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The disaggregation theory of signal transduction revisited: Further evidence that G proteins are multimeric and disaggregate to monomers when activated

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ABSTRACT **We have compared the sedimentation rates on sucrose gradients of the heterotrimeric GTP-binding regulatory (G) proteins G_s, G_o, G_i, and G_q extracted from rat brain synaptoneurosomes with Lubrol and digitonin. The individual α and β subunits were monitored with specific antisera. In all cases, both subunits cosedimented, indicating that the subunits are likely complexed as heterotrimers. When extracted with Lubrol all of the G proteins sedimented with rates of about 4.5 S (consistent with heterotrimers) whereas digitonin extracted 60% of the G proteins with peaks at 11 S; 40% pelleted as larger structures. Digitonin-extracted G_i was cross-linked by *p*-phenylenedimaleimide, yielding structures too large to enter polyacrylamide gels. No cross-linking of Lubrol-extracted G_i occurred. Treatment of the membranes with guanosine 5′-[γ-thio]triphosphate and Mg^{2+} yielded digitonin-extracted structures with peak sedimentation values of 8.5 S—i.e., comparable to that of purified G_o in digitonin and considerably larger than the Lubrol-extracted 2S structures representing the separated α and $\beta\gamma$ subunits formed by the actions of guanosine 5′-[γ-thio]triphosphate. It is concluded that the multimeric structures of G proteins in brain membranes are at least partially preserved in digitonin and that activation of these structures in membranes yields monomers of G proteins rather than the disaggregated products (α and $\beta\gamma$ complexes) observed in Lubrol. It is proposed that hormones and GTP affect the dynamic interplay between multimeric G proteins and receptors in a fashion analogous to the actions of ATP on the dynamic interactions between myosin and actin filaments. Signal transduction is mediated by activated monomers released from the multimers during the activation process.**

Great progress has been made in understanding the structure and function of GTP-binding regulatory (G) proteins and their role in signal transduction. Much of this recent progress stems from the powerful tools of molecular biology that have provided knowledge of the primary structure of their components and the fact that there is a growing family of these proteins. Reconstitution studies with purified components have provided major support for the theory that receptor activation causes exchange, in a catalytic fashion, of tightly bound GDP with GTP on the α subunits of heterotrimeric G proteins; the GTP-bound α subunit is then thought to dissociate from the $\beta\gamma$ complexes leading ultimately to regulation of a variety of effector systems; turnover of the reactions occurs through hydrolysis of GTP to GDP (for review, see ref. 1). Although these propositions have been widely accepted, some of the underlying assumptions have been challenged (2) in part because of apparent discrepancies between the structures determined by target analysis of G-protein-mediated systems in their native membrane environment (3) and those found in detergent extracts. Based on target analysis, G proteins in the native membrane environment are thought to be higher-ordered structures, termed multimers, that are converted to monomers by the combined actions of hormones and guanine nucleotides (4). Further support for this hypothesis stems from recent findings that the major heterotrimeric G proteins in rat brain synaptoneurosomes are cross-linked in their native membrane environment, yielding very large structures compatible with their being multimeric proteins (5). Different structures of G proteins and their products of hormone and guanine nucleotide activation have also been obtained, depending on the types of detergents employed for extraction from membranes (6, 7). Extraction of G proteins from these membranes with octyl glucoside yields polydisperse structures that are suggestive of multimeric proteins. Glucagon activation of the G protein G_s in hepatic membranes involves structures that sediment in octyl glucoside extracts (6, 7).

Possibly because of the broad range of S values for G proteins extracted by octyl glucoside, attempts to purify the multimeric forms of octyl glucoside-extracted G proteins proved unsatisfactory. Moreover, the β-subunit antibodies employed in those studies were subsequently found to be insensitive for detecting these proteins. Supplied with high-titer antibodies to all of the G-protein subunits in synaptoneurosomes and prompted by earlier studies (8) showing that G_s extracted from reticulocyte membranes with digitonin yielded very large structures (>600 kDa) that are not associated with either receptor or adenylyl cyclase, we have investigated the sedimentation rates of various types of G proteins (G_s, G_i, G_o, and G_q) present in rat brain synaptoneurosomes by comparing Lubrol and digitonin as extracting agents. The results suggest that digitonin at least partially preserves the multimeric structures of G proteins observed in membranes. Moreover, the products of activation by guanosine 5′-[γ-thio]triphosphate (GTP[γS]) are monomers rather than dissociated α and $\beta\gamma$ subunits observed as products of activation in Lubrol.

MATERIALS AND METHODS

Materials. Goat anti-mouse IgG, goat anti-rabbit IgG, and peroxidase anti-peroxidase (rabbit) were purchased from Organon Teknika–Cappel. Rabbit polyclonal antisera 8129 ($G\beta_{35}$ specific) and 8132 ($G\beta_{36}$ specific) (9) were generously supplied by David Manning (University of Pennsylvania). Rabbit polyclonal antisera W082 ($G\alpha_q$ specific) and X384 ($G\alpha_q$ and $G\alpha_{11}$ specific) (10) were kindly supplied by Paul Sternweis (University of Texas). Rabbit polyclonal antiserum QL ($G\alpha_q$ and $G\alpha_{11}$ specific) (11, 12) was supplied by Allen Spiegel (National Institute of Diabetes and Digestive

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Abbreviations: GTP[γS], guanosine 5′-[γ-thio]triphosphate; G protein, GTP-binding regulatory protein.

and Kidney Diseases, National Institutes of Health). Sources of rabbit polyclonal antisera specific for $G\alpha_s$, $G\alpha_i$, and $G\alpha_o$ have been described (6). In all cases the antibodies were raised against selective peptide regions of the G-protein subunits. Mouse anti-α-tubulin monoclonal antibody was purchased from Amersham, and peroxidase anti-peroxidase (mouse) was procured from Accurate Chemicals.

Detergents and Other Reagents. Lubrol PX [10% (wt/vol) aqueous solution] was from Pierce. An aqueous stock solution of digitonin [5% (wt/vol)] was prepared by boiling followed by cooling to room temperature for 2–3 days and filtration through a 0.45-μm (pore size) filter to remove insoluble material; the stock solution was then stored at room temperature. Working solutions of detergents were generally prepared fresh just before use. GTP[γS] was purchased from Boehringer Mannheim. All other reagents were of the highest quality available.

Preparation and Treatment of Synaptoneurosomes. Rat brain synaptoneurosomes were prepared (6) and stored as aliquots in liquid nitrogen until use. Synaptoneurosomes (1 mg) were incubated at 30°C for 5 min in buffer A (20 mM Hepes·NaOH, pH 7.4/150 mM NaCl/2 mM $MgSO_4$/1 mM EDTA/0.4 mM phenylmethylsulfonyl fluoride), without or with an appropriate concentration of GTP[γS] as indicated in figure legends, in a total volume of 0.2 ml. Reactions were stopped by the addition of detergent stock solutions to obtain a concentration of 1%. Extractions were carried out on ice for 1 h.

Sucrose Density Gradient Fractionation. After detergent extraction on ice for 1 h, 0.2 ml of total reaction mixture was layered over a prechilled 5–20% (wt/wt) linear sucrose gradient in buffer B (20 mM Hepes·NaOH, pH 7.4/150 mM NaCl/1 mM EDTA/1 mM dithiothreitol) and either 1% Lubrol or 0.5% digitonin. After centrifugation at 50,000 rpm for 15 h at 4°C in a Beckman SW60 rotor, about 22 fractions (0.2 ml) were collected (fraction 22 being the pelleted material). Alternate fractions were then examined for α and β subunits of G proteins by SDS/PAGE and Western blot analysis (13, 14). As markers for relative S values ($s^0_{20,w}$) cytochrome *c* (2.1 S, 12.5 kDa), bovine serum albumin (4.4 S, 68 kDa), aldolase (8.5 S, 158 kDa), and catalase (11.2 S, 240 kDa) were added in parallel to the same gradients used with the detergent extracts of synaptoneurosomes. Both cytochrome *c* and catalase also functioned as indicator proteins due to their colors and thus could be observed directly. For each marker protein only minor changes in S values were observed in the various detergent-containing sucrose gradients used in this study, thus permitting direct comparisons of fraction numbers and S values from experiment to experiment.

Image Analysis. For analysis of the distribution of GTP-binding proteins, immunoblots were scanned with a flatbed scanner (Microtek ScanMaker model 600ZS) at a resolution of 75 dots per inch (1 inch = 2.54 cm) in the gray scale mode. Scanned images were stored as TIFF files by using the public domain software IMAGE Version 1.4 from the National Institutes of Health. The mean area of a selected protein band in pixels and the average gray level of the pixels in the selected protein band (mean density) were measured for each individual G-protein band on a given blot. This mean density is plotted on the *y* axis in various figures and is labeled as density (arbitrary units). The results are representative of at least three experiments with different preparations of synaptoneurosomes.

Cross-Linking. Fractions collected from sucrose gradients were subjected to cross-linking by the addition of 75 μM *p*-phenylenedimaleimide in dimethylformamide at room temperature for 1 h. Sample loading buffer containing 2% (wt/vol) SDS was then added to each fraction and boiled for 5 min, and the fractions were examined by SDS/PAGE and Western blot analysis.

RESULTS

The sedimentation velocity measurements (S values) of G proteins, monitored by immunoblot analysis of the fractions obtained from sucrose gradients, are shown in Fig. 1. When extracted with Lubrol, the most extensively used detergent for purification of G proteins, all of the G-protein subunits ($G\alpha_s$, $G\alpha_i$, $G\alpha_q$, $G\alpha_o$, Gβ-1, and Gβ-2) displayed sedimentation values with peaks in the range of 4.0–4.5 S, which approximates that of heterotrimeric G proteins (15). In contrast, digitonin extraction of all the G proteins invariably yielded structures that distributed in the soluble fractions as structures of 10 to >12 S; an estimated 40% of the G proteins pelleted at the bottom of the gradient tube (fraction 22). Note that both α subunits and β(γ) subunits gave similar sedimentation rates, indicating that the structures most likely contain complexes of heterotrimeric G proteins. Identical results were obtained using liver membranes and various cell lines extracted with digitonin (data not shown).

An explanation for the large differences in hydrodynamic properties of the G proteins in digitonin and Lubrol is that multimers are stabilized in digitonin but not in Lubrol. To test this possibility, the sucrose gradient fractions were treated with 75 μM *p*-phenylenedimaleimide under conditions identical to those used for cross-linking native membrane forms of G proteins; the products were subsequently examined by

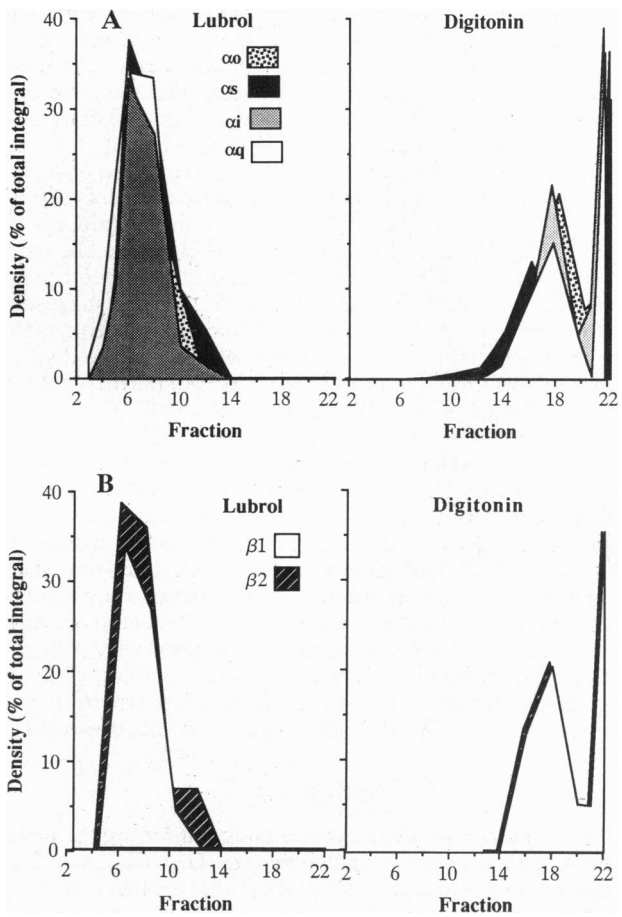

FIG. 1. Comparative distribution of G-protein α subunits (*A*) and β subunits (*B*) on sucrose gradients after extraction of membranes with indicated detergents. Methods of analyses are described in text. For these area plots, the mean density was normalized to 100% of the maximum and plotted on the *y* axis.

SDS/PAGE followed by Western blot analysis as described (5). In Lubrol extracts, essentially no cross-linking was observed; i.e., the electrophoretic patterns given by both α and β subunits were indistinguishable from controls. In contrast, all of the G proteins extracted with digitonin and treated with the cross-linking agent failed to enter the stacking gel and, hence, could not be immunodetected on the blots (data not shown). These results are consistent with those obtained from cross-linking of G proteins in native synaptoneurosomes and indicate that part of the difference in the hydrodynamic properties observed in digitonin versus Lubrol reflects the stabilization of multimers of G proteins in digitonin but not in Lubrol.

Digitonin has been reported (16) to form micelles of from 70 kDa to 400 kDa; in contrast, the aggregate molecular mass of Lubrol micelles is ≈60 kDa (17). Hence, part of the difference between the S values for G proteins in these detergents could be attributed to the micellar contribution of the bound detergent.

The current view of the activation of G proteins by guanine nucleotides and hormones is that the heterotrimers undergo dissociation into α and $\beta\gamma$ subunits (1). In part this concept stems from the dissociative effects of GTP[γS] on purified heterotrimeric G proteins in detergents such as Lubrol. Not known, however, is the nature of the product(s) formed by activation of G proteins in their native membrane environment. Since Lubrol and digitonin extract G proteins as heterotrimers and multimers, respectively, it was of interest to compare the products of GTP[γS] activation in the native membranes followed by detergent extraction. By using either G_o or G_i as examples, in Lubrol extracts a significant shift was observed to lower S values (2 S) relative to the control (4.5 S), as shown in Fig. 2*A*. Note that both α and β subunits were shifted concomitantly, indicating that the heterotrimeric forms of G_i (and, data not shown, G_o) were converted to the dissociated products α and $\beta(\gamma)$, as originally reported (15). Decreases in S values for G_o and G_i were also observed in digitonin extracts of membranes pretreated with GTP[γS]. However, even at 1 mM GTP[γS], the S values obtained were no less than 8 S (Fig. 2*B*). Purified heterotrimeric G_o, when subjected to gradient centrifugation in the presence of digitonin, gave the same 8S value (data not shown), suggesting that the product of activation of G_o (or G_i) in native membranes is a heterotrimeric structure rather than the dissociated products observed in Lubrol extracts of the membranes. Similar studies were also carried out with G_s and G_q. GTP[γS], even at 1 mM, failed to cause significant shifts in hydrodynamic values of G_s and G_q with either Lubrol or digitonin extracts. The same relative insensitivity of G_s to the activating effects of GTP[γS] has been shown in liver membranes extracted with octyl glucoside (7). In the absence of glucagon, 1 mM GTP[γS] was required for significant shifts to lower S values whereas glucagon-activated G_s displayed shifts in the presence of 1 μM GTP[γS]. Recently, we have found that G_q in rat liver plasma membranes is very sensitive to the effects of GTP[γS]; after extraction with octyl glucoside, a significant shift from a 4S to a 2S structure was observed with 1 μM GTP[γS] (unpublished observations).

DISCUSSION

In agreement with other studies, heterotrimers are the major structures of G proteins, including $G\alpha_s$, $G\alpha_i$, $G\alpha_o$, and $G\alpha_q$, extracted with Lubrol. As reported (6), extracts of these proteins in octyl glucoside sediment over a broad range of S values, suggesting polydisperse structures larger than heterotrimers. As shown here, these same proteins extracted with digitonin appear as larger structures exhibiting higher S values and a portion (40%) that sediments in the pellet. A reasonable explanation for these differences is that Lubrol and digitonin bind to and induce selective structural changes in the native membrane-bound form of the G proteins. Consistent with this interpretation is our finding (unpublished observations) that tubulin, which in its dimeric structure is a lipophilic protein that binds octyl glucoside and other detergents (18, 19), is converted from its native synaptoneurosomal multimeric structure by Lubrol to give a mixture of products ranging from the individual subunits (55 kDa) to heterodimers (110 kDa) and multimers whereas digitonin extracts primarily heterodimers. From such observations, it is evident that the native structures of tubulin and G proteins cannot be derived solely from the products of detergent extraction. Cross-linking of digitonin-extracted G proteins suggests large structures of the G proteins that are not observed in Lubrol. Since all of the G proteins detected in synaptoneurosomes with the antibodies employed here are cross-linked by *p*-phenylenedimaleimide to form similar large structures (5), we interpret these findings as evidence for multimeric forms of G proteins that are stable during extraction with digitonin but not with Lubrol.

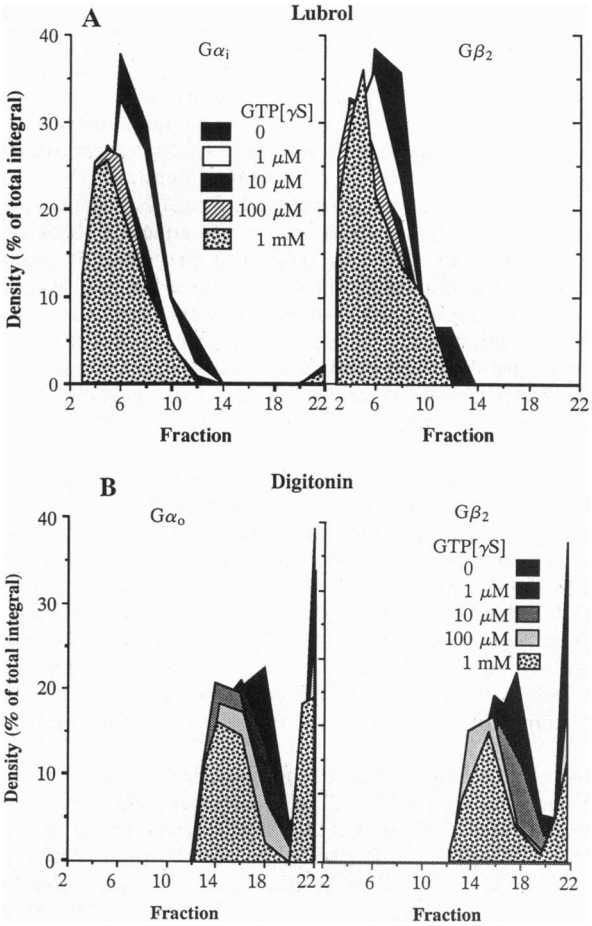

FIG. 2. Effects of GTP[γS] on the distribution of $G\alpha_i$ (*A*), $G\alpha_o$ (*B*), and Gβ2 on sucrose gradients after extraction with Lubrol or digitonin. Synaptoneurosome membranes were incubated without or with the indicated concentrations of GTP[γS] for 5 min at 30°C and were extracted with either Lubrol or digitonin, as indicated.

Target size analysis of hormone-stimulated and -inhibited adenylyl cyclase in rat hepatic and adipocyte membranes (3, 20) revealed that the structures involved in hormonal and guanosine nucleotide regulation of adenylyl cyclase are very large (≈1500 kDa for the hepatic glucagon-sensitive system). When the masses contributed by adenylyl cyclase [≈120 kDa (21)] and the glucagon receptor [64 kDa (22)] are subtracted from the total and the remainder (≈1200 kDa) is assumed to represent the contribution of heterotrimeric G_s (≈90 kDa), an

estimated 12-mer multimer of G_s is required for cyclase activation by glucagon. A structure of this size would pellet on sucrose gradients, which is consistent with the finding that glucagon primarily activates a G_s-containing structure in hepatic membranes that pellets on sucrose gradients (7). Thus, based on evidence obtained with target analysis, cross-linking of G proteins in membranes, and the hydrodynamic studies with either digitonin or octyl glucoside, multimers appear to be the major structure of G proteins associated with membranes. A major question is the nature of the products formed by hormonal and guanosine nucleotide activation of multimeric G proteins.

Target-size analysis of the products of guanosine 5′-[β,γ-imido]triphosphate or fluoride activation of hepatic adenylyl cyclase yielded a mass of ≈250 kDa (3). When the contribution of adenylyl cyclase (≈120 kDa) is subtracted from this mass, the remainder (≈125 kDa) approximates the mass of heterotrimeric G_s, not that of α_s (≈50 kDa), which is the commonly held view of the actions of hormones and guanine nucleotides (1). The findings reported here that the major products of GTP[γS] activation of G_o and G_i multimers in synaptoneurosomes are the size of monomers (i.e., heterotrimers) are consistent with target-size analysis of the activation products. It is noteworthy that adenylyl cyclase in turkey erythrocyte and brain membranes is associated, after activation of membranes with guanosine 5′-[β,γ-imido]-triphosphate, with both α_s and $\beta\gamma$ subunits of G_s, suggesting that some form of activated G_s associates during activation of the enzyme (23).

The original "disaggregation theory" of hormonal activation of adenylyl cyclase postulated that the "signal transduction" unit required for activation of adenylyl cyclase by hormone and GTP consists of equivalent amounts of both receptor and G protein entwined in an oligomeric structure (4). To account for the fact that minimal occupation of the total repertoire of receptors is required for near complete activation of the enzyme, it was postulated that, through the concerted actions of hormone and GTP, this unit may be allosterically disrupted to liberate a GTP-bound monomer, the reactive species responsible for activating the enzyme. However, with the exception of the rhodopsin/transducin system, G-protein/receptor-coupled systems seem to have many more G proteins than receptors (24) and, indeed, a single activated receptor can activate many G proteins (25). A plausible explanation for this phenomenon is that a single receptor—possibly as a dimer (26)—interacts with a multimer so that hormone-induced exchange of GTP and bound GDP causes the receptor to move along the multimer chain in a pulsatile fashion, each "hit" resulting in a change in the type of bound nucleotide on each successive monomer—i.e., a sequence leading from GDP to GTP followed by hydrolysis to GDP plus P_i and accompanying structural alterations. In this scheme, the end result is the release of an activated monomer. The activated monomer may react with an effector such as adenylyl cyclase or recycle in its inactive GDP-bound state back to a terminus of the multimer. These ideas are presented in Fig. 3 and have, as counterpart, many of the features proposed for other systems in which dynamic interactions between molecules are driven by the binding and hydrolysis of purine nucleotides. For example, interactions between myosin and actin are controlled in a ratchet-like or sliding movement by the myosin-induced exchange of bound ADP with ATP, followed by on-site hydrolysis of ATP serving as the energy source, and the release of P_i as the rate-limiting step in the overall dynamic process (ref. 27 and references therein). By substituting GTP for ATP and replacing myosin with membrane receptors, a similar scenario can be constructed for multimeric G proteins (2, 28). In this context, it should also be noted that G proteins, like actin or tubulin, attach to membranes through hydrophobic domains

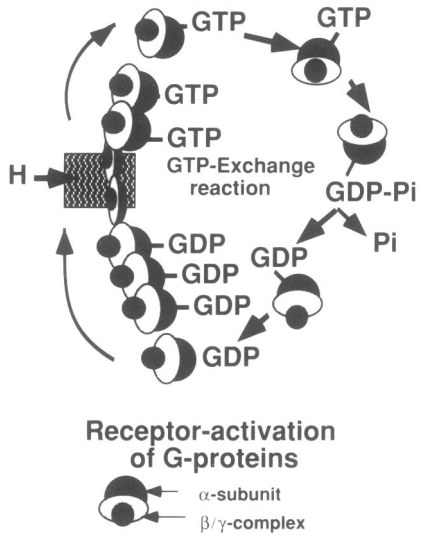

FIG. 3. Schematic illustration of receptor activation of G proteins. All of the components represented remain bound to the surface membrane during the entire dynamic process. Multimeric G proteins are depicted in the following three states: GDP-bound; unoccupied by nucleotide and bound to hormone (H)-occupied receptor; and GTP-bound. The nucleotide exchange reaction occurs as each "monomer" in the chain becomes attached to the high-affinity form of the receptor; "release" of receptor occurs when the G protein becomes occupied by GTP. This results in progressive movement of the receptor, in ratchet-like fashion, along the multimeric structure. Release of monomers occupied by GTP from multimers occurs in "quantal" fashion. On-site hydrolysis of GTP to GDP plus P_i, followed by release of P_i controls, in conjunction with the hormone-activated exchange reaction, is the overall dynamics of the reaction cycle.

with both lipids and selective membrane proteins (29–31). By placing multimeric G proteins in the same cytoskeletal framework as actin and tubulin, one can readily envision that regulation of the multimer–monomer states of these proteins may be largely responsible for the structural and metabolic changes in cells responding to external signals (32).

In conclusion, based on the properties of G proteins gleaned from their hydrodynamic properties, from target-size analysis, and from cross-linking of structures in biological membranes, there is now a reasonable basis for explaining both the catalytic role of receptors in activating G-protein multimers and the dynamic control of the activating process through the binding and degradation of GTP. If the multimers of G proteins are constructed of more than one type of G protein (i.e., heterogeneous multimers), it can be readily understood how a single receptor can activate two or more G-protein-controlled signaling pathways, an increasingly common phenomenon (for examples, see refs. 33–35). Testing this possibility will require some means of extracting and purifying multimeric forms of G proteins. Digitonin extraction appears to be a positive step toward this goal.

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