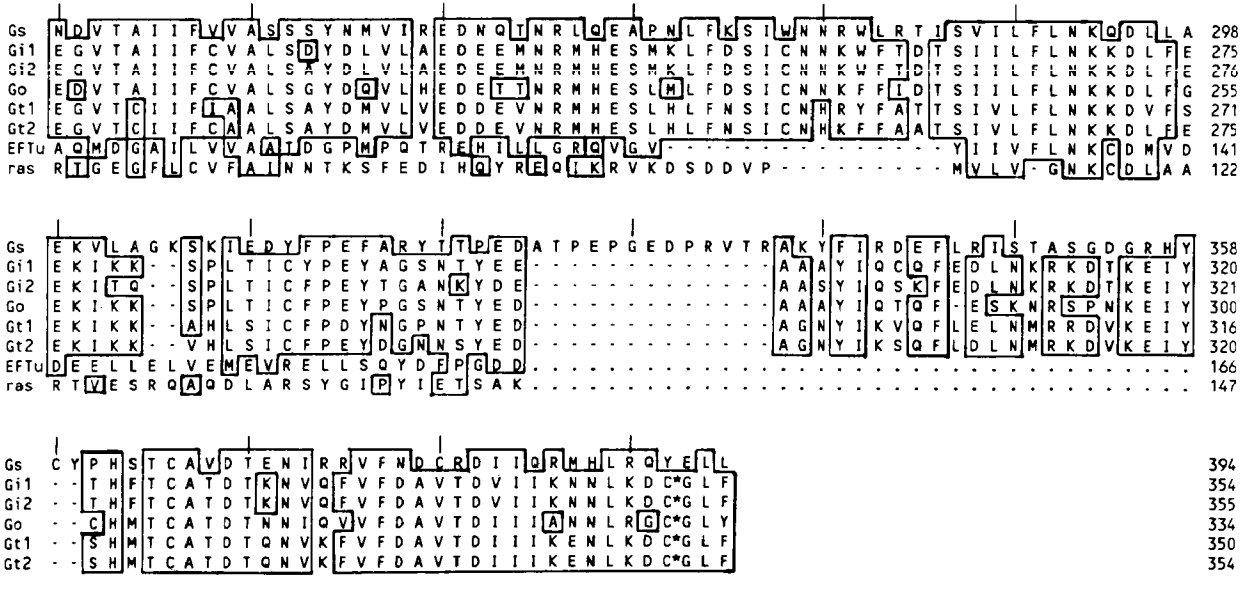


Figure 1 Amino acid sequences of G protein α subunits and the presumptive GTP binding regions of EF-Tu and c-Ha-ras. $G_{s\alpha}$, $G_{i\alpha 1}$, $G_{t\alpha 1}$, and $G_{t\alpha 2}$ are bovine. $G_{i\alpha 2}$ and $G_{o\alpha}$ are rat. The amino-terminal sequence of $G_{o\alpha}$ has not yet been determined; the first 11 residues shown are from the bovine protein. Presumed and proven sites of ADP-ribosylation are indicated by*. Boxes surround residues that are identical or conserved among any four of the six G_α subunits and residues in EF-Tu and c-Ha-ras that are identical or conserved with corresponding boxed residues of G_α subunits. Conserved residues are defined as follows: C; S, T, P, A, G; N, D, E, Q; H, R, K; M, I, L, V; F, Y, W.

G_t, the β subunit was apparently identical (40). The small γ subunit, not detected initially because of its poor staining qualities, was detected soon thereafter (41, 76). The functional attributes ascribable to G_i were deduced by reconstitution of the rabbit liver protein into platelet and S49 cell membranes (42, 43, 83). A protein with superficially indistinguishable features has since been purified from bovine (84, 85) and rat (86) brain and is frequently termed G_i. However, there is heterogeneity of substrates for pertussis toxin, which became grossly obvious when brain was studied. Furthermore, inhibition of adenylyl cyclase may not be the exclusive property of "G_i." Caution in nomenclature is thus mandated. (The terminology α_{Mr} serves this purpose.)

Nukada et al (87) purified $G_{i\alpha}$ (α_{41}) from bovine brain and determined the amino acid sequence of several of its tryptic peptides. These sequences are represented faithfully in that deduced from a cDNA clone isolated from a bovine brain cDNA library. The cDNA encodes a protein with 354 amino acid residues and a calculated molecular weight of 40,400. Northern analysis reveals an RNA with approximately 3900 nucleotides.

Itoh et al (80) screened a rat C6 glioma cDNA library with an oligonucleotide probe based on amino acid sequence data obtained with purified rat brain $G_{i\alpha}$ (α_{41}). With the exception of two residues, amino acid sequences deduced from one of the cDNA clones isolated by these investigators matched those determined for seven tryptic peptides derived from the protein. However, the entire sequence of 355 amino acid residues deduced by Itoh et al (80) differs significantly (~11%) from that of Nukada and coworkers (87). Is this difference due to species? The extreme similarity between bovine and rat $G_{s\alpha}$ suggests not, but this argument is hardly definitive. More interesting is the fact that the rat protein sequence obtained by Itoh et al differs from Nukada and associates' bovine cDNA sequence by only three residues in 78. It is suggested that the predominant α_{41} ($G_{i\alpha}$) corresponds to that purified from bovine and rat brain by these two groups and that its sequence is represented by the cDNA of Nukada et al (87). Itoh et al are presumed to have cloned a similar cDNA, but one that encodes a distinct entity. For the moment, we refer to these proteins as $G_{i\alpha 1}$ (Nukada et al) and $G_{i\alpha 2}$ (Itoh et al) or $\alpha_{41,1}$ and $\alpha_{41,2}$.

$G_{o\alpha}$ Sternweis & Robishaw encountered surprising [^{35}S]GTPγS binding activities (10-fold greater than anticipated) during initial attempts to purify G_i from brain. Protein fractionation, ADP-ribosylation with pertussis toxin, and electrophoresis revealed the explanation. Brain contains a plentiful substrate for pertussis toxin in addition to G_i (84). This protein proved to be an obvious member of the G protein family, since, in addition to serving as a substrate for pertussis toxin, it has a guanine nucleotide–binding α subunit (M_r = 39,000) and β and γ subunits that are apparently identical to those of G_s and G_i. This

new G protein was dubbed G_o (o = other G protein), and evidence was presented that it was not a proteolytic product of the larger G_i (also abundant in brain). Neer and associates detected similar heterogeneity of substrates for pertussis toxin in brain at essentially the same time (85). In addition to α_{41} ($G_{i\alpha}$) and α_{39} ($G_{o\alpha}$), these investigators also noted a 40-kd toxin substrate in their purified preparations. The question of possible proteolytic origin of α_{40} and α_{39} was not settled in this report, and this remains an issue for α_{40}. However, it is possible that α_{40} corresponds to $G_{i\alpha 2}$, as defined above.

Although the function of G_o remains to be determined, its discovery has had a major impact. Since bidirectional regulation of adenylyl cyclase activity had presumably been settled with the discovery of G_s and G_i, the existence of another G protein in brain implied a broader role for the family. Furthermore, the abundance of G_o (and G_i) in brain has greatly facilitated experimentation on a number of fronts.

When Itoh and coworkers (80) screened their rat C6 glioma cDNA library for $G_{i\alpha}$ as described above, the first clone detected turned out to correspond to $G_{o\alpha}$. This identification was based on perfect agreement of sequence predicted from the cDNA with that obtained from six tryptic peptides derived from purified rat brain $G_{o\alpha}$ (80). The cDNA for $G_{o\alpha}$ described by Itoh et al lacks nucleotides corresponding to the amino terminus of the protein (probably about 30 amino acid residues); 15 of these are available for the bovine protein (81).

$G_{t\alpha}$ G_t or transducin, purified at about the same time as G_s, has been studied extensively. It is a major component of the disks of the retinal rod outer segment; disks are prepared easily from bovine retina, and mg-quantities of G_t can be purified in one or two days after selective elution of the protein from the disk membrane with GTP (in the absence of detergent) (50). The availability of antibodies to $G_{t\alpha}$ and partial amino acid sequence (81) permitted the essentially simultaneous cloning of cDNAs corresponding to $G_{t\alpha}$ in four laboratories (88–91). Perhaps not surprisingly, at least retrospectively, two sequences, which differ in approximately 20% of the encoded amino acid residues, were elucidated ($G_{t\alpha 1}$ and $G_{t\alpha 2}$). The clones characterized by three of these groups (89–91), which were selected using expression vectors and antibodies to purified "G_t," encode identical sequences of 350 amino acid residues (M_r = 40,000). The odd clone out was selected with an oligonucleotide probe and encodes a protein of 354 residues (88). Antibodies to purified "G_t" detect immunoreactivity in retinal rods but not in cones (92); the same is true of an antipeptide antibody when the peptide sequence was chosen to be specific for the 350-residue protein (93). However, antibodies raised against a peptide synthesized according to sequence specific for the 354-residue protein show reactivity exclusively with cone photoreceptor outer

segments (93). It is assumed that there exist two isoforms of G_t—one that activates the photosensitive cyclic GMP–specific phosphodiesterase of rod outer segments (G_{t1}) and one that plays the analogous role in cones (G_{t2}).

THE STRUCTURE OF G_a The availability of essentially complete amino acid sequences for seven G protein α subunits obviously invites comparisons (Figure 1). Overall, the relationship is striking. $G_{i\alpha 1}$ and $G_{i\alpha 2}$ are most alike (approximately 95% of the residues identical or homologous), in keeping with the tentative designation of both of these molecules as G_is. The two G_t α subunits are also very similar (88% identical or homologous). More surprising is the strength of the relationship between $G_{i\alpha}$s, $G_{t\alpha}$s, and $G_{o\alpha}$ (roughly 80% identical or homologous for all of these comparisons). $G_{s\alpha}$ differs the most (about 50%) from the other α subunits. Its larger mass is due to two discrete "inserts" (residues 72–86 and 324–336) and to additional residues at the amino terminus. A less extensive relationship between G protein α subunits, EF-Tu, and the ras oncogene products is also obvious; the regions of greatest similarity form portions of the guanine nucleotide-binding domain of EF-Tu (75, 94).

Variability among the α subunits is concentrated in three "hot spots": the amino terminus [residues 1–40 of α_{avg}, as defined by Masters et al (95)], residues 120–150, and residues 340–360. Most significant differences between the two $G_{i\alpha}$s are in the second of these regions; nonhomologous differences between the $G_{t\alpha}$s are largely confined to the amino terminal 30 residues. When G_s is compared with any of the other α subunits, the variability that is seen at residues 120–150 extends back to (but not beyond) the region where amino acid residues are inserted in the larger form of $G_{s\alpha}$ (residues 72–86). The variable region near the carboxy terminus of α_{avg} is also immediately adjacent to a G_s-specific insert.

Masters et al (95) have made predictions about the secondary structure of α_{avg}, and the agreement with the crystal structure of the GDP-binding domain of EF-Tu (75) was sufficiently good to inspire a gamble (as defined respectably by one of the authors—see 96). Constraint of the four regions of α_{avg} that are believed to contribute to the guanine nucleotide–binding site (75, 94) with the three-dimensional structure determined for this region of EF-Tu divides α_{avg} into three domains. Two—the amino (1–41) and carboxy (298–396) termini—are obviously mandatory; the third (60–208) results from a long insertion between the first two of the four regions involved in guanine nucleotide binding. Each of the three domains contains one of the "hot spots" mentioned above. Regions of greatest homology are focused around the guanine nucleotide–binding site, and variability increases as one moves away from this core in any direction (with the exception of the extreme carboxy terminus). The authors have speculated on functional roles that might be

assigned to these domains. I consider these arguments in the context of protein-protein interactions, below.

SUBUNIT-SPECIFIC AND NONSPECIFIC ANTIBODIES Elucidation of primary sequence has permitted generation of a number of antipeptide antibodies with predetermined specificity for a given α subunit or for all known α subunits (82, 93, 97). These antibodies have in general been useful for immunoblotting, immunohistochemistry, and immunoprecipitations. There is little information on their reactivities with native subunits or oligomers. Sequences of proven utility are listed in Table 1.

β SUBUNITS Purification of G_s, G_t, and G_i revealed apparently similar 35-kd polypeptides associated with the more distinctive α subunits. The amino acid composition of β prepared from the three oligomers is indistinguishable; the three proteins yield the same electrophoretic pattern of peptides after proteolysis (40, 98). $\beta\gamma$ subunit complexes are functionally interchangeable: for example, $\beta\gamma$ from G_s or G_i appears to interact identically with $G_{s\alpha}$ (42, 99); $\beta\gamma$ from G_i or G_t can interact with $G_{i\alpha}$ or $G_{t\alpha}$ to reconstitute rhodopsin-stimulated GTPase activity (100). Thus arises the issue (not yet resolved) of the identity or nonidentity of β subunits and, if the latter, their total number. It is a particularly pertinent question in the context of possible dissociation (and mixing) of G protein subunits as part of their mechanism of activation (see below).

It is now clear that there is some level of heterogeneity of β. The "35-kd" subunit of G_s, G_i, and G_o can be resolved into a doublet by SDS-polyacrylamide gel electrophoresis (32, 84). The terminology β_{36}/β_{35} has arisen to define this situation. $G_{t\beta}$, by contrast, displays only one component of this doublet. Its electrophoretic mobility corresponds to that of the upper band, but it is not known if $G_{t\beta}$ is identical to β_{36}. β_{36} and β_{35} are also distinguishable immunologically. Polyclonal antisera to purified $G_{t\beta}$ (β_{36}?) or β_{36}/β_{35} react almost exclusively with β_{36} (97, 101). Antipeptide antibodies prepared against a sequence common to $G_{t\beta}$ and a mixture of β_{36}/β_{35} have great preference for β_{36} (97). This situation is confusing. It is possible that β has very few strong antigenic determinants and that crucial sites may be altered between β_{36} and β_{35}. It is difficult to believe that β_{36} and β_{35} are grossly different.

Evans et al (102) have recently characterized a form of β_{35} from human placental membranes. The protein was resolved (by DEAE) from oligomeric G proteins, wherein the β subunit has the typical β_{36}/β_{35} doublet structure. β_{35} and the β subunit doublet preparation have similar abilities to inhibit adenylyl cyclase activity, presumably by virtue of interaction with $G_{s\alpha}$ (see

below). An apparently identical γ subunit is associated with both β_{35} and the β_{35}/β_{36} doublet.

Sugimoto et al (103) have cloned a cDNA for $G_{t\beta}$. The protein has 340 amino acid residues (M_r = 37,400). Two bands were detected when the cDNA was utilized for Northern hybridization with retinal, brain, and liver RNA (~1.8 and 3.3 kb). Two β subunit clones were also isolated from a bovine brain cDNA library. Restriction mapping with seven endonucleases revealed differences only in the 5' noncoding region. It was concluded that the mRNAs for β have the same coding sequence.

An apparently identical cDNA clone for $G_{t\beta}$ was also isolated by Fong and coworkers (104). The authors noted that the entire sequence of $G_{t\beta}$ consists of a reiterated pattern of about 86 amino acid residues; each of these can be divided into two similar 43-residue segments. In addition, there is a resemblance between $G_{t\beta}$ and the carboxy-terminal portion of the yeast CDC4 gene product. (CDC4 is a cell-division-cycle gene of unknown function.) Northern analysis of several tissues revealed 1.8- and 2.9-kb mRNAs.

Robishaw has isolated a cDNA that includes β-subunit-specific sequences from a bovine adrenal library (unpublished observations). The nucleotide sequence of this clone is quite different from those reported previously (103, 104), and the deduced amino acid sequence also differs significantly. This cDNA hybridizes with a 1.8-kb mRNA. Thus there appear to be at least two genes for β. Their relationship to β_{36}/β_{35} is unknown. Nevertheless, there is as yet no reason to believe that there are differences among the β subunits of G_s, G_i, and G_o.

γ SUBUNITS Ignorance becomes more obvious with regard to γ. This subunit of G_t was recognized early (25, 50), but its detection as a component of G_s and G_i was delayed because of poor avidity for stain (41, 76). β and γ remain tightly associated under nondenaturing conditions. They dissociate as a complex from G_α in the presence of activating ligands (see below).

cDNAs that encode $G_{t\gamma}$ have been cloned and sequenced (105, 106); the protein has been sequenced as well (107). $G_{t\gamma}$ has 74 amino acid residues (M_r = 8400) and is very hydrophilic and acidic. Two-dimensional peptide mapping of γ subunits from human erythrocyte G_s and G_i and from bovine brain failed to reveal differences; $G_{t\gamma}$ (bovine or frog) could be distinguished from these other polypeptides (98). Antibodies to $G_{t\gamma}$ fail to recognize γ subunits from other sources (101, 108). Thus the situation with regard to β and γ may be similar. The specialized retinal rod may have distinct β and γ subunits, while those G proteins that are coincidentally expressed in essentially all (G_s, G_i) or several (G_o) cells may share a common $\beta\gamma$ complex. However, there are hints of greater complexity. There may be multiple γ subunits in evidence in the brain G protein preparations of Sternweis & Robishaw (84). An antibody that recognizes a human placental G protein γ subunit apparently

fails to visualize γ in rabbit liver G_i, bovine brain G_i/G_o, bovine G_t, or human platelet G_i (102).

G_p A novel entity, termed G_p by its discoverers (109), has been described recently. Purified from placenta (and visualized in platelets; thus the designation p), G_p may be a member of the immediate G protein family. There are uncertainties, however, which is why its discussion has been postponed to this point.

Purified preparations of G_p contain a GTPγS-binding polypeptide with an apparent molecular weight of 21,000. They also contain approximately equimolar concentrations of an apparently "conventional" $\beta\gamma$ subunit complex. However, evidence for association of putative $G_{p\alpha}$ with $\beta\gamma$ is not yet at hand. G_p is not an obvious substrate for pertussis or cholera toxin, GTPase activity has not yet been demonstrated, and it is not recognized by antibodies to highly conserved domains of the α subunits described above. (It is also not recognized by anti-ras antibodies.) G_p is of obvious interest; given its size and, perhaps, a low affinity for $\beta\gamma$, it may resemble ras more than do the other signal transducing G proteins. The simultaneous choice of the letter p to designate this entity and that hypothetical G protein responsible for regulation of phospholipase C has generated some confusion. The function of G_p, as defined by Evans et al (109), is unknown.

LIGAND–G PROTEIN INTERACTIONS

Studies of the interactions of G protein α subunits with nucleotides have focused particularly on GTPγS (or other nonhydrolyzable triphosphate analogues), GTP, and GDP. The characteristics of the binding reactions are influenced by Mg^{2+}, anions, and proteins that interact with α (particularly receptors and $\beta\gamma$). Given the existence of at least four purified G proteins available for study, the potential for accumulation of data is large.

Binding of GTPγS to oligomeric G proteins or to their resolved α subunits is clearly not a diffusion-controlled process, and it proceeds at a rate that is independent of nucleotide concentration (41, 84, 110, 111). This anomaly is explained by the fact that the proteins, as purified, contain stoichiometric amounts of GDP, obviously bound with high affinity (49, 111). GDP can be removed from G_i or G_o by chromatography in the presence of 1 M $(NH_4)_2SO_4$ and 20% glycerol (111). The kinetics of GTPγS binding to these nucleotide-free α subunits or oligomers is then bimolecular and apparently diffusion-controlled.

There is negative cooperativity of the binding of GTPγS and $\beta\gamma$ to G protein α subunits; thus, GTPγS promotes G protein subunit dissociation (32, 33, 38, 39, 112).

$$G_{\alpha\beta\gamma} + GTP\gamma S \leftrightarrows G_{\alpha}\cdot GTP\gamma S + G_{\beta\gamma} \quad 1.$$

Mg^{2+} shifts the equilibrium for this reaction far to the right. The rate of dissociation of GTPγS from G_α is slow, but measurable, in the absence of Mg^{2+} (0.4 min^{-1} for $G_{o\alpha}$; 0.2 min^{-1} for $G_{i\alpha}$). βγ increases the rate of dissociation of GTPγS by about threefold in the absence of the divalent cation (113).

The effect of Mg^{2+} on the binding of GTPγS is striking; the rate of dissociation of the nucleotide from $G_{o\alpha}$ or $G_{i\alpha}$ is reduced to near zero (113). The apparent K_d for interaction of Mg^{2+} with G_α·GTPγS is extremely small—about 5 nM. Low concentrations of Mg^{2+} have a similar capability to slow dissociation of GTPγS from oligomeric G_o or G_i. However, the rate of nucleotide dissociation remains measurable until the concentration of Mg^{2+} exceeds 1 mM. At higher Mg^{2+} concentrations subunit dissociation occurs and, as mentioned, G_α·GTPγS·Mg^{2+} is extremely stable. The significance of the subunit dissociation reaction will be discussed further below.

The intrinsic fluorescence of G_α is enhanced modestly on binding of GTPγS and more dramatically in the presence of nM concentrations of Mg^{2+} (114). F^-, Al^{3+}, and Mg^{2+} cause a similar effect. This change in fluorescence is presumed to reflect the activated state of the G protein α subunit.

The interactions of GDP with G protein α subunits provide a contrast with those of GTPγS (113). Brandt & Ross first reported the differing effect of βγ on dissociation of GTPγS and GDP from $G_{s\alpha}$ (115); βγ inhibits the dissociation of GDP (unless the Mg^{2+} concentration is high; see below). This phenomenon has been studied in more detail with G_o and $G_{o\alpha}$. The affinity of GDP for $G_{o\alpha}$ is high (K_d ~40 nM in the absence of Mg^{2+}) and is increased markedly by βγ (K_d ~0.1 nM). This effect appears to result from both a substantial increase in rate of association of GDP with the protein (surprisingly) and a decrease in the rate of dissociation. In the presence of 10 mM Mg^{2+} the effect of βγ on the affinity of $G_{o\alpha}$ for GDP is not as great, but it is still substantial (K_d ~100 nM for $G_{o\alpha}$, 10 nM for oligomeric G_o). The effect of Mg^{2+} is to decrease the rate of association of GDP with $G_{o\alpha}$ or G_o and to increase the rate of dissociation from G_o; however, there is no effect of the metal on the dissociation of GDP from $G_{o\alpha}$ (0.3 min^{-1}). It seems probable that the extreme high affinity of Mg^{2+} for the nucleotide-protein complex noted above is a property only of the GTP- (or GTPγS-) bound form of the protein. Thus Mg^{2+} and GTPγS promote dissociation of oligomeric G proteins and the formation of an "activated" state of G_α; GDP stabilizes the oligomer and, at modest concentrations of Mg^{2+}, dissociates from it extremely slowly.

The interaction of G proteins with their physiological activator, GTP, is of course more complex, in that nucleotide hydrolysis is involved. The basal

GTPase activity, which is extremely low, has been evaluated for G_s (115), G_i (86, 116, 117), and G_o (85, 86, 113, 118). I will ignore modest quantitative discrepancies between these studies, particularly because most are uninterpretable. A typical molar turnover number is 0.3 min^{-1}; the K_m for GTP is low (0.3 μM), and nM concentrations of Mg^{2+} satisfy the requirement for divalent cation. GTP increases the intrinsic fluorescence of G_α, apparently in the same manner as does GTPγS (118). Fluorescence intensity declines as the bound nucleotide is hydrolyzed. Rate constants and relative steady-state concentrations of G·GTP and G·GDP can thus be measured by quantitation of intrinsic fluorescence (118) or with radioactive nucleotides (115, 119). During steady-state hydrolysis the great majority of the protein exists as the GDP-bound form, since k_{cat} exceeds k_{off} for GDP (0.3 min^{-1}) by an order of magnitude. The rate of dissociation of GDP thus limits the basal GTPase activity. As mentioned, $\beta\gamma$ inhibits the dissociation of GDP from $G_{o\alpha}$ at low concentrations of Mg^{2+} and thereby inhibits GTPase activity (113). As the concentration of Mg^{2+} is increased, the rate of dissociation of GDP from G_o (but not from $G_{o\alpha}$) increases and can exceed the value observed with $G_{o\alpha}$. Under such conditions $\beta\gamma$ activates the GTPase activity of $G_{o\alpha}$ (113). The concentration of Mg^{2+} required for this effect on the dissociation of GDP from oligomeric G proteins is high and is dependent on the protein in question. Although not studied systematically, one can estimate that the effect occurs in the range of 1–10 mM for G_o, 5–50 mM for G_i, and 10–100 mM for G_s (86, 113, 115).

These effects of Mg^{2+} are complicated further by the counter ion, since high concentrations of Cl^- appear to inhibit GTPase activity directly (120). Other effects of relatively modest (mM) concentrations of Cl^- have also been noted, including the ability to inhibit the rate of dissociation of GTPγS and GTP (but not GDP) from $G_{o\alpha}$ (120). Lubrol inhibits the steady-state rate of GTP hydrolysis by interfering with the dissociation of GDP (115). Variations in concentrations of Cl^- and Lubrol account for some of the quantitative discrepancies that are apparent in the literature.

The anomalous ability of F^- to activate adenylyl cyclase was found to be a result of interaction of the anion with G_s (14, 121), and it has since become clear that there is a characteristic effect of F^- on all G proteins (38, 122). Manifestations of these interactions closely resemble those with nonhydrolyzable guanine nucleotide analogues: G proteins become "activated" (i.e. capable of fruitful interaction with their effector molecules), G_α dissociates from $\beta\gamma$, and there is an enhancement of intrinsic fluorescence, at least of G_o (114). Curiously, Al^{3+} (or Be^{2+}) was found to be required for activation of G_s by F^-, and it was suggested that the activating ligand was AlF_4^- (123). Bigay et al (124) have suggested that AlF_4^- interacts only with the GDP-bound form of G_α, and that the anion mimics the role of the γ-phosphate of GTP. It should

be possible to verify this very attractive hypothesis by rigorous demonstration of a requirement for bound GDP for AlF_4^--stimulated interaction between G_α and an appropriate effector.

LIGAND-REGULATED PROTEIN-PROTEIN INTERACTIONS

Characteristics of the interactions of ligands with isolated G proteins have been presented above for the sake of simplicity. Their effects on the protein-protein interactions that characterize transmembrane signaling systems are obviously at the heart of mechanism, and these are now being studied in detail with purified reconstituted systems (Figure 2). It should be noted that crucial features of many of these interactions were deduced correctly by study of impure or intact systems, in some cases even before the components had been unambiguously identified. The most important of these deductions were noted above. Of course, this early phase of research was also characterized by many incorrect mechanistic interpretations.

Receptor–G Protein Interactions

The interaction of receptor with a G protein is driven by an appropriate agonist (hormone, photolyzed retinal, etc). This was implied by the comigration of crude β-adrenergic receptors and G_s after solubilization in the presence of agonist (and absence of guanine nucleotide) (125) and by study of the interactions of G_t and rhodopsin. The interaction between R and G is antagonized by guanine nucleotide, either GTP or GDP.

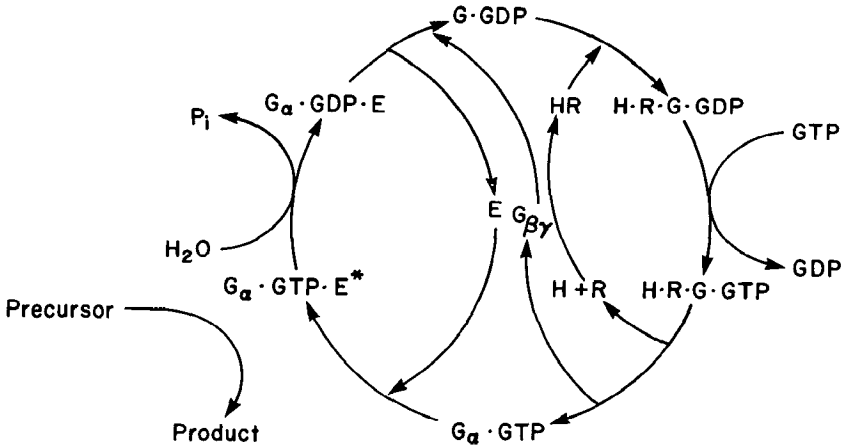


Figure 2 Interactions of receptor, G protein, GTP, and effector. See text for explanation. Modified from Stryer (5).

Pedersen & Ross (126) developed the first successful reconstitution of β-adrenergic receptors and G_s in phospholipid vesicles, and the basic approach has now been utilized extensively for this hormone receptor system and for others. Detailed study of the properties of these interactions has revealed their essential regulatory features (119, 127–132). I will concentrate on the β-adrenergic receptor and G_s in this discussion, since this system has been studied most extensively.

1. HR stimulates dissociation of G·GDP (119). Dissociation of GDP obviously must precede binding of GTP if there is but one site for nucleotide, and only one such site has been detected (however, see 133).
2. HR stimulates nucleotide binding, even when most of the bound GDP has been induced to dissociate by interaction of G·GDP with HR (119). Thus, release of GDP per se is required but is not necessarily sufficient for hormone-stimulated, GTP-mediated activation of G_s. A similar conclusion had been reached by Tolkovsky et al (134), who examined the rate of activation of adenylyl cyclase by Gpp(NH)p and epinephrine in membranes where G_s had hypothetically been cleared of GDP by incubation with hormone. It is perhaps simplest to envision a guanine nucleotide–binding site that is "closed" in the absence of HR and "open" (allowing nucleotide exchange) in its presence.
3. HR stimulates the steady-state GTPase activity of G_s (1–2 min^{-1}) without affecting k_{cat} (4 min^{-1}). This effect is due exclusively to HR-stimulated dissociation of GDP and association of GTP and the resultant accumulation of significant levels of G·GTP.
4. HR functions catalytically (126, 131); one receptor can interact with ~10 molecules of G_s over a period of a few seconds in a single phospholipid vesicle. These observations verify the same conclusion by Tolkovsky & Levitzki (135, 136), who had studied the kinetics of activation of adenylyl cyclase after inactivation of receptor with an irreversible antagonist.
5. HR-stimulated nucleotide exchange requires the $\beta\gamma$ subunit complex of the G protein (131). This observation was made initially with G_t and rhodopsin (137).
6. There are at least two requirements for Mg^{2+} for maximal catecholamine-stimulated GTPase activity: low (nM) concentrations of Mg^{2+} are necessary for nucleotide hydrolysis per se and higher (10 μM) concentrations maximize HR-catalyzed nucleotide exchange (119). This latter requirement is consistent with the initial observation of Iyengar & Birnbaumer (138), who demonstrated that glucagon lowered the concentration of Mg^{2+} necessary for activation of G_s by GTPγS from 25 mM to 10 μM.
7. Reconstitution of R and G results in the establishment of guanine nucleotide–sensitive agonist binding to the receptor (128). Low-affinity agon-

ist states are R and its presumed equivalent, R in the presence of G·GTP or G·GDP (i.e. R and G not associated); the high-affinity state is R·G (nucleotide dissociated). Rojas & Birnbaumer (139) have highlighted the importance of GDP in this negative binding interaction with agonist; participation by GDP seems mandatory. They have also suggested that GTP may not have a similar effect. If true, binding of GTP would apparently not cause dissociation of HR from G, and the active complex of G and effector would then incarcerate HR—drastically reducing its catalytic efficiency.

To summarize, most would agree to the following model. The affinity of HR for G·GDP is sufficient to drive their interaction and to promote dissociation of the nucleotide. HRG is presumably a relatively stable intermediate, but its lifetime is brief in the presence of a normally high concentration of GTP. Binding of GTP causes dissociation of HR (see 133). The lifetime of G·GTP (or G_α·GTP, see below) is many seconds. The catalytic action of HR and the relatively long lifetime of G·GTP provide considerable amplification.

MULTIPLE EFFECTS OF MG^{2+} Effects of Mg^{2+} have been described just above and in the preceding section. It may be useful to summarize these observations and to speculate on their significance. The list of effects and approximate concentrations required is as follows: 1. GTPase, ~5 nM; 2. slow dissociation of GTPγS from G_i or G_o, ~5 nM; 3. fluorescence enhancement of $G_{o\alpha}$, < 100 nM; 4. HR-stimulated G_s activation, ~10 μM; 5. HR-stimulated GTP binding and, by inference, GDP dissociation, ~10 μM; 6. βγ-stimulated GDP dissociation, 1–100 mM; 7. GTPγS-induced subunit dissociation, 1–100 mM.

Once GTP or GTPγS is bound, interaction of Mg^{2+}, presumably with both protein and nucleotide, occurs with extremely high affinity. Effects 1–3 above are all believed to reflect interaction at this site, and this is presumably sufficient to "activate" a resolved G protein α subunit. I speculate that effects 4–7 all reflect interaction of Mg^{2+} at a second site, whose location of G (α, β, or γ) is unknown. The apparent affinity of this site for Mg^{2+} is relatively poor in the absence of HR (1–100 mM). Interaction of Mg^{2+} at this site is necessarry for βγ-facilitated "opening" of the guanine nucleotide–binding site to permit dissociation of GDP, association of GTP or GTPγS, and nucleotide-induced subunit dissociation. HR lowers the concentration requirement for Mg^{2+} at this hypothetical single site; Iyengar & Birnbaumer have stressed the importance of such an interaction (138). Viewed in this context, HR shifts the dependency on Mg^{2+} from a concentration range where βγ stabilizes the binding of GDP to a range where βγ actually facilitates guanine nucleotide exchange.

SPECIFICITY OF R-G INTERACTIONS The availability of purified G proteins and receptors has permitted tests of specificity of the functional interactions between R and G by reconstitution. Prototypical receptors have been the β-adrenergic (adenylyl cyclase stimulator), α_2-adrenergic (adenylyl cyclase inhibitor), muscarinic cholinergic (cyclase inhibitor or phospholipase C stimulator), and rhodopsin.

G_s appears to be rather specific, in that it interacts selectively with receptors that stimulate adenylyl cyclase activity. The ability of rhodopsin or the α_2-adrenergic receptor to stimulate nucleotide binding to this G protein is minimal (140, 141). Similarly, the interaction between transducin and the β-adrenergic receptor is difficult to detect; however, there is a measurable reaction between transducin and the α_2-adrenergic receptor (~20% as effective as rhodopsin). G_i and G_o are more promiscuous. It is presumed that their interactions with muscarinic (71, 72, 142) and α_2-adrenergic (141) receptors in vitro reflect their physiological activities. Surprising was the observation of a very significant level of interaction between G_i and β-adrenergic receptors in vitro (143); rhodopsin also stimulates the GTPase activity of G_i and G_o to about the same extent as that of G_t (100, 141).

The unexpected extent of cross-reactivity between receptors and G proteins almost certainly speaks to conservation of structure among the receptor-binding domains of the G proteins and the G protein-binding domains of the receptors. The ability to compare the primary structures of G protein–linked receptors was acquired recently with the cloning of cDNAs for the second such entity, the β-adrenergic receptor (144, 145); the first sequence was, of course, that of rhodopsin (146). The two receptors display an intriguing level of overall similarity, including the fact that both appear to span the bilayer seven times. Interestingly, the most conserved sequences in the two receptors are in the transmembrane spanning regions. It has been suggested that cytoplasmic loop 1-2 of rhodopsin is involved in the interaction with G_t (147).

Masters et al (95) have suggested that the carboxy-terminal domain of G_α is responsible for interaction with receptors. The most compelling argument is that the carboxy-terminal 21 residues of $G_{t\alpha}$ are homologous with an internal region of arrestin (148), a retinal protein that binds to phosphorylated rhodopsin (149). ADP-ribosylation of G_i and G_t on a cysteine residue four removed from the carboxy terminus prevents G protein–receptor interactions (150, 151). Analysis of the UNC mutant of the S49 lymphoma may also reveal a modest amount of information on the receptor-binding domain of G_α. This specific lesion eliminates interaction between G_s and receptors, leaving the other functions of the G protein intact (152).

Lack of the expected specificity for receptor–G protein interaction has stimulated investigation of heretofore unsuspected physiological regulatory mechanisms. Although rhodopsin and G_i presumably never have the opportu-

nity to interact in vivo, the β-adrenergic receptor and G_i presumably do. The questions, therefore, are whether this interaction occurs in vivo; if so, to what purpose; and if not, why not? Ligand-binding studies carried out by Abramson & Molinoff strongly suggest an interaction between the β-adrenergic receptors of cyc$^-$ S49 cells and a G protein (153). These cells contain G_i (83, 154, 155); they lack $G_{s\alpha}$ activity, protein, and mRNA (77); they do not appear to contain G_o. Murayama & Ui (156) have suggested that an interaction between β-adrenergic receptors and G_i is responsible for β-adrenergic agonist-mediated inhibition of adenylyl cyclase in adipocyte membranes. Treatment of many cell types with pertussis toxin potentiates the effects of stimulatory hormones on adenylyl cyclase activity (37). Perhaps this is due in part to elimination of an interaction between G_i and stimulatory receptors (143). Also interesting is that pertussis toxin can prevent homologous desensitization of adenylyl cyclase, at least in some systems (157, 158). Cerione et al (159) have attempted to demonstrate a role for G_i in hormonal stimulation of adenylyl cyclase by reconstitution of β-adrenergic receptors with G_s, G_i, and a crude preparation of the cyclase itself. Hormonal stimulation of cyclic AMP synthesis increased as a percentage of basal activity, but absolute activities decreased (basal > hormone stimulated) as G_i was added. This effect is presumably due to $\beta\gamma$ (see below) and probably has little to do with any interaction between the receptor and G_i.

G Protein–Effector Interactions

Two G protein–effector interactions are relatively well defined—G_t-phosphodiesterase and G_s-adenylyl cyclase.

PHOSPHODIESTERASE The cyclic GMP–specific phosphodiesterase of the rod outer segments is also a heterotrimer (α: 88 kd; β: 84 kd; γ: 11 kd)(160). It is loosely associated with the rod outer segment disks and can be purified in the absence of detergent. The native trimer is essentially inactive, but catalysis is increased markedly after limited tryptic digestion (161). This release from inhibitory constraint is apparently due to proteolysis of the γ subunit (162). γ has a K_d of 0.1 nM for $\alpha\beta$, and the activity of $\alpha\beta$ can be titrated over a broad range (i.e. inhibited) by addition of purified γ. The fully active phosphodiesterase has a ratio of k_{cat}/K_m ($6 \times 10^7\ M^{-1}\ sec^{-1}$) equal to those of catalase and carbonic anhydrase—near the diffusion-controlled limit (5). $G_{t\alpha}$·Gpp(NH)p (resolved from $\beta\gamma$) activates the phosphodiesterase to the same extent as does trypsin, presumably by alteration of the interactions of the subunits or by displacement of γ (25). A recent study by Sitaramayya et al (163) suggests that the composition of the activated complex may be $PDE_{\alpha\beta}$·$G_{t\alpha}$ or $PDE_{\alpha\beta\gamma}$·$G_{t\alpha}$. In view of the catalytic prowess of the phosphodiesterase, it is clear that a significant amount of cyclic GMP can be hydrolyzed during the lifetime of $G_{t\alpha}$·GTP.

Gpp(NH)p causes dissociation of the subunits of G_t (25, 137), and, as noted, $G_{t\alpha}$·Gpp(NH)p activates the phosphodiesterase in the absence of $G_{t\beta\gamma}$. It is not possible to do the same experiment with GTP because of hydrolysis of GTP by $G_{t\alpha}$. However, Fung studied the dependence of the rhodopsin-catalyzed GTPase activity of $G_{t\alpha}$ on the ratio of $G_{t\alpha}$ to $G_{t\beta\gamma}$ (137). The subunits bind to rhodopsin in equimolar quantities, and both α and $\beta\gamma$ are required for rhodopsin-stimulated GTPase activity. However, under conditions where GTPase activity was linearly dependent on $G_{t\alpha}$, the requirement for $\beta\gamma$ was saturated at a $G_{t\alpha}$:$G_{t\beta\gamma}$ of approximately 20:1. This important experiment indicates that the subunits can be mostly dissociated and function maximally as a receptor-stimulated GTPase. One $G_{t\beta\gamma}$ can catalyze the binding of GTP to many $G_{t\alpha}$ subunits. Thus, subunit dissociation is driven by the binding energy of GTP and Mg^{2+}. The phenomenon is not a unique property of the interaction of G protein α subunits with nonhydrolyzable guanine nucleotide analogues.

ADENYLYL CYCLASE Adenylyl cyclase exists as multiple molecular species. At least one major form of the enzyme in brain is activated by calmodulin, probably directly (164–166). Most species are also stimulated directly by an unusual diterpene, forskolin, isolated from the roots of the aromatic herb *Coleus forskohlii* (167). The cyclase has been purified from heart (168) and brain (166, 169, 170) using affinity chromatographic techniques with immobilized calmodulin or forskolin, pioneered by Storm and colleagues (171) or Pfeuffer & Metzger (24), respectively. Although some differences among these preparations are apparent, the enzyme appears to be a single polypeptide with a molecular weight of approximately 150,000; it also appears to be a glycoprotein (166, 168). Preparations of the enzyme from Gpp(NH)p-treated membranes (which treatment greatly stabilizes activity) contain $G_{s\alpha}$, but little or no $G_{\beta\gamma}$ (168, 169). Some preparations from untreated membranes appear to be rather free of G protein subunits (166, 170); some apparently are not (169). However, Arad et al (172) have indicated that adenylyl cyclase and G_s copurify during the initial stages of fractionation and appear to be associated even when they have not been exposed to nonhydrolyzable guanine nucleotide analogues. Differences in conditions (particularly in detergent) are presumed to account for these discrepancies (see below).

Levitzki has proposed that adenylyl cyclase is always coupled to G_s in vivo. This argument is based on the work just described (172) and on kinetic analysis, which indicates that the interaction between G_s and C is not rate limiting (134). I find it difficult to conclude that all of the cyclase is always associated with G_s, based on its behavior in detergent. However, this is not to deny the possibility. The suggestion then opens the question of the role of the considerable excess of G_s over adenylyl cyclase and how free G_s interacts with HR compared with the interactions of HR with G_s·AC.

Stimulation Adenylyl cyclase is activated by G_s (Figure 3). In the absence of the regulatory protein catalytic activity is nearly undetectable in the presence of the usual substrate, MgATP (15); some activity is observable with MnATP. A hormone-sensitive adenylyl cyclase activity has been reconstituted in phospholipid vesicles from three purified proteins—the β-adrenergic receptor, G_s, and the cyclase itself. Thus, these three proteins suffice to constitute a primary pathway for hormonal stimulation of cyclic AMP synthesis (173). Since the turnover number of adenylyl cyclase is probably about 1000 min^{-1}, several hundred molecules of cyclic AMP can be made during the lifetime of a single G_s·GTP.

Adenylyl cyclase is activated by $G_{s\alpha}$·GTPγS (112). This interaction is direct, and, as mentioned, a complex of $G_{s\alpha}$·Gpp(NH)p associated with adenylyl cyclase is sufficiently stable to survive purification to homogeneity (168). The capacity of $G_{s\alpha}$ to activate adenylyl cyclase accounts for the activity of oligomeric G_s (112). $G_{\beta\gamma}$ inhibits activation of G_s by GTPγS (110). Activation of G_s by AlF_4^- appears to occur by a similar mechanism: subunits dissociate (32); $G_{\beta\gamma}$ increases the rate of deactivation of F^--activated G_s (99).

Inhibition Elucidation of the mechanisms of inhibition of adenylyl cyclase by G_i has been less straightforward. Purification and reconstitution of the oligomer indicate that it can indeed mediate hormonal inhibition of the enzyme, and incubation of G_i with GTPγS causes characteristic "activation" of the inhibitory capacity of the protein, accompanied by dissociation of its subunits to $G_{i\alpha}$·GTPγS and $G_{\beta\gamma}$ (42, 43). However, resolution of the subunits, followed by their individual reconstitution with platelet or wild-type

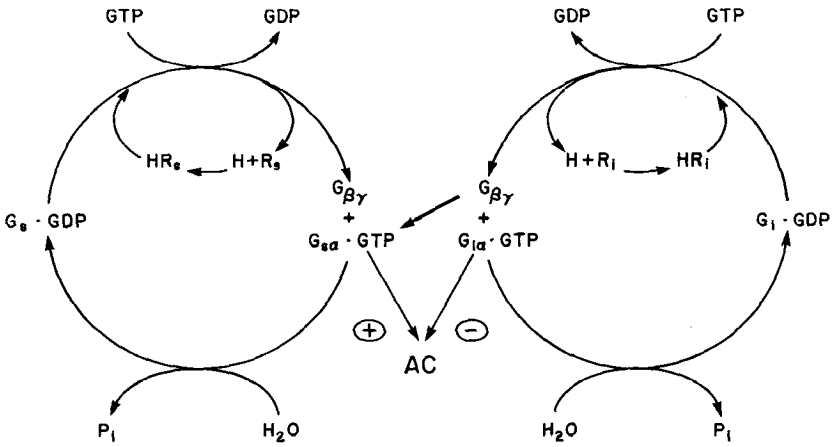


Figure 3 Mechanisms of receptor-mediated stimulation and inhibition of adenylyl cyclase.

S49 cell membranes, revealed only modest inhibitory activity associated with $G_{i\alpha}$·GTPγS; the $\beta\gamma$ subunit complex was the primary source. [The profound inhibitory effect of $\beta\gamma$ (from G_s, G_i, G_o, or G_t) on the adenylyl cyclase activity of normal plasma membranes from a variety of cells has subsequently been observed in several laboratories (174, 175); it is a fact and it has implications (see below).] The inhibitory activity of $\beta\gamma$ is dependent on the presence of G_s (83, 166). Direct inhibition of adenylyl cyclase by $\beta\gamma$ seems unlikely. Although the possibility of this interaction was raised by Katada et al (176), Smigel's data (166) suggest that this effect was due to contamination of crude adenylyl cyclase with G_s. It should be noted that G_i is in considerable excess of G_s in all tissues that have been examined. Thus, G_i can serve as a reservoir of $\beta\gamma$, available to buffer the release of $G_{s\alpha}$.

Based on these observations it was proposed that $\beta\gamma$ can mediate hormonal inhibition of adenylyl cyclase by interaction with $G_{s\alpha}$ (Figure 3):

$$G_{s\alpha} + G_{\beta\gamma} \rightleftarrows G_{s\alpha\beta\gamma} \qquad 2.$$

Several additional observations support this hypothesis; I will mention one here (43). When platelet membranes are treated briefly with GTPγS and an α_2-adrenergic agonist at low Mg^{2+} concentrations, adenylyl cyclase is "irreversibly" inhibited. This inhibition is of the same magnitude as that produced by maximally effective concentrations of $\beta\gamma$, and it is not additive with the effect of $\beta\gamma$. The inhibition is overcome completely by reconstitution of the membranes with physiological concentrations of $G_{i\alpha}$·GDP. The most reasonable explanation for this fact is interaction between $G_{i\alpha}$·GDP and $G_{\beta\gamma}$ to relieve the inhibition caused by free $\beta\gamma$ in the membrane.

It was never proposed that inhibition of adenylyl cyclase by the indirect action of $\beta\gamma$ was the only possible mechanism, and this notion was untenable from the beginning because of the observation of hormonal inhibition of adenylyl cyclase activity in the cyc^- S49 cell mutant (154). These cells lack all traces of $G_{s\alpha}$, and, logically, $\beta\gamma$ is not inhibitory when reconstituted with cyc^- membranes (83). The relatively modest inhibitory effect of $G_{i\alpha}$ was invoked to explain this situation (83). This inhibitory effect of $G_{i\alpha}$·GTPγS has also been observed by Roof et al (101). It does not appear to be a property of $G_{o\alpha}$·GTPγS (101, 176). The effect of $G_{i\alpha}$·GTPγS on adenylyl cyclase is competitive with that of $G_{s\alpha}$ (176). It should be noted, however, that inhibition of adenylyl cyclase in cyc^- is assayed under unusual conditions; in the absence of $G_{s\alpha}$, forskolin is included to observe a significant level of enzymatic activity.

G PROTEIN SUBUNIT DISSOCIATION The hypothesis of indirect inhibition of adenylyl cyclase by $G_{\beta\gamma}$ is surrounded by a certain level of controversy,

which centers in particular around the issue of dissociation of G protein subunits. When G_s and G_t were the only two G proteins in the picture, the possibility of subunit dissociation was of modest interest to a few. However, there are important implications if an inhibitory, shared subunit is released on activation of any of several G proteins. In particular this would provide a mechanism for coordination of the activity of opposing pathways of transmembrane signaling (3). Activation of one pathway would inhibit others, depending on the relative strength of signal input and the relative concentrations of the pertinent reactants in a given cell. A corollary is that G proteins that shared a $\beta\gamma$ subunit complex with G_s would all be "G_is" in terms of regulation of adenylyl cyclase. Differences in $\beta\gamma$ among G proteins would be a mechanism to partition their reciprocal interactions. A G protein without a $\beta\gamma$ subunit complex (? ras) would be immune to such regulation. Dissociation of G protein subunits thus provides a literal branch point in pathways for regulation of cell function: G_α initiating certain actions and $G_{\beta\gamma}$ terminating others. In view of these considerations, the issue of the reality of G protein subunit dissociation in the bilayer has assumed some importance.

The subunit dissociation model is based in part on the fact that the phenomenon occurs in solution (and, therefore, usually in the presence of detergent) with all G proteins examined when exposed to GTPγS, Gpp(NH)p, or AlF_4^- and Mg^{2+}. Evidence discussed above indicates that GTP has the same effect on G_t. G protein α subunits are sufficient to activate their effectors. The $\beta\gamma$ subunit complex interferes with these effects. The $\beta\gamma$ subunit complex is distinctly inhibitory to hormone-stimulated adenylyl cyclase activity in normal membranes. This effect does not appear to be exerted directly on adenylyl cyclase and is dependent on the presence of G_s. Thus, it implies that there exists a steady-state concentration of free $G_{s\alpha}$ in the bilayer. Based on these facts, the hypothesis is a reasonable one. Whether it is true is another matter. It has always been recognized that subunit dissociation in the bilayer has not been demonstrated directly. This is an obvious deficiency, although one that is difficult to remedy. Until it is, the question remains open. If such a demonstration is to be taken seriously, I believe that it must occur in a normal membrane, where the concentrations of the reactants are physiological and the environment, although unknown, is relevant.

Trivial criticisms of the subunit dissociation hypothesis include the notion that adenylyl cyclase is always associated with G_s, as discussed above. This possibility does not deny the potential for dissociation of subunits; it is easily accommodated by GTP-induced dissociation of $\beta\gamma$ from the complex of G_s and the enzyme, as noted by Levitzki (4). Similarly, disagreement about the relative abilities of $G_{i\alpha}$ and $G_{\beta\gamma}$ to cause inhibition does not speak to the issue of whether or not subunits actually dissociate.

GTPγS can cause a conformational change of G_s or G_i at 0°C, which was

detected as a change in their sedimentation coefficients (177). However, actual subunit dissociation did not occur until the protein was warmed. The authors described the altered form of the G protein as "preactive." Others have mistakenly stated that Codina et al (177) claimed that the ability of G_s to activate adenylyl cyclase preceded subunit dissociation.

It has also been suggested that the lifetime of G·GTP is too short to permit a cycle of subunit dissociation. Recent estimates of k_{cat} suggest a value of approximately 4 min^{-1}. It is difficult to understand how this relatively long lifetime constitutes evidence against the hypothesis without knowledge of the actual rate of dissociation in situ. The status of the proposed $\alpha\beta\gamma \leftrightarrows \alpha + \beta\gamma$ steady state must be measured in the presence and absence of relevant potential perturbants.

G PROTEIN EFFECTOR INTERACTION DOMAIN Masters et al (95) speculate that the long domain (residues 60–208 of α_{avg}) that is inserted between the first two of the four regions involved in guanine nucleotide binding is involved in G protein–effector interactions. The argument is based particularly on analogy with the corresponding (smaller) regions of EF-Tu and ras. It is also reasonable to suggest that the conformation of this domain is likely to be regulated by GTP. H21a is an S49 cell mutant, wherein G_s is capable of interaction with receptors but incapable of interaction with adenylyl cyclase (178). Elucidation of the molecular basis of this defect may shed light on the domain of G_α necessary for interaction with effectors.

ARF ADP-ribosylation of $G_{s\alpha}$ by cholera toxin requires the presence of another protein, which has been termed ADP-ribosylation factor or ARF. Distinguishable soluble (179) and membrane-bound (180) forms of this activity have been characterized, and the latter has been purified (181, 182). Although it is certainly intriguing that ARF is a 21-kd GTP-binding protein, it is not ras (182, see also 183). The tight association of purified ARF with endogenous GDP suggests that the protein is a GTPase, although this activity was not detected under the conditions utilized. Evidence indicates that ARF·GTP (or GTPγS) in association with $G_{s\alpha}$·GDP (but not GTPγS) $\pm$ $G_{\beta\gamma}$ is the substrate for cholera toxin. The significance of the apparent association of G_s with ARF is an important (and elusive) question.

G PROTEIN–MEMBRANE INTERACTIONS

This potentially interesting subject has been neglected. Detergent is required for solubilization of G_s, G_i, and G_o and for their behavior as distinct entities in solution. G_t can be eluted from disk membranes with GTP or nonhydrolyzable triphosphate analogues and does not aggregate in the absence of detergent.

Classical transmembrane spanning sequences have not been found for any G protein subunit. It is assumed that the proteins are associated with the inner face of the plasma membrane.

Sternweis (184) has noted that $G_{i\alpha}$·GDP and $G_{o\alpha}$·GDP behave as soluble monomers in the absence of detergent; $G_{\beta\gamma}$ aggregates. $G_{\beta\gamma}$ associates readily with phospholipid vesicles; the α subunits do not. Of interest, the α subunits interact in a saturable fashion with $G_{\beta\gamma}$-containing phospholipid vesicles. The binding of α is essentially stoichiometric with $\beta\gamma$ and is reversed on addition of GTPγS. It is possible that $G_{\beta\gamma}$ serves as the membrane anchor for G_α and that G_α subunits dissociate from this anchor when activated. If true, their sites of action need not be confined to the plasma membrane. Rodbell has speculated boldly on this subject (185).

COVALENT MODIFICATION OF G PROTEINS

Well-characterized covalent modifications of G proteins are the ADP-ribosylation reactions carried out by toxins elaborated by *V. cholerae* and *B. pertussis* (18–21). Elucidation of the molecular basis of intoxication by these important pathogens is an important landmark, and, of course, the toxins have been of great experimental value. Despite occasional claims to the contrary, there is no convincing evidence for ADP-ribosylation of G proteins as a physiological event.

The complex substrate for ADP-ribosylation of G_s by cholera toxin has been mentioned above. It is not clear if ARF is a requirement for ADP-ribosylation of G_t by the toxin. An arginine residue is the site of modification in G_t (186), and the analogous arginine is presumably ADP-ribosylated in G_s (79). It is not obvious why G_i and G_o are poor substrates for cholera toxin. Differences in sequence surrounding the site of modification or differences in their ability to interact with ARF are the obvious possibilities. The characteristic effect of the ADP-ribosylation is usually described as inhibition of the receptor-stimulated GTPase activity of the G protein (10, 187). This is consistent with the fact that GTP activates receptor-free G_s almost as well as does GTPγS. However, ADP-ribosylation also appears to decrease the affinity of $G_{s\alpha}$ for $G_{\beta\gamma}$, which could account for loss of receptor-dependent GTPase activity (188). The basal GTPase activity of ADP-ribosylated G_s is unimpaired. Further experimentation is necessary.

Pertussis toxin–catalyzed ADP-ribosylation of G_i, G_o, and G_t is somewhat more straightforward, in that ARF is not required; however, resolved α subunits are not substrates. A cysteine residue four removed from the carboxy terminus of the α subunit is the site of modification (81, 189). It is interesting that an analogous cysteine residue in ras is acylated (190). As mentioned above, ADP-ribosylation by pertussis toxin appears to block interactions between G proteins and receptors.

Exposure of cells to phorbol esters causes alterations of their abilities to respond to hormones that interact with G protein–linked receptors. There is, however, no clear picture of mechanism. For example, there are indirect data that are consistent with both enhanced interaction of G_s with adenylyl cyclase (191) and impaired function of the inhibitory pathway (192); these possibilities are obviously not mutually exclusive. Protein kinase C can phosphorylate $G_{i\alpha}$ in vitro, and this reaction is suppressed by $G_{\beta\gamma}$ (193). Unfortunately, there are no data to indicate that this occurs in vivo. This interesting subject is best left for future discussion. Hints of possible covalent modification of G proteins are provided by their isoelectric heterogeneity (194). Variations in the relative quantities of such differing forms have also been noted as a function of development or transformation (195, 196).

Although it is beyond the scope of this review, it is worth mentioning that knowledge of covalent modification of G protein–linked receptors has evolved very impressively in recent years. Rhodopsin is phosphorylated on multiple sites by a rhodopsin kinase, and phosphorylated rhodopsin is then apparently "capped" by interaction with another protein, arrestin (149). The β-adrenergic receptor inspires lavish attention from a hoard of well-known and previously uncharacterized kinases. At least one of these is presumed to be specific for this and related receptors (197). It is predictable that other G protein–linked receptors will be treated similarly. G proteins themselves may be more aloof.

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