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BIOCHEMICAL AND MOLECULAR GENETIC ANALYSIS OF HORMONE-SENSITIVE ADENYLYL CYCLASE

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INTRODUCTION AND SCOPE

The hormone-sensitive adenylyl cyclase of vertebrate cells detects external hormonal signals and transduces them across the plasma membrane into changes in the rate of cAMP synthesis. Each of these events—detection, transduction, and cAMP synthesis—involves a distinct class of membrane proteins. Receptors, integral membrane proteins with specific binding sites for endogenous ligands and drugs, detect the signal. Depending upon the type of receptor, the ligand–receptor interaction results in inhibition or stimulation of cAMP synthesis by the catalytic adenylyl cyclase (C)[1], an integral membrane protein whose enzymatic site faces the cytoplasm. A family of GTP-binding regulatory (G) proteins conveys information between the liganded receptor and the catalyst.

The G-proteins comprise the central element of this signal transduction system. These proteins interact with the receptor–ligand complex, bind GTP, and become activated. They then interact with the catalyst to alter the rate of cAMP synthesis; finally, they terminate their effect on adenylyl cyclase and return to the basal state by hydrolyzing GTP to GDP. Our understanding of

[1]Abbreviations: β-AR, β-adrenergic receptor; GTPγS, Guanosine-5'-O-(3-thiodiphosphate); Gpp[NH]p, guanylyl imidodiphosphate; G_s, G_i, G_o, holo-G-proteins consisting of α, β, and γ subunits; α_s, α_i, α_o, alpha subunits of the respective G-proteins; $\beta\gamma$, beta and gamma subunits of the G-proteins; R*, photoexcited rhodopsin; PDE, cGMP phosphodiesterase; SDS, sodium dodecyl sulfate.

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the functioning of this complex system derives from biochemistry, genetics, and, more recently, from molecular biology. We discuss the biochemistry of the G-proteins in light of information gained from study of the nucleic acid sequences that encode their components. We discuss only briefly earlier work, such as the discovery of GTP effects on adenylyl cyclase and of the G-proteins themselves. The pharmacology and regulation of the receptors themselves similarly fall outside the scope of this review.

THE G-PROTEINS

Both stimulation and inhibition of adenylyl cyclase by hormones require GTP. Indeed, hydrolysis-resistant analogs of GTP (GTPγS and Gpp[NH]p) in many cases exert these effects without hormone. The sites of action of guanine nucleotides (and of aluminofluoride ion, which mimics the effects of GTP analogs) are G_i and G_s. These proteins couple inhibitory and stimulatory receptors, respectively, to the catalytic adenylyl cyclase. The function of a third G-protein, G_o, discussed below, is not known.

Recent purification of G_s, G_i, and G_o (1–4) and subsequent study of the purified proteins (for review see 5) showed that each is an $\alpha\beta\gamma$ heterotrimer. The α subunits contain a single GTP binding site, GTPase activity, and sites for modification by either of two bacterial exotoxins: cholera toxin for the α subunit of G_s (α_s) and pertussis toxin for α_i and α_o. The α subunits differ among the various G-proteins, whereas β (35 or 36 kd) and γ (6–10 kd) appear the same.

Figure 1 shows a generalized scheme for the G-protein regulatory cycle, first proposed by Cassel & Selinger (6, 7) and later elaborated by Gilman and coworkers (5, 8). The hormone-receptor complex promotes GTP binding and activation of the G-proteins. Activated α-GTP dissociates from $\beta\gamma$, and the dissociated form of the G-protein affects the catalyst. Hydrolysis of GTP to GDP by α, and reassociation of α with $\beta\gamma$, deactivates the G-protein and returns the system to the basal state.

G_s, The Stimulatory Guanine Nucleotide–Binding Regulatory Protein

Somatic-cell genetics laid the groundwork for the discovery and subsequent purification of G_s. Bourne and coworkers (9) isolated *cyc*$^-$, a hormone-resistant mutant of the S49 mouse lymphoma cell line. Cyc$^-$ membranes lack G_s activity (10) and the 52- and 45-kd cholera toxin substrates (11). Gilman and coworkers subsequently exploited this system by utilizing *cyc*$^-$ membranes as a bioassay to purify G_s (see 8). G_s, purified from rabbit liver, contains either the 52-kd or the 45-kd cholera toxin substrate as its α subunit, associated in 1:1:1 stoichiometry with β and γ (3, 12). The 52-kd α_s is not

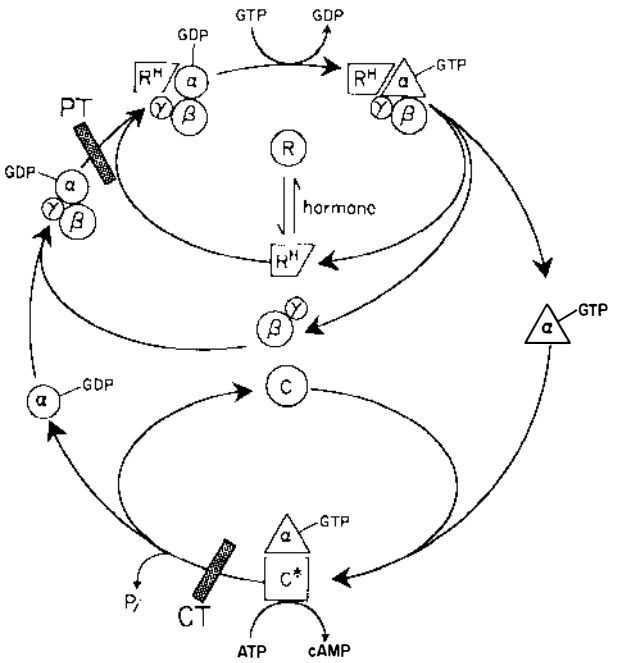

Figure 1 The G-protein regulatory cycle. Abbreviations: R, unliganded receptor; R^H, liganded receptor; C, inactive catalyst; C*, activated catalyst; α, β, γ, G-protein subunits. Stippled bars indicate the points at which cholera toxin (CT) and pertussis toxin (PT) block the cycle by ADP-ribosylation of α.

found in all tissues (3, 13, 14); we discuss its relationship to the 45-kd α_s in a later section.

G_s binds hydrolysis-resistant GTP analogs in a magnesium-dependent manner (3). Binding of GTP analogs, in vitro, results in activation of G_s, as assessed by its ability to stimulate cAMP synthesis in *cyc*$^-$ membranes. Activation of G_s closely parallels GTP analog binding. Both require guanine nucleotide, Mg^{2+}, and have a similar time course (15, 16). Ligands that do not activate purified G_s (GTP, GDP, GMP, and ITP) compete for GTPγS binding and activation with similar affinities (16). Thus, binding of GTP analogs and activation of G_s occur more or less simultaneously. Several other events occur concomitantly with GTP binding and activation. G_s releases bound GDP (7, 17), and α_s dissociates from $\beta\gamma$ (3, 12, 18) and undergoes conformational change (19). The precise mechanism and timing of these events are not yet known.

Whatever the mechanism of G_s activation, stimulation of the catalyst requires only α_s. Activated α_s, separated from $\beta\gamma$, stimulates cAMP production in *cyc*$^-$ membranes (13) and stimulates purified C in phospholipid

vesicles (20). $\beta\gamma$ is not necessary for stimulation of adenylyl cyclase; indeed, it antagonizes the stimulation (21).

Upon removal of activating ligand, deactivation of G_s occurs very slowly and is accompanied by reassociation of α_s with $\beta\gamma$ (13, 16, 21). Addition of purified $\beta\gamma$ to activated G_s or to resolved, activated α_s increases the rate of deactivation (21); indeed, deactivation of α_s-GTPγS absolutely requires the presence of $\beta\gamma$. $\beta\gamma$ may also serve as the membrane anchor for α_s (and α_i and α_o as well). Without $\beta\gamma$, the α subunits do not associate with phospholipid vesicles; $\beta\gamma$, on the other hand, associates readily with vesicles in the absence of α (22).

G_i, The Inhibitory Guanine Nucleotide–Binding Regulatory Protein

Discovery of G_i came not from study of adenylyl cyclase, but rather from research into the mechanism of action of a bacterial toxin secreted by *Bordetella pertussis,* the causative agent in whooping cough. Pertussis toxin (also called *islet-activating protein*) blocks hormonal inhibition of adenylyl cyclase by catalyzing ADP-ribosylation of the α subunit of G_i (23, 24). This toxin-labeling assay facilitated purification of G_i (25, 26).

Like G_s, G_i dissociates into its α (41-kd) and $\beta\gamma$ subunits when activated by hydrolysis-resistant GTP analogs (27–29). Purified G_i reconstitutes α_2-adrenergic inhibition of adenylyl cyclase in platelet membranes whose endogenous G_i has been inactivated by pertussis toxin (27). Experiments in which purified α and $\beta\gamma$ subunits of G_i were added to platelet membranes suggested that G_i inhibited adenylyl cyclase by releasing $\beta\gamma$, which then deactivated α_s (27, 29). This model fails to account for the findings that α_i inhibits adenylyl cyclase when added to cyc^- membranes (28) and that somatostatin inhibits adenylyl cyclase in cyc^- membranes via G_i (28) even though cyc^- lacks detectable α_s or indeed any α_s mRNA (30). Further study indicates that $\beta\gamma$ may directly inhibit the purified catalyst and that α_i competes with α_s for binding to C (T. Katada, personal communication). Thus G_i may inhibit adenylyl cyclase through both its α and $\beta\gamma$ subunits.

G_o, The "Other" G-Protein

G_o was also discovered and purified because pertussis toxin ADP-ribosylates its α subunit (39 kd) (4, 31). Like G_s and G_i, G_o dissociates into α and $\beta\gamma$ subunits on binding of GTP analogs. G_o couples in vitro to muscarinic receptors (32), but thus far has no demonstrated role in hormonal control of adenylyl cyclase.

THE β-ADRENERGIC RECEPTOR AND THE CATALYST

The β-adrenergic receptor (β-AR) and C have now been purified and reconstituted with G-proteins into phospholipid vesicles. β-ARs are single, glycosy-

lated polypeptides with protein molecular weights of about 49,000 (33). The catalyst is a single polypeptide with a molecular weight of 120,000 (34).

β-ARs in intact membranes show biphasic agonist binding curves, which indicate two classes of binding sites with differing affinities. Activation of G_s with GTP analogs converts nearly all receptors to the low-affinity form. Two purified proteins, G_s and β-AR, are sufficient to reconstitute this effect in phospholipid vesicles. Receptors reconstituted alone into phospholipid vesicles exhibit low affinity for agonist. β-ARs reconstituted with G_s show biphasic agonist binding curves characteristic of intact membranes; activation of G_s with GTP analogs restores the receptors to their low-affinity form (35, 36).

This shift in receptor affinity results from dissociation of the β-AR:G_s complex. In the absence of guanine nucleotide, agonist-bound (high-affinity) β-AR and G_s exist as a stable complex. Activation of G_s by guanine nucleotide causes dissociation of the complex and a decrease in receptor affinity for agonist (37).

GTP analogs activate purified G_s slowly (3). In phospholipid vesicles containing β-AR and G_s, agonist occupancy of the receptor greatly increases the rate of GTP analog binding and of activation of G_s (38–40), and stimulates GTP hydrolysis (35, 36, 40–42). The presence or absence of the catalyst has little effect on G_s activation or stimulation of GTPase activity by liganded receptors (42). Addition of C to vesicles containing β-AR and G_s forms a complete system for positive regulation of cAMP synthesis. In the reconstituted system, β-adrenergic agonists stimulate cAMP synthesis in a GTP-dependent fashion (20).

Thus three proteins, β-AR, C, and G_s, form a functional adenylyl cyclase system. When reconstituted into phospholipid vesicles, these proteins display all the properties of the β-adrenergic hormone-sensitive adenylyl cyclase in intact membranes. They demonstrate hormonal stimulation of GTPase activity and cAMP synthesis, hormone- and GTP-dependent G-protein activation, and the effects of GTP analogs on affinity of the β-AR for ligands. The relatively modest hormonal stimulation of cAMP synthesis in the reconstituted system, however, may indicate that optimal performance of the system requires other proteins (20).

THE G-PROTEINS: FUNCTION VERSUS STRUCTURE

Any discussion of the G-proteins must include transducin, the protein that couples photoexcited rhodopsin (R*) to stimulation of a cGMP-phosphodiesterase (PDE) in vertebrate rod outer segments. Like G_s, G_i, and G_o, transducin is an $\alpha\beta\gamma$ heterotrimer. The α and γ subunits of transducin have molecular weights of 39,000 and 8,000, respectively; the β-chain behaves as a single 36-kd polypeptide on SDS-polyacrylamide gels (43, 44). Like

hormones and β-AR, light and rhodopsin stimulate GTP binding and activation of transducin. The α subunit of transducin (α_t) dissociates from $\beta\gamma_t$ when activated by guanine nucleotide and, like α_s, stimulates its target enzyme, PDE (see 45 for review).

The G-proteins share more than their GTP regulatory cycle. Cholera toxin ADP-ribosylates the α subunits of transducin (46, 47) and of G_s (11, 48). In each case, the preferred toxin substrate is the activated, GTP form of the G-protein; light and GTP analogs enhance modification of transducin, just as hormone and GTP analogs increase labeling of G_s. Modification of both proteins by cholera toxin inhibits GTPase activity and results in persistent activation. Pertussis toxin catalyzes ADP-ribosylation of the α subunits of transducin (49, 50), G_i (23, 24, 26), and G_o (2, 4). In contrast to cholera toxin, pertussis toxin preferentially modifies the deactivated form of these proteins. Light or hormone and GTP analogs inhibit the toxin-catalyzed modification. Pertussis toxin–modified G-proteins remain persistently deactivated because they cannot interact with activated receptor (see Figure 1).

Several observations indicate that the α subunits of transducin, G_i, and G_o comprise a structurally related subgroup. Transducin and G_i interact much more effectively with R* than does G_s, whereas the reverse is true of the β-AR (40, 51). Antibodies against α_t cross-react with α_i and α_o but not with α_s (52). Similarly, antibodies to α_o cross-react with α_i but not α_s (31).

The 36-kd β subunits of all four G-proteins are very similar and may be identical. These β-chains are immunologically indistinguishable (31, 52, 53) and have similar peptide maps (54). The 35-kd β subunits differ immunologically from the 36-kd proteins (52). Transducin contains only the 36-kd β-chain.

PRIMARY STRUCTURE OF THE G-PROTEINS AND THE β-ADRENERGIC RECEPTOR

With the isolation and sequencing of cDNAs (55–61), the primary structures of the G-proteins can now be directly compared. Figure 2 shows pairwise comparisons of amino acid homologies among the G-proteins. The degree of homology between transducin, G_i, and G_o (about 65% allowing for conservative substitutions) confirms the conclusion, based on immunological and functional evidence, that these proteins form a closely related subgroup. G_s, the most divergent of the G proteins, shares about 45% homology with each of the others and contains extra sequences.

Examination of cDNAs encoding the 45- and 52-kd α_s chains resolves the question of their origin (58; A. G. Gilman, personal communication). The two cDNAs are identical except for one stretch of bases within the coding region; this stretch in the 52-kd α_s cDNA encodes 15 amino acids not encoded

by the cDNA that specifies the 45-kd polypeptide. The mRNAs that correspond to these two cDNAs are probably derived by alternative splicing of exons from a single α_s gene.

Two other families of GTP-binding proteins, the bacterial elongation factors and the ras proteins, contain four regions of homology implicated in GTP binding and hydrolysis (62). The G-proteins contain sequences homologous to these regions (Figure 2), indicating a common origin for the three groups of proteins. Especially striking is the finding that the predicted secondary structures of these regions in the G-protein α chains closely resemble the solved crystal structure (63) of elongation factor-Tu (EF-Tu; S. Masters, R. Stroud & H. Bourne, unpublished).

The function of other portions of the G-proteins can also be predicted. Part

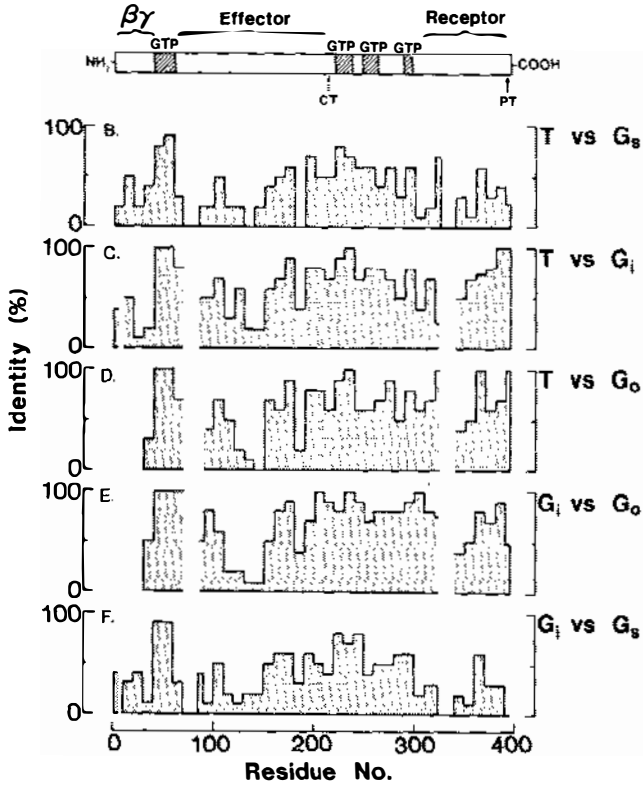

Figure 2 Comparison of amino acid sequences of the G-protein α subunits. The percentage of identical amino acid residues, averaged over blocks of 10 residues, is plotted versus position for pairs of α chains. The X-axis coordinates refer to residue numbers in α_s. Because α_s is longer than the other G-protein α chains, the plots have gaps. Regions implicated in GTP binding (62) and in interactions with $\beta\gamma$, effectors, and detectors are indicated in the diagram at the top.

of the area between the first and second GTP-binding regions (α_s residues 61–132) is poorly conserved among the G-proteins and may contain the effector binding domain. The highly conserved area preceding the second GTP contact site (α_s residues 133–204) is a good candidate for interaction with $\beta\gamma$. The analogous region of EF-Tu participates in aminoacyl tRNA binding (63). Just as GTP regulates the affinity of EF-Tu for aminoacyl tRNA (64), this region of the G-proteins may participate in GTP-dependent interaction with both the effector and $\beta\gamma$. The amino terminus of α_t is clearly required for interaction with $\beta\gamma$ because no interaction occurs when the N-terminal 18 amino acids are proteolytically removed (65). Poor conservation of amino acid scquence among the G-proteins in this region suggests, however, that it may not contain the actual $\beta\gamma$ contact site (Figure 2).

The 25 carboxy-terminal amino acid residues of the α chains may participate in receptor binding. The degree of homology in this region between pairs of G-proteins (Figure 2) correlates very well with their preference for interaction with R* or β-AR. Both α_t and α_i interact well with R* and are highly homologous in this region, whereas α_s interacts poorly with R* and is quite divergent. Pertussis toxin–catalyzed modification of a cysteine near the C-terminus blocks functional interaction of α_t with R* (49). Furthermore, this region of transducin is highly homologous to that of an internal sequence of another retinal protein, arrestin (66), which competes with transducin for binding to R*.

Recently, Dixon et al (67) isolated the gene and cDNA encoding the hampster β-AR. The predicted amino acid sequence of the hampster β-AR shows striking similarities to that of the rhodopsins. Like the rhodopsins, the β-AR contains 7 hydrophobic amino acid sequences of 20–25 residues. These regions are thought to span the membrane. Similarities in amino acid sequence in these regions between the β-AR and opsin suggests a common mode of action (67). Thus, similarities between GTP-dependent signal-transduction systems extend beyond the G-proteins themselves to receptors for such disparate signals as photons and catecholamines.

THE YEAST ADENYLYL CYCLASE SYSTEM

The adenylyl cyclase system of the yeast *Saccharomyces cerevisiae* deserves brief mention because of its similarity to vertebrate adenylyl cyclase and because of the enormous potential for genetic exploitation of the yeast system.

Matsumoto and his coworkers isolated yeast mutants with alterations in cAMP metabolism (see 68 for review). Their approach illustrates the power of the yeast system. Because wild-type yeast take up cAMP poorly, these investigators began by isolating mutants from an adenine-requiring strain that could use exogenous cAMP as an adenine source. From these mutants

(designated *cam*), Matsumoto et al then isolated cells with mutations causing them to require cAMP for growth. Mutations in three genes resulted (69). One of these, *CYR1*, encodes the catalytic adenylyl cyclase (70, 71).

Biochemically, yeast adenylyl cyclase strikingly resembles that of vertebrate cells. Initial biochemical characterization of the yeast system showed that at the minimum it contains distinct stimulatory guanine nucleotide–binding regulatory and catalytic proteins (72). The genes encoding the yeast catalyst and regulatory proteins have now been isolated: the catalyst *(CYR1)* by complementation of *cyr1*$^-$ mutants (70, 73, 74) and the regulatory proteins (*RAS1* and *RAS2*) by virtue of their homology to the viral *Ha-ras* oncogene (75, 76). *CYR1* encodes a potential polypeptide of 2026 amino acid residues with a molecular weight of 225,000. The catalytic activity resides in a C-terminal domain of about 60 kd (74).

RAS1 and *RAS2* encode proteins of 308 and 322 amino acids, respectively. The amino acid sequences of the amino-terminal half of the yeast RAS proteins exhibit considerable homology (80% over the N-terminal 80 residues and 60% over the next 80) to the mammalian ras proteins (76), including the four GTP-binding regions (62). Interestingly, the yeast RAS proteins show no more homology to vertebrate G-proteins than do the mammalian ras proteins. Also, $p21^{ras}$ substitutes for yeast RAS proteins in supporting GTP-dependent cAMP synthesis (77), but clearly does not regulate adenylyl cyclase in vertebrates (see 78).

The yeast adenylyl cyclase system thus resembles two different signal-transduction systems in higher organisms: the hormone-sensitive adenylyl cyclase system and the $p21^{ras}$ system, whose effector and detector elements have not yet been identified.

FURTHER GENETIC STUDY OF HORMONE-SENSITIVE ADENYLYL CYCLASE

Biochemical studies reviewed here describe an overall scheme for hormonal control of adenylyl cyclase. Isolation of the genes encoding components of the system allows investigators to address more specific questions concerning the molecular mechanisms of signal transduction. In vitro mutagenesis of these genes will provide information on the structure and function of the proteins and provide answers to such questions as: How does the liganded receptor facilitate activation of the G-proteins? What is the nature of the activation event? How does the activated G-protein effect a change in the rate of cAMP synthesis?

The yeast system will be particularly useful for such studies. Techniques available in yeast allow replacement of a gene in the chromosome with a modified version produced by selective in vitro mutagenesis. The function of

the mutant protein may then be studied in vivo and in native membranes. For example, substitution of valine for glycine at position 19 in *RAS2* results in constitutive activation of adenylyl cyclase (77). Substitution of valine for glycine at the analogous position in the mammalian ras gene products activates their oncogenic potential (79). The question of additional, undiscovered components of the adenylyl cyclase system may also be addressed in yeast. Our knowledge of the physiological role of cAMP in this organism should allow isolation of mutations in any component of the system.

G-proteins function as signal transducers in systems other than adenylyl cyclase (see 80 for review). How many G-proteins are there? Which of these regulate newly discovered effectors such as phospholipase C and potassium channels? Which are involved in tyrosine kinase–mediated processes? What, if any, is the role of developmental regulation of G-protein expression? With the tools at hand we may now address such questions.

A final set of questions concerns the evolutionary origin of signal transduction. The remarkable conservation of structure and function in receptors such as rhodopsin and the β-AR, and in the GTP-binding proteins, including bacterial elongation factors, transducin, and the yeast and vertebrate G-proteins, through eons of evolutionary time demonstrates their utility as signal detectors and transducers. Understanding the evolution of these systems will provide us with a more precise knowledge of their design and function.

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