The Gβγ Dimer Drives the Interaction of Heterotrimeric Gᵢ Proteins with Nonlamellar Membrane Structures*

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Heterotrimeric G proteins are peripheral membrane proteins that propagate signals from membrane receptors to regulatory proteins localized in distinct cellular compartments. To facilitate signal amplification, G proteins are in molar excess with respect to G protein-coupled receptors. Because G proteins are capable of translocating from membrane to cytosol, protein–lipid interactions play a crucial role in signal transduction. Here, we studied the binding of heterotrimeric G proteins (Gαβγ) to model membranes (liposomes) and that of the entities formed upon receptor-mediated activation (Gα and Gβγ). The model membranes used were composed of defined membrane lipids capable of organizing into either lamellar or nonlamellar (hexagonal HII) membrane structures. We demonstrated that although heterotrimeric Gᵢ proteins and Gβγ dimers can bind to lipid bilayers of phosphatidylcholine, their binding to membranes was markedly and significantly enhanced by the presence of nonlamellar phases of phosphatidylethanolamine. Conversely, activated G protein α subunits showed an opposite membrane binding behavior with a marked preference for lamellar membranes. These results have important consequences in cell signaling. First, the binding characteristics of the Gβγ dimer account for the lipid binding behavior and the cellular localization of heterotrimeric G proteins. Second, the distinct protein–lipid interactions of heterotrimeric G proteins, Gβγ dimers, and Gα subunits with membrane lipids explain, in part, their different cellular mobilizations during signaling upon receptor activation. Finally, their differential interactions with lipids suggest an active role of the membrane lipid secondary structure in the propagation of signals through G protein-coupled receptors.

G protein-coupled receptors (GPCRs) constitute the main class of membrane receptors and form the widest gene family known in the mammalian genome. Therefore, signal transduction via G proteins represents one of the most important ways of cell signaling (for a review, see Ref. 1). Upon activation by agonists, GPCRs undergo conformational changes that induce the activation of heterotrimeric G proteins, promoting the exchange of the GDP bound to the Gα subunit for GTP. This exchange provokes the dissociation of the Gα subunit from the Gβγ dimer and enables both molecular entities to modulate the activity of specific effectors or other signaling proteins (e.g. G protein receptor kinases).

The association of both GPCRs and G proteins to the plasma membrane makes them susceptible to their lipid environment so that lipid-protein interactions are crucial to their function. In recent years, evidence has accumulated showing that the plasma membrane organization is more complex than a simple “chaotic sea” of bipolar lipid molecules in a liquid-crystalline state that only serves to support membrane proteins (2). In fact, differentiated membrane domains with specific protein and lipid compositions can exist in a cell such as the basal and apical membranes of epithelial/endothelial cells, the pre- and postsynaptic membranes of neuronal synapses, or lipid rafts and caveolae. Moreover, the extracellular and cytosolic leafletts of the plasma membrane differ in their lipid composition (3), further demonstrating the relevance of lipids in the organization and function of the membrane.

Natural membranes are composed of different amphitropic molecules that differ in their propensity to form secondary lipid structures that influence the structural properties of the membrane (for a review, see Ref. 4). In this context, hexagonal (HII) structures regulate the localization and activity of some key membrane signaling proteins (5, 6). Indeed, we have recently been able to show that changes in the lipid composition of erythrocyte cell membranes can influence the membrane association of G proteins and protein kinase C in vivo (7). Moreover, the neural membranes of cold-adapted fishes contain higher levels of phosphatidylethanolamine (PE) species in winter than in summer (8). These lipid changes probably lower the solid-to-liquid and lamellar-to-hexagonal phase transition temperatures of membranes to maintain protein function and cell signaling in neurons and other cells. These data suggest that hexagonal phase propensity plays a major role in regulating physiological processes and might also participate in the control of other pivotal cellular events influenced by membrane proteins, such as cell growth or energy metabolism.

Our main goal was to elucidate the role of the membrane lipid structure in the association to membranes of heterotrimeric G proteins and the molecular entities formed after their receptor-mediated activation. For this purpose we used synthetic membranes (liposomes) and purified G proteins as model systems because their lipid composition can be precisely controlled. We show that heterotrimeric Gᵢ proteins (αβγ) and Gβγ
dimers have protein-membrane lipid interactions distinct from those of Goi monomers. These differences may explain their distinct cellular localizations after dissociation. We also show that the binding of the Gβγ dimer accounts for the binding characteristics and cellular localization of the complete heterotrimeric G protein. To our knowledge, this is the first work describing this new role of the G protein βγ dimer.

EXPERIMENTAL PROCEDURES

31P NMR—Multilamellar lipid vesicles (15%; w/w) were prepared mixing 56 mg of phospholipids with deuterated water (D2O). Either egg phosphatidylcholine (PC), bovine brain PE, or mixtures of both were prepared, keeping constant the lipid phosphorus content. The lipid suspensions were hydrated and homogenized with a pestle-type mini-homogenizer (Sigma) and submitted to vortex shaking until homogeneity. The suspensions were then submitted to 10 heating (60 °C) and freezing (−80 °C) cycles and equilibrated prior to data acquisition, as reported previously (9). 31P NMR measurements were made in 5-mm tubes on an Advance-300 multinuclear NMR spectrometer (Bruker Instruments). Samples were equilibrated at the assay temperatures (4 or 25 °C) for 15 min before data was acquired. 31P NMR free induction decays were accumulated for 64 transients by employing a 4.4-μs, 90° radio-frequency pulse, a 24.3-KHz sweep width, and 65,500 data points. The delay between transients was 2 s, and the spectra were obtained by scanning from lower to higher temperatures.

Liposome Preparation and G Protein Binding—Liposomes containing PC were prepared, with or without PE, by dissolving the lipids in chloroform and mixing the appropriate volumes in glass vials. The solvent was removed under argon, and the residue was dried for at least 3 h under vacuum. Subsequently, the lipid films were hydrated in 20 mM Tris-HCl at pH 7.4 containing 100 mM KCl and 0.1 mM EDTA at 42 °C for 1 h and subjected to vigorous vortex shaking every 15 min. Following five freeze/thaw cycles, this lipid emulsion was passed 11 times through a 400-nm polycarbonate membrane using a mini-extruder (Avanti Polar Lipids). The lipid emulsions were then diluted to a final concentration of 600 μM in lipid phosphorus, as determined by the method of Chen et al. (10). Subsequently, the liposomes were incubated with either 500 ng of purified heterotrimeric G proteins, 100 ng of the Gβγ dimer, or 150 ng of the Goi subunit in a total volume of 300 μl for 30 min at 25 °C. Incubation of heterotrimeric G proteins or the Goi subunit was carried out in the presence of 50 μM GDPβS or GTPγS, respectively. The unbound G proteins were then separated from the membranes by centrifugation at 62,000 × g for 45 min at 25 °C. Finally, the membrane pellets were resuspended in electrophoresis loading buffer (84 mM Tris-HCl (pH 6.8), 4% SDS, 1% 2-mercaptoethanol, 5% glycerol, and 0.01% bromphenol blue) and boiled for 5 min.

Immunoblot Analysis and Quantification of Specific Immunoreactivity—Immunoblotting was performed as described elsewhere (11). Briefly, proteins were submitted to electrophoresis on SDS-polyacrylamide gels and then transferred to nitrocellulose membranes. These membranes were blocked with phosphate-buffered saline containing 5% nonfat dry milk, 0.5% bovine serum albumin, and 0.02% Tween 20. The membranes were then incubated with the specific primary antibodies and the secondary antibodies as visualized with the ECL Western blot detection system and ECL Hyperfilm (Amersham Biosciences). Quantification was performed by image analysis of the immunoreactive bands on the film. Values from liposomes composed of PC:PE were normalized to the values of pure PC liposomes, which were considered 100%.

Materials—Egg PC and liver PE were both purchased from Avanti Polar Lipids. Recombinant, purified, myristoylated Goi protein subunit, purified heterotrimeric G protein, and purified Gβγ protein dimers were from Calbiochem. The polyclonal anti-Goi antibody was from PerkinElmer Life Sciences and the monoclonal antibody anti-Gβγ, peptide was obtained from BD Biosciences. All other reagents were supplied by Sigma.

Data Analysis—The data shown are the mean values ± S.E. from the number of experiments indicated (n). Values were compared by one-way analysis of variance followed by a Bonferroni comparison test against the control values. Differences between experimental groups were considered statistically significant at p < 0.05.

RESULTS

Temperature-dependent Formation of Nonlamellar Phases in Model Membranes—For G protein-membrane binding experiments, model membranes of PC or PE or a combination of both (see “Experimental Procedures”) at different proportions were used. At 25 °C, egg PC membranes were organized forming lipid bilayers (lamellar structures), whereas bovine brain PE was mainly organized into hexagonal (HII phase) structures (Fig 1A). Liposomes formed with different proportions of both lipids behaved as non-ideal mixtures, as has been described for many binary systems with two membrane lipids or more (for example, see Ref. 12) where membrane domains enriched in one lipid species are formed. In this context, mixtures containing a higher content of PC (PC:PE at 10:0, 8:2, and 6:4; mol/mol) exhibited the peaks corresponding to the lamellar organization of each phospholipid (L1 and L2) (Fig 1A). In addition, the shoulder at positive chemical shift values indicates the presence of a small proportion of nonlamellar phases in PE-containing samples. At greater proportions of PE (PC:PE at 4:6, 2:8 and 0:10; mol/mol), the occurrence of nonlamellar structures (NL1 and NL2, defined by the peaks at 0.10 and 5.94 ppm) increased at 25 °C with a concomitant decrease of the peaks corresponding to the lamellar phases (Fig 1A). In contrast, at 4 °C there were only observed two peaks corresponding to the lamellar phases of PC and PE, respectively (−17.20 ppm and −12.20 ppm) (Fig 1B). Therefore, at 4 °C PC, PE, and their mixtures are organized into lamellar phases but not into nonlamellar phases.

Binding of the G, Heterotrimer to Model Membranes—We have studied the binding of heterotrimeric Gai proteins to model membranes (liposomes) when the dissociated state was stabilized by the addition of GDPβS (50 μM). We found that the lipid composition of the liposome greatly influenced the binding affinity of G proteins. Particularly, the PE content of the membrane favored Goiβγ protein binding to membranes. The PE ratios used in these model membranes reflected conditions in different biological situations and natural membranes (PC:PE at 10:0, 8:2, 6:4, 4:6, and 0:10; mol/mol). In mammalian cells the overall percentage of PE is ~20%, although the inner leaflet of the plasma membrane contains ~40%. However, in some bacteria the PE content can reach up to 80% of the total membrane phospholipids.

The presence of the hexagonal phase phospholipid PE, which organizes into HII phases under the experimental conditions used, induced an increase in the binding of Gai heterotrimeric to liposomes in a concentration-dependent manner (Fig 2A). Vesicles containing a ratio of 8:2 (120 μM PE) or 6:4 PC:PE (240 μM PE) (resembling mammalian plasma membranes or their inner leaflets) bound 338.5 ± 59.8 and 479.5 ± 51.7% of Gai protein heterotrimers, respectively. This was between 3- and 5-fold greater binding than that of vesicles composed of only the lamellar phase phospholipid PC (100%; Fig 2A). Higher concentrations of PE (that may resemble certain membrane domains or bacterial membranes) induced even greater binding of heterotrimeric Gai proteins, reaching 705.5 ± 25.1% at a PC:PE molar ratio of 4:6 (360 μM PE) and 898.9 ± 58.6% for pure PE liposomes (600 μM PE). On the other hand, at 4 °C only lamellar and not hexagonal phases were formed in our model systems. Under these conditions, the binding of Gi-heterotrimers to liposomes was approximately the same for all ratios of PC:PE used (8:2, 6:4, 4:6, and 0:10 at 116.0 ± 9.2, 116.3 ± 9.4, 122.1 ± 12.8, and 99.5 ± 11.3% with respect to PC:PE 10:0 as 100% control; p > 0.05 in all cases; Fig 2B). This result points out that the observed increase in binding of the Gi-heterotrimer is indeed caused by its binding preference to inverted hexagonal membrane structures and not by its preference for PE phospholipids.
**Binding of Go subunit to Model Membranes**—Similarly to the study with heterotrimeric G proteins, we also studied the binding of G protein subunits to liposomes to determine the membrane-lipid interactions that might occur upon G protein activation and dissociation. The purified Go subunit protein used was myristoylated and therefore able to bind to membrane bilayers in a natural manner (13). Additionally, the presence of 50 μM GTP-γS ensured that the protein remained in the conformation that corresponds to its activated state. By contrast to the heterotrimeric G proteins, the presence of the nonlamellar prone lipid PE (HII phases) did not increase the binding of monomeric Go subunits to liposomes (Fig. 3). In fact, an increase in the membrane content of PE strongly inhibited the binding of Go subunits to membranes. Thus, vesicles containing 8:2 or 6:4 PC:PE (mol/mol) bound only 50.6 ± 15.5 or 33.0 ± 15.4% the amount of Gi monomer bound by pure PC membranes (lamellar) (Fig. 3A). Greater proportions of PE further reduced Gi protein binding to 35.6 ± 5.4% for a ratio of 4:6 (mol/mol) and 26.1 ± 1.6% of control values for pure PE liposomes (Fig. 3A). At 4 °C, where only lamellar and not hexagonal phases are formed in our model system, binding of the Gi monomer to liposomes did not change with the ratios of PC:PE (8:2, 6:4, 4:6, and 0:10; mol/mol; 90.6 ± 6.4, 93.7 ± 4.3, 91.0 ± 1.5, and 85.3 ± 4.1%, with respect to PC:PE at 10:0 as 100% control; Fig 3B), showing that the observed decrease in binding of the Gi subunit was indeed caused by its binding preference for lamellar membrane structures and not by its preference for PC species. These results demonstrate that the Gi subunit interacts better with lamellar phases (i.e. planar membranes and lipid bilayers) than with hexagonal (HII phase) structures (i.e. membrane structures with negative curvature).

**Binding of Gβγ dimer to Model Membranes**—Finally, we investigated the protein-lipid interactions of the Gβγ dimer, which revealed lipid binding characteristics more similar to those of heterotrimeric Gi proteins than to those of monomeric Go subunits. Thus, the binding of Gβγ subunits was significantly and markedly augmented as the PE content in the model membrane increased (Fig. 4). Gβγ subunit binding to model membranes with molar ratios of PC:PE at 6:4 and 2:8 increased to 403.2 ± 58.8 and 1575.0 ± 316.5% that of pure PC lipid bilayers, respectively. These results clearly show that the Gβγ dimer has a marked preference for hexagonal (HII phase) structures.

**DISCUSSION**

Receptor-mediated signaling pathways operate as signal amplification cascades. In signals propagated from GPCRs, the first amplification step occurs at the membrane where each receptor can activate several G proteins upon agonist binding. However, rather than being homogeneously distributed on the cell surface, GPCRs and many other membrane receptors are concentrated or clustered in defined membrane regions (14, 15). Therefore, a large number of inactive, GDP-bound, heterotrimeric G protein molecules must be available in these receptor-rich domains associated with membrane lipids. Although the protein-lipid interactions between Ga subunits and lamellar membranes have been thoroughly studied (16), little emphasis had been placed on the possible interactions with nonlamellar lipid phases. Here, we have investigated the different interactions of Ga, Gβγ, and Goβγ proteins with lamellar and nonlamellar (HII) lipid phases. As a result, we have demonstrated that the presence of nonlamellar phases formed by PE favors the binding of heterotrimeric G, proteins and Gβγ dimers to membranes, whereas Ga subunits prefer lamellar membrane structures formed by PC and/or PE.

Cell membranes are usually rich in PE, which is mainly
distributed on the protoplasmic face of the plasma membrane where high concentrations of G proteins are bound (17). Indeed, our results show that the greater the hexagonal phase propensity, the higher the affinity of heterotrimeric G proteins for membranes. Thus, PE-rich domains or regions with negative curvature strain, in either a stable or a transient manner, could act as membrane reservoirs for these peripheral proteins. This finding is in agreement with the observed loss of G proteins from brain plasma membranes after the disruption of nonlamellar HII structures by the anthracycline daunomycin (18).

Nonlamellar prone lipids modulate the physical properties of lipid bilayers, inducing membrane "defects" such as changes in the intrinsic monolayer curvature, the surface pressure, and the hydration of membranes (19). The relative amounts of PEs in membranes under physiological conditions could justify the presence of HII structures or regions with negative curvature in vivo, which might be stabilized or induced by certain membrane proteins (20). In this context, it is noteworthy that peptides with α-helical structures, such as the transmembrane regions of GPCRs, promote the formation of hexagonal phases under certain conditions (21). Moreover, it has been shown that PEs facilitate the conformational change of the GPCR rhodopsin to its activated state in model membranes (22). Thus, regions enriched in hexagonal phase-forming membrane lipids like PE could provide a platform for the spatial co-localization of GPCRs and heterotrimeric G proteins, suggesting a dual role for hexagonal prone lipids in signal transduction, namely membrane co-localization of the signaling components and participation in GPCR activation (see below).

In contrast to G protein heterotrimers, the activated monomeric Ga1 subunit shows a marked preference for lamellar structures. This would provoke the rapid exit of activated Ga1 monomers from the receptor environment where the hexagonal phase propensity is increased by the transmembrane domains of GPCRs and the isoprenyl modifications of the Gβ subunit peptides. This mobilization of the Ga protein subunit away from the receptor environment may facilitate its interaction with effector proteins (e.g. adenyl cyclase) that might be present in other membrane domains. Indeed, Ga1 and Gaα have been found in lipid rafts (23), domains enriched in sphingolipids and cholesterol, with a highly ordered lamellar state and almost gel-like physical properties. Our findings demonstrate...
that monomeric Go1 subunits have a much greater affinity for microdomains with lamellar organization, such as those formed in lipid rafts. However, the lipid preferences of G proteins could be attributed to specific interactions with phospholipid polar heads in a lipid structure-independent manner. Therefore, we also studied the binding of these proteins to the same membrane systems at 4 °C. Whereas at 25 °C PE forms nonlamellar phases, at 4 °C it is only organized into lipid bilayers both alone or in combination with PC. At this temperature, neither Goβγ nor Go had a preference for PE or PC, respectively. This binding behavior is markedly distinct from that observed at 25 °C and demonstrates that G protein binding to membranes is regulated by the membrane structure and not by interactions with specific phospholipids (e.g. PC or PE).

In keeping with this suggestion, the G protein transducin (Go) that couples to the GPCR rhodopsin also translocates to lipid rafts when activated (24). It therefore seems feasible that the opposite binding preferences of activated Ga subunits target them away from the receptor environment to other membrane areas where they may interact with their effectors. The fact that a variety of proteins of the signal transduction machinery are located in lipid rafts supports this idea (25, 26). Thus, lipid rafts could serve as platforms to enhance the effectiveness of the second step of the signaling cascade by promoting the spatial association between Ga subunits and their effectors.

The high affinity of Gβγ subunits for hexagonal prone membranes also influences their distribution in native membranes. The present results are in agreement with the exclusion of Gβγ subunits from synthetic lipid rafts reported by Moffet et al. (27). Our data strongly suggest that the Gβγ dimer is responsible for the lipid-protein interaction properties of heterotrimeric G proteins. In this sense, the Gβγ dimer defines the hexagonal phase preference of complete Goβγ heterotrimers, thereby masking the lamellar membrane affinity of the Ga subunit. Therefore, one of the functions of the Gβγ dimer could be to carry the Ga subunit to the vicinity of the GPCR, similar to the “piggyback” transport to nuclei of proteins lacking a nuclear localization sequence by another proteins containing such a sequence (28, 29).

The hexagonal (HII) phase preference of the Gβγ dimer could also be important for GPCR desensitization, which occurs after agonist-mediated GPCR activation. Dissociated Gβγ dimers can bind G protein-coupled receptor kinases, which phosphorylate and inactivate GPCRs. The affinity of the Gβγ dimer for nonlamellar membrane regions (where membrane receptors are concentrated) would guide these kinases to their targets, provoking the termination of the signaling process initiated by the binding of the agonist to the receptor. The involvement of membrane lipids other than PC in the regulation of GPCR phosphorylation and desensitization strongly supports this hypothesis. Interestingly, it has been demonstrated that, with the exception of PC, membrane lipids can indeed regulate G protein-coupled receptor kinase activity (30).

In summary, the results presented here introduce new concepts about GPCR-associated signal propagation. Both GPCRs and prenylated G protein γ subunits facilitate the formation of hexagonal phase membrane domains (Fig. 5). Thus, these nonlamellar membrane domains would be enriched in Goβγ proteins because of the higher affinity of these heterotrimeric complexes for membranes with negative curvature driven by the Gβγ dimer. Therefore, a pool of G proteins would be generated near GPCR membrane receptors. Upon agonist-mediated activation, each GPCR receptor molecule can activate several G proteins, and the activated Ga subunit dissociates from the Gβγ complex. Because of its reduced affinity for nonlamellar membrane structures, activated Ga protein monomers would translocate away from the receptor area to planar bilayer membrane regions (i.e. highly ordered lamellar phases) such as lipid rafts. In these specialized microdomains, the accumulation of effector proteins would facilitate their interaction with activated Ga subunits and, therefore, the efficient propagation of the transmitted signal. Because the Gβγ dimer exhibits a higher affinity for nonlamellar lipid phases, it may regulate signaling effectors or enable G protein-coupled receptor kinases to interact with the membrane receptors. The latter process is associated with receptor phosphorylation, which induces GPCR desensitization. In general, our results highlight the functional relevance of structural biology for signal transduction, not only in terms of proteins and protein-protein interaction but also with respect to lipid organization and lipid-protein interactions.

REFERENCES
