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Structural Features of Heterotrimeric G-Protein-Coupled Receptors and Their Modulatory Proteins

Harry LeVine III

Parke-Davis Pharmaceutical Research Division of Warner-Lambert Company, Ann Arbor, MI 48105

Abstract

Over the past 20 years, the general mechanism for signaling through 7-transmembrane helix receptors coupled to GTP hydrolysis has been worked out. Although similar in overall organization, subtype variability and subcellular localization of components have built in considerable signaling specificity. Atomic resolution structures for many of the components have delineated the domain organization of these complex proteins and have given physical form to the idea of subtype specificity. This review describes what is known about the physical structures of the 7-transmembrane helix receptors, the heterotrimeric GTP binding coupling proteins, the adenylate cyclase and phospholipase C effector proteins, and signaling modulatory proteins, such as arrestin, phosducin, recoverin-type myristoyl switch proteins, and the pleckstrin homology domain of G-protein receptor kinase-2. These images allow experimenters to contemplate the details of the supramolecular organization of the multiprotein complexes involved in the transmission of signals across the cellular lipid bilayer.

Index Entries: X-ray structures; NMR, reconstitution; peptides; arrestin; phosducin; G-protein receptor kinases; RGS proteins; adenylate cyclase; phospholipase C.

Introduction

Structure/functional studies of the proteins involved in transmembrane signaling events through heterotrimeric GTP-binding protein coupled seven-transmembrane helix receptors (GPCRs) paint an increasingly detailed picture of functionally important regions and residues involved in signaling *(1)*. This functional knowledge is now being supplemented with detailed physical structure determinations of the components of GPCR systems. The ability to clone and express domains of interacting components provides the tools for atomic resolution determinations. Relationships of the isolated domains to each other in the intact molecules can then be worked out and the structure of the overall complex inferred from the appropriate physical and biochemical information. The intent of this review is to

summarize the work that bears on the three-dimensional structure of components of the GPCR system, as well as the intra- and intermolecular organization of the proteins that modulate that network.

Seven-Transmembrane Helix Receptors

Physical Structure of Receptors

The seven hydrophobic domains of the GPCR family of proteins embedded in the membrane lipid bilayer are the signature of the recognition component of these transmembrane signaling systems. The three-dimensional arrangement of residues of the receptor ligand binding domain are much sought after for the design of pharmacological agents. Unlike growth factor (nerve growth factor [NGF], epidermal growth factor [EGF], insulin) and cytokine receptors whose binding sites are extracellular, most GPCR's appear to sequester the ligand binding domain within the helices immersed in the lipid bilayer, exceptions being the larger polypeptide hormone GPCRs, the gonadotropins *(2)*, and the metabotropic glutamate receptors *(3)*. Because of their relevance to pharmacological manipulation of GPCRs, mutagenesis and chimera studies of the ligand binding sites have attracted considerable attention, although for the reasons outlined below, physical structure characterization is largely lacking.

X-ray and electron crystallography of integral membrane proteins in general, and of GPCRs in particular, has not achieved the high resolution obtained for soluble proteins. Although limited, this lower resolution has permitted the definition of two general classes of membrane protein structures; the hollow antiparallel β-barrel (e.g., porin *(4)*, and the α-helix class (e.g., bacterial protein–chlorophyll complex *(5,6)*. The nicotinic acetylcholine receptor seems to combine features of both of these classes *(7)*. Two-dimensional protein crystals in natural photosynthetic membranes have provided the only submacromolecular structures. Bacteriorhodopsin, a non-G-protein-coupled seven transmembrane helix light-dependent proton pump, resembles a hypothetical minimal GPCR with the shortest possible helix-connecting loops. Electron microscopy *(8)*, electron cryomicroscopy *(9)*, and electron diffraction of halorhodopsin, a related light-driven chloride pump *(10)*, have established low- to medium-resolution structures (3.5–7 Å) for bacterial rhodopsin-like ion pumps. Neutron diffraction with specifically deuterated residues in the protein defined the positions of the N-terminal bacteriorhodopsin helices A and B *(11)*.

The first three-dimensional electron density map of a true GPCR, bovine rhodopsin, oriented in a two-dimensional crystalline arrangement in rod outer segments (9-Å resolution) *(12,13)*, displayed an overall similarity to bacteriorhodopsin. Some differences between the two molecules in the tilt and arrangement of the helices and kinks within the helices were evident. From low-angle X-ray and neutron scattering studies, approx 50% of the mass of rhodopsin is buried within the bilayer *(14)*. In neither the bacteriorhodopsin nor the rhodopsin structure were the loops between helices or the N- or C-terminal polypeptide chains observable, owing to their mobility. Although the ends of the helices were not distinct, the core helix structures were visible. Individual amino acid residues generally, however, could not be identified. The resolved structures did suggest a markedly precise arrangement and alignment of the helices. Theoretical studies probing the basis of helix–helix interactions within a lipid bilayer *(15,16)* suggested that specific interactions could be favored. The theoretical studies indicated that certain protein–protein interactions could be promoted by isolation within a lipid environment *(17)* and would be capable of orienting the complex to generate vectorial processes *(18)*. Consideration of these principles and detailed sequence comparisons among a large number of G-protein-coupled receptors *(19,20)* have suggested a counterclockwise arrange-

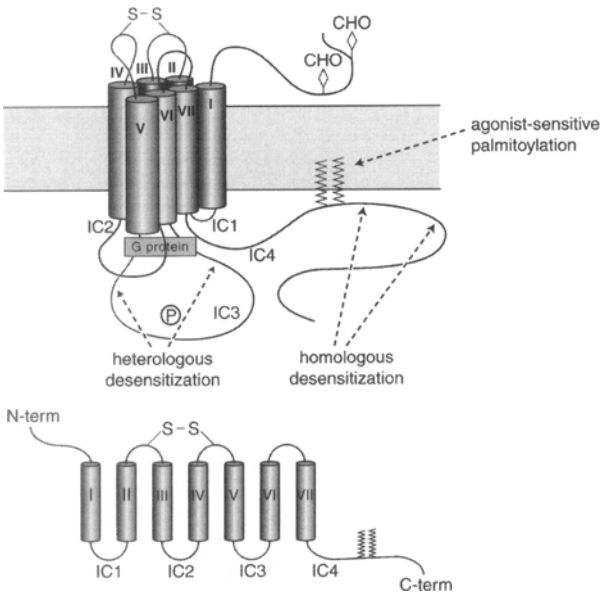

Fig. 1. Anatomy of a generic G-protein-coupled receptor (GPCR). Membrane topography. CHO, carbohydrate; I–VII transmembrane helices; -S-S-, disulfide bond; ICn, intracellular loops; P, phosphorylation site; G protein, G protein interaction region of receptor; zig-zag lines, attached palmitate alkyl chains. Schematic arrangement of GPCRs N-term, N-terminus; C-term, C-terminus.

ment of the helices when viewed from the external membrane surface. Figure 1 is a generic diagram of a GPCR illustrating the transmembrane helix (TMH) and loop nomenclature and functionally defined regions of these receptors.

Because of the formidable technical barriers of size and interactions with the lipid bilayer, high-resolution structural analysis has instead been applied to fragments of the receptor molecule that can assume folded structures in solution. Although most successfully applied to soluble peptides or domains derived from the extramembrane loops discussed below, under *Loops Connecting Transmembrane Helices*, some ingenious methodologies have yielded insight into the association of the transmembrane helices with each other and the bilayer, as well as changes that occur upon receptor activation. Specialized nuclear magnetic resonance (NMR) techniques have established an equilibrium between two conformers, (1) a type I/III β-turn around the N–P linkage, and (2) a *trans*-proline kink between N- and C-terminal α-helices within the neurokinin-1 transmembrane helix TMH VII acetyl-AMSSTMYNPIIYSSL-amide in solution. This TMH VII sequence, S or T-X1-X2-N-P-X3-X4-Y, where X is any amino acid, is conserved among many GPCRs. This interconversion between conformers (1) and (2) could potentially be perturbed by agonist binding *(21)*. Helix–helix interactions *(22)* and conformational equilibria *(23)* can be monitored in the solid state (membrane-embedded) for transmembrane proteins or fragments of the protein of <10,000 M_r by high-speed magic angle spinning solid-state NMR techniques. Structures of larger functional fragments of GPCRs in membranes are beyond the limits of present technology.

Arrangement of the Transmembrane Helices

Experimental support for the arrangement of the helices comes from indirect evidence inferring proximity of two amino acid residues from compensating mutation of residues on two helices in rhodopsin, such as TMH I and VII *(24)* or TMH II and VII *(25,26)*. The effects of the rhodopsin substitutions are expressed clinically in a form of night blindness *(27)*. Accessibility of substituted cysteines to hydrophilic modifying reagents in the dopamine D2 receptor *(28)* or to disulfide crosslinking in rhodopsin *(29)* probe the membrane/helix interface and interhelix distances. Engineered *bis*-His zinc binding sites in rhodopsin *(30)*, the κ-opioid receptor *(31)*, and tachykinin NK-1 receptor *(32)* have probed the relative proximity of residues on facing helices and seem to corroborate a counterclockwise organization. The interpretation of the effects of these modifications assumes minimal perturbation of the system by the imposed changes. More biophysically informative techniques employing bulky spin-labeled COOH

terminal residues with bacteriorhodopsin showed an immobilized C-terminal tail and buried COOH residues residing 16 Å from the bilayer surface *(33)*.

The evolutionary preference for membrane-bound protein systems exploiting specific transmembrane helix–helix interactions combined with theoretical considerations of binding energy suggests that a multihelical membrane protein might be composed of two or more substructural units of associated helices. Such organizational paradigms are also found in multitransmembrane helix proteins other than GPCRs, *(15,34)*. There is considerable evidence that the subdomain concept may hold for the 7-transmembrane helix non-G-protein-coupled photoreceptor, bacteriohodopsin. Membrane-bound bacteriorhodopsin has been cleaved within the exposed loops connecting transmembrane helices into fragments containing the transmembrane helices I–II, III–VII with chymotrypsin *(35,36)*, and I–V; VI–VII with *Staphylococcus aureus* V8 protease *(37)*. These fragments can be purified under denaturing conditions and reconstituted into liposomes to regenerate the retinal chromophore and light-activated proton pumping. Reconstituting with TMH III–VII and synthetic peptides corresponding to TMH I and II also regenerates function *(38)*.

An analogous scheme appears to function in eukaryotic cells as well. Similar, but less detailed, observations have been made employing proteolysis of membrane-bound forms of rhodopsin *(39)*, β_2-adrenergic receptor *(40)*, and turkey $\beta^1/_2$-adrenergic receptor *(41)*. Biochemical studies with receptor fragments generated by proteolysis in natural systems have been augmented and extended to lower abundance receptors by coexpressing recombinant fragments of receptors in cell culture and assaying function *(42)*. It is unknown at what point the helix bundles become associated. When the fragments are expressed and are stable, this obviates the difficult isolations. Bovine opsin (rhodopsin) transmembrane helix combinations [I–III;IV–VII], [I–V; VI–VII], and [I–III; IV–V; VI–VII] coexpressed in COS-1 cells with retinal supplement reconstituted the appropriate 11-*cis*-retinal chromophore and activated transducin (Gt) in vitro *(43)*. Coexpressed fragments [I–V; VI–VII] of the β_2-adrenergic receptor were functional as assayed by ^{125}I-cyanopindolol binding in *Xenopus* oocytes *(44)*. Muscarinic receptor subtype covalent chimeras m2/m3 or m3/m2 for [I–V; VI–VII] generated subtype-specific ligand binding in COS-7 cells. Only the m3/m3 fragments and the m3 full-length (wild-type) sustained agonist-stimulated polyphosphoinositide hydrolysis *(45)*. This finding suggests that portions of the m2 and m3 muscarinic receptors in addition to IC3 are required for functional coupling to G protein. Coexpression of the [I–III; IV–VII], [I–IV; V–VII], and [I–V; VI–VII] fragments (as with the chimeras) in COS-7 cells yielded high-affinity agonist and antagonist binding, accompanied by PI hydrolysis for the fragment combinations of the m3 muscarinic receptor *(46)*. Other combinations of expressed fragments failed to reconstitute, determined to be largely attributable to improper processing of the receptor fragments by the cells. A number of mutant vasopressin (V2) receptors responsible for nephrogenic diabetes insipidus contain defects in IC3 or TMH VII. These nonsignaling receptors could be complemented by coexpression of the TMH VI-VII to C-terminus fragment of normal receptors *(47)*.

Another avenue of evidence supporting a modular structure for GPCRs comes from studies of receptor subtype covalent chimeras. Receptor subtypes are sequence-variant, and therefore presumably structurally variant, forms of receptors that interact with a particular biological ligand, often distributed in tissue-specific patterns. While interacting similarly with the same biological ligand, they can be distinguished by a series of pharmacologic ligands optimized to bind selectively to individual receptor subtypes. The α_2- and β_2-adrenergic receptors can bind the same ligand, epinephrine, but the receptors are distinct gene products coupling to different G proteins, Gi and Gs, which inhibit and stimulate adenylate cyclase activity, respectively. Early chimeric

studies with selective ligands found that the G-protein coupling specificity was contained within a region of the receptors containing the TMH V–VI *(44)*. This also included IC3, the third intracellular loop, which has since been demonstrated to bear major determinants for G-protein activation *(48)*. TMH VII was found to control functional determinants of subtype-specific agonist and antagonist ligand binding.

Evidence for domain swapping between GPCRs was obtained with chimeras formed from the exchange of the C-terminal TMH VI-VII regions of the α_{2c}-adrenergic and m_3 muscarinic acetylcholine receptors. When expressed individually in COS-7 cells, these chimeric receptors failed to either bind radioligands or to stimulate phosphoinositide hydrolysis *(49)*. However, when the α_{2c}/m_3 and m_3/α_{2c} chimeras were coexpressed in the same cell, binding and PI hydrolysis responses to both ligands were restored to near normal. Such results suggest that I–V; VI–VII bundles from the two different receptors can reform the appropriate associations with the cognate receptor domains to restore biological function. The ability to effect the exchange appears dependent on the presence of a long IC3 loop *(50)*. Ligand-binding but nonsignaling muscarinic receptor mutants could also complement one another to partially restore PI hydrolysis. Distinct coexpressed non-ligand binding site point mutants of the angiotensin II AT_{1A} receptor, [K102 (TMH III) and K199 (TMH V)] were shown to complement each other in binding assays but did not couple to inositol phosphate metabolism *(51)*. Such results suggest that receptors may multimerize in the plane of the membrane and can exchange helix bundles with appropriate partners. Experimental support for this type of interaction comes from muscarinic receptor dimers detected by photoaffinity labeling in rat heart *(52)* and brain *(53)*. Epitope-tagging experiments with the β_2-adrenergic receptors demonstrated homodimerization of receptors which can be blocked by a β_2-TMH VI peptide, residues 276–296, but not a TMH VII peptide from the dopamine D2 receptor or other peptides *(54)*. In prevailing models of helix arrangement of GPCRs, TMH VI is a highly membrane-exposed helix, and thus would be a candidate for an interaction domain within the bilayer. The vasopressin V2 receptor was shown to homodimerize under similar conditions, whereas β_2-adrenergic receptors do not heterodimerize with muscarinic m2 acetylcholine receptors *(54)*. The relationship of such associations to receptor biology is unknown. Models involving multimeric complexes of receptors and G proteins have been invoked to reconcile binding isotherms with functional response coupling *(55)*.

Loops Connecting Transmembrane Helices

With the capacity for self-assembly demonstrated by the GPCR transmembrane helices, one might wonder about the role of the polypeptide chain connecting the transmembrane helices in receptor structure and function. In some cases, it appears to impose geometrical constraints on the helical bundle. A disulfide bond between extracellular loops EC1 and EC2 restricts the orientation of TMH I, II and III, IV in many G-protein receptors and may be required for correct folding during synthesis and membrane insertion as well as for stabilizing high-affinity binding *(56)*. Some receptors, such as those for cannabinoids, appear to lack this disulfide. The consequences for receptor structure are unknown.

In many respects, the parts of the receptor connecting the transmembrane helices support the most interesting interactions and functionality of the receptor. Figure 1 identifies the established functionalities of a generic GPCR. In particular receptors, some loops are quite large, such as the IC3 of the m3 muscarinic cholinergic receptor with 242 amino acids, which probably have structure of their own, whereas others may be too short to maintain a structure in solution. Conformations of the short loops may be influenced by other parts of the receptor, or be modified by proteins that interact with the receptor. Cellular expression of intracellular interhelix loops *(57)* and muta-

genesis experiments *(58)* have implicated multiple intracellular loops in G-protein interactions. Synthetic peptide *(59)* and loop region antibody competition studies *(60,61)* have also provided evidence for interactions with receptor loop regions. The effects of these added peptides were sequence-specific, although they often required 10^{-5}–10^{-4} *M* concentrations. The poor efficacy may be the result of a requirement for conformational selection from a random coil population of conformers. Gs, but not Gi/Go, was activated by 15-amino acid peptides from the N- and C-terminal parts of the hamster β_2 adrenergic receptor IC3, but not by other peptides from this loop or the C-terminal receptor tail. Activation occurred only when the G protein was incorporated into phospholipid vesicles *(62)*. The G-protein receptor kinase (GRK), involved in short term homologous desensitization of some GPCRs, bound to and was influenced by peptides derived from multiple loops of the β_2-adrenergic receptor *(63)*. Peptides from IC2, IC3, and IC4 (TMH VII to the palmitoylation sites) of rhodopsin, but no other extracellular or intracellular exposed regions of the receptor, synergistically competed for the interaction of transducin (Gt) with metarhodopsin II *(64)*. A 13-amino acid peptide from the C-terminal portion of IC3 interfered directly with Gi activation by the human α_{2A}-adrenergic receptor and a 12-amino acid peptide from IC2 interfered with Gi-dependent high-affinity agonist binding to the receptor, whereas other loop peptides had no effect *(65)*.

Biophysical measurements similarly argued for interactions among the intracellular loops. Site-directed mutagenesis substituted cysteines modified by nitroxide spin labels at strategic positions argued for partial immobilization of the small EC1 loop, as well as the large IC3 loop in the non-G-protein coupled seven TMH analog, bacteriorhodopsin *(66)*. In the G-protein-coupled rhodopsin, nitroxide spin labels attached to H65C (histidine 65 substituted with cysteine) near the cytoplasmic face of TMH I and C316 in the C-terminal tail of the receptor near TMH VII indicated that these residues, and hence the helices, were within 10 Å of each other in dark-adapted rhodopsin, consistent with the GPCR helix arrangement in Figure 1. Light activation caused them to move apart, implying a considerable conformational change *(67)*. Similar labeling experiments suggest rigid body motion between TMH III and TMH VI upon receptor activation *(68)*, as well as reorientation of both TMH III and TMH IV with respect to IC2 *(69)*. Comparison of the exposure of nitroxide spin labels to aqueous- and lipid-localized paramagnetic probes showed that the membrane hydrophobic/aqueous boundary is near V138 in TMH III and H152 in TMH IV *(69)*.

NMR studies of the synthetic peptides corresponding to IC1 (17 aa), IC2 (16 aa), and IC3 (22 aa) of rhodopsin showed that although short, all three peptides adopted stable β-turn structures in solution. The TMH V segment leading into IC3 showed some α-helical character extending into the loop, as did TMH VII, which extended into the C-terminal tail of the receptor. The other helices did not appear to extend into the IC1 or IC2 cytoplasmic loops *(70)*.

Extracellular Receptor Surface

Much less is known about the extracellular loops of the GPCRs. Although many receptors are glycosylated on the N-terminal polypeptide preceding TMH I, ligand binding and signal transduction are generally are not critically dependent on the presence of the carbohydrate. The N-terminal peptide may be deleted with little effect for the small molecule ligand receptors. The carbohydrate may play a role in other aspects of receptor regulation, such as turnover and processing during internalization cycles. By contrast, the gonadotropin family of peptide hormone GPCRs possess an atypically large N-terminal domain that participates in binding the large protein ligand *(2)*, and in this case glycosylation was important in receptor function *(71)*. The extracellular face of the receptor was also responsive to the functional state of the receptor. Antibodies to the EC2 loop stabilized the activated agonist-liganded

state of the β_2-adrenergic receptor *(72)*. The development of such antireceptor antibodies spontaneously (autoantibodies) leads to a pathological state in Grave's disease (thyroid-stimulating hormone receptor) *(73)* and asthma *(74)*. An intact disulfide between cysteine residues in the EC1 and EC2 loops connecting TMH III and TMH IV conserved among many GPCRs is required for ligand binding (signaling) *(56)*.

These least constrained parts of the receptor have not yielded to physical structure determination of the entire receptor. In the diffraction images of bacteriorhodopsin and rhodopsin, the loops were unresolved, implying considerable mobility. Modeling studies simply omit the connecting loops for lack of data. This flexibility does not imply a lack of interactions or deny the organizational importance of the connection. Interactions with other proteins or the lipid bilayer may stabilize particular conformations of the loops. What is certain is that the connecting loops are critically important in specifying membrane insertion of GPCRs after their synthesis. The polypeptide chain that will become the connecting loops codes a portion of the stop-transfer positive charge clusters that, in concert with the membrane and cytosolic factors, including ATP and one or more high-molecular mass (>30-kDa) factors *(75)*, organize the insertion of the transmembrane segments into the bilayer *(18)*. The connectivities and restricted lengths of some loops place limits on the arrangement of the helices, a criterion useful in modeling *(20)*. Their length was used to constrain the interpretation of the electron diffraction data on rhodopsin and bacteriorhodopsin *(9,10)*. Other transmembrane proteins have evolved analogous mechanisms to control insertion and orientation of membrane-penetrant domains.

G-Protein Coupling Region

The interhelix loops are the portions of the GPCRs directly accessible to cytosolic or extracellular proteins. Both the N- and C-terminal ends of the third intracellular loop connecting TMH V and VI adjacent to the bilayer are responsible for the coupling to, and specificity for, the heterotrimeric G-proteins (recent references include *(76,77)*. Crosslinking studies with a photoaffinity labeled peptide (residues 361–373) from the N-terminal region of the porcine α_{2A}-adrenergic receptor IC3 loop indicated an association between this region of the receptor, as well as both the α and β subunits of purified Go/Gi reconstituted with receptor in liposomes *(78)*. This peptide also stimulated Go/Gi GTPase activity of the heterotrimer, but not of the separated Gα subunit, whereas an N-terminal peptide from IC3 was less effective. The C-terminal region is believed to be an amphipathic helix, and alanine insertional mutations implicated the hydrophobic face of this helix in the G-protein recognition surface *(79)*. Transmembrane helix boundaries estimated by nitroxide label exposure to paramagnetic probes in the aqueous and membrane phases place the cytoplasmic ends of TMH V (V227–K231) and TMH VI (V250–V254) at the bilayer surface. Periodic variations in the exposure and mobility of the nitroxide labeled sidechains support the extension of the transmembrane α-helical conformation from 1.5–3 turns out of the membrane into IC3 *(80)*, which is also the part of that loop thought to interact with Gt.

In other GPCRs, the human dopamine D1, D2, and β1-adrenergic receptors, however, experiments with synthetic peptides, $\leq$20 amino acids, derived from the N- and C-terminal ends of the IC3 loop indicated that formation of an α-helix was not needed for activity *(81)*]. Only the N-terminal D2 peptide antagonized G-protein activation by the cognate receptor and stimulated Gi/Go GTPase activity. Circular dichroism showed that the D2 peptide possessed little α-helix, whereas the C-terminal D1 peptide was α-helical but inactive in other assays. These discrepancies may be explained by the inability of an isolated short peptide to manifest a stable secondary structure in solution, although the isolated loops of rhodopsin are capable of forming stable β-turn solution structures *(70)*.

Specialized Functional Domains of GPCRs

GPCR-mediated signaling is modulated through the receptor at several levels: (1) by controlling the number and accessibility of receptors to the ligand on the cell surface, (2) by limiting signal amplification, (3) by weakening the coupling to G proteins, and (4) by modulating interactions with cellular proteins that actively terminate signaling. Except for transcriptional regulation of the number of receptor molecules, all these events are mediated through posttranslational reversible modifications of segments of intracellular loops. Figure 1 indicates their location generically, although in some receptors the functional domains appear to have been translocated to other loops. While homology in primary amino acid sequence allows prediction of these motifs, no three-dimensional structural data are available.

Phosphorylation Sites

GPCRs contain multiple sites for phosphorylation by various serine- and threonine-modifying protein kinases, which are distinguished by the primary amino acid sequence surrounding the residue bearing the primary alcohol sidechain. Those sites most readily identified are those recognized by the second messenger-regulated protein kinases, protein kinase C, cAMP- and cGMP-dependent protein kinases, and calmodulin-dependent protein kinases. Activation of these kinases is a response to the activation of signaling events, through G-protein-coupled receptors or other stimuli. Since the general effect of phosphorylation by these kinases is to uncouple the receptor from the G proteins, this feedback regulation tends to reset the signaling system so that it will be ready for the next stimulus. Regulation by these protein kinases tends to be heterologous, because receptor activation does not influence the accessibility of these phosphorylation sites. A rise in second messenger levels in the cell (cyclic nucleotides, calcium, inositol phosphates/diacylglycerol) is sufficient to activate the kinases. The activation state of the protein kinases and the accessibility of the receptor at the plasma membrane seem to account for phosphorylation, so unactivated receptors and unrelated GPCRs with the appropriate primary amino acid sequence can also be modified.

A family of serine-threonine kinases whose substrate recognition signature contains multiple acidic amino acid residues, and that requires agonist-occupied receptor for phosphorylation, is the family of G-protein-coupled receptor kinases (GRKs). The substrate sequences are typically in the C-terminal peptide after TMH VII, although the acidic target sequence in the m2 muscarinic acetylcholine receptor appears to have been "translocated" to the middle of IC3. These protein kinases confer homologous regulation, only acting on agonist-occupied receptors. This mechanism competes effectively with heterologous regulation only under conditions of high receptor occupancy, and hence high agonist concentration. The nervous system, principally synaptic regions with high concentrations of GPCRs and restricted volume synaptic clefts, is enriched in the GRKs, particularly with two family members bearing a domain that interacts with G protein βγ subunits, β-adrenergic receptor kinase-1 (βARK1) and βARK2. Because of its specialized intimate association with GPCRs, and because of some recent NMR structural studies of its Gβγ-binding Pleckstrin homology (PH) domain, βARK1 is considered in more detail in a later section. Reviews on the GRKs and desensitization are available *(82–86)*.

Internalization

The primary amino acid sequence NPXXY at the junction between the C-terminal α-helix of TMH VII and the C-terminal tail is conserved in many GPCRs as well as other types of receptors has been shown by mutation of tyrosine (Y326; β_2-adrenergic receptor) *(87)* to be one of several sites on receptors involved in coupling GPCRs to the cellular machinery responsible for sequestering the receptor away from the plasma membrane and disruption of the signaling process. Other parts of the receptor also contribute to sequestration *(88)*. Exposure to

agonist ligand leads to internalization of the receptor and eventually either recycling of the receptor to the plasma membrane, or direction to the lysosome for destruction. β-Arrestin, a well-described signal termination protein that binds to GRK-phosphorylated GPCR's, blocking further G-protein activation also has been implicated in targeting the phosphorylated receptors to clathrin coated pits *(89)*. The activation cycle is completed by removing the regulatory phosphates, regenerating the ground state of the signaling system. This resensitization process occurs within acidic endosomes containing the internalized receptors in which a member of the PP-2A family of protein phosphatases dephosphorylates the β_2-adrenergic receptors *(90)*. Once again, no physical structural detail is available.

Palmitoylation

Many GPCRs and their associated proteins and other signaling systems are subject to lipid modification, which presumably facilitates their interactions at the lipid interface *(91)*. Fatty acylation also modulates receptor internalization *(92,93)*. Some of these modifications are dynamic, responding to the agonist state of the system *(94)*. A number of GPCRs are modified 13–15 residues C-terminal to TMH VII on one or more cysteine residues flanked by multiple N-terminal hydrophobic residues and followed by multiple C-terminal basic amino acid residues. Rhodopsin, the β1- and β2-adrenergic, α1- and α2-adrenergic, m2 muscarinic cholinergic, dopamine 1, serotonin 1A, 1B, 1C, and 2, endothelin A and B, substance K, vasopressin-2, luteinizing hormone/human choronic gonadotropin, and metabotropic GluR4 receptors all undergo palmitoyl modification. The palmitoyl-thioesters are both hydroxylamine-labile and subject to enzymatic removal, and the deacylation alters the functional properties of the receptor *(95)*. Palmitoylation of some receptors have been shown to parallel an increase in G-protein coupling efficiency and palmitoylation can be modulated by agonist occupancy of the receptor *(96)*. The fatty acyl chains have been shown by dynamic quenching studies of rhodopsin labeled with a fluorescent substitution for the palmitic acid to insert deeply in the lipid bilayer *(97)*. This adds another receptor attachment point to the lipid bilayer, creating a fourth IC loop. A synthetic peptide corresponding to this loop derived from the avian β-adrenergic receptor is an antagonist of receptor-stimulated adenylate cyclase activity *(59)*. In the presence of lipid micelles, this peptide, residues 345–359, was shown by two-dimensional NMR to be entirely α-helical and adsorbed to the bilayer surface *(98)*. The relationship of this loop to the other cytoplasmic receptor sequences is unknown.

Heterotrimeric G Proteins

The GTP-binding coupling proteins are the most abundant component of the GPCR signaling system and have been most amenable to purification and study by a variety of biochemical and biophysical techniques. Although not integral membrane proteins, they are post-translationally modified to target them to membranes with a series of lipid moieties. They have also been the first to yield atomic resolution structures by X-ray crystallography, initially with the α subunits of Gt and Gαi, followed by the heterotrimer complexes of Gαi–Gβ1γ2 and Gαt–Gβ1γ1. The structures provide physical evidence for the diversity in signal transduction function so well documented by biochemical and molecular biological studies.

Gα–GTP-Binding Subunit

Some 20 Gα subunit subtypes have been defined by molecular cloning and have been grouped into families by sequence homology and biochemical properties including sensitivity to the ADP-ribosylating bacterial toxins cholera toxin and pertussis toxin. Although receptor subtype selectivity is observed in vivo *(99)*, biochemical reconstitution or cellular overexpression within Gα subunit families fails to achieve a comparable subtype selectivity.

There are clearly additional constraints on the supramolecular organization of G-protein-coupled systems in cells that are not present in vitro and that may be overwhelmed in overexpression systems. Distinct subtype selectivity between receptors and effectors also has been demonstrated in intact cells with antisense nucleotides to Gα subtypes *(100)*. The core structures of Gα subunits map onto the primary sequence of the proteins defining G regions categorized as to subfamily (e.g., Gαs; Gαi/o/t; Gαq; Gα 12/13). Subtypes (e.g., Gs family = Gs and Golf; Gi/o/t family = Gil-3, Go, G z, Gt1, Gt2, Ggust; Gq family = Gq, G11, G14, G15; G12/13 family = G12, G13) differ primarily in insert regions, which extend outside the core protein fold *(99,101)*. In the Gα subunits, this core comprises the GTP-binding site and associated motifs that propagate the conformational change associated with GTP hydrolysis to the C-terminal peptide involved in interactions with receptors. Although poorly resolved in crystal structures because of their flexibility, NMR studies of the isolated C-terminal 10-amino acids from Gt (340–350) showed differences in structure of the peptide in the presence of light-excited rhodopsin (agonist condition) (metarhodopsin II) and dark-adapted rhodopsin (antagonist condition). An N-terminal helix-like turn was relaxed, whereas the C-terminal β-turn disappeared upon binding to metarhodopsin II *(102)*. This structural transition may explain the subfamily specificity of Gα subunits. Substituting three C-terminal residues of Gαq by those of Gαi converted its receptor specificity to that of Gαi. The amino acid sequences of the C-terminus in the two subfamilies predict different types of β-turn *(103)*. Deletion of the N-terminal six amino acid residues of Gαq allowed the truncated Gαq to interact productively with receptors that normally couple only to Gαi/o or to Gαs *(104)*. The interaction of Gα's with phospholipase Cβ1 (Gq), cGMP phosphodiesterase (Gt), and adenylate cyclase (Gs) all involve residues in α-helices 3 and 4 of the respective Gα-subunits, a region in which there is a corresponding divergence in amino acid sequence *(105)*.

The X-ray structures of p21ras *(106)* and the bacterial protein synthesis elongation factor EF-Tu *(107)* provided a template of the guanyl nucleotide binding/GTPase domain that predicted *(101)*, with appropriate insertions, the subsequently determined Gα structures. The structural features of the GTPase domain are highly conserved within the family, with insertions accounting for the idiosyncrasies of the individual signal switch proteins *(108)*. Comparisons of the X-ray structures of the activated (GTP-γS)- and (GDP)-bound forms of Gt *(109)* and Giα1 *(110)* showed that the structural changes initiated by the terminal γ-phosphate of GTP binding extended to regions thought to be involved in effector activation some distance from the nucleotide-binding pocket. The structure of Giα1 complexed with the transition state analog GDP-AlF_4^- revealed a pentacoordinate intermediate just before cleavage of the terminal phosphate bond *(111)*. A mutant Giα1 (G203A) with reduced Mg^{2+} affinity for the GTP-protein complex, inhibiting a key conformational change upon GTP binding, failed to release the Gβγ subunits, thereby blocking effector activation. The structure of the GDP–Pi complex of this mutant resembled a transition state after GTP hydrolysis to GDP but before the release of phosphate *(110)*. The switch II region conformation that interacts with Gβγ is stabilized by the mutation.

The crystal structure of Gαs in complex with GTPγS has also been determined *(112)*. A ribbon diagram of the structure is presented in Fig. 7C. Superimposing the corresponding previously determined Gαi-GTPγS X-ray structure indicates that the differences in effector specificity lie in the binding surface formed by the switch II helix and the α3–β5 loop, although the primary sequences are quite homologous in this region. The lack of regulation of Gαs GTPase activity by most G-protein modulators is likely to reside in the sequence divergence. Receptor specificity, the other target of the coupling action of the Gα subunit, is probably determined by differences in the surface made up of the C-terminal helix and the α4–β6 loop.

While the physical structure of the Gαs subunit and the complex with the catalytic portion of adenylate cyclase has been solved *(113)*, much less is known about the physical basis of the interaction with receptors. The crystal structure of Gt suggested that a surface loop containing residues 212–215 contacts the C-terminus of Gαt, which interacts with rhodopsin. This is within the switch II region whose conformation is altered upon activation of Gt by GTP. Other residues contacting the guanine ring and the GTPase domain also are good candidates for receptor contact regions *(114)*.

Lipid Modification

Gα subunits are differentially covalently modified by lipids that serve to alter protein–protein interactions, membrane binding and subcellular localization *(115)*. Palmitoylation of Gαs, Gαo, αGil-3, Gαz, and αGq (on two cysteine residues) occurs on N-terminal internal cysteines within the consensus sequence (N-terminal M)-G or S-X-X-S or C, where X is any amino acid and is dynamically regulated by receptor interactions *(116)*. In the G αo, αGil-3, and Gαz subtypes, myristoylation on the N-terminal glycine also occurs and is permissive for subsequent palmitoylation, whereas Gαs and Gαq are not myristoylated. These lipids also are required for full biological function, including second messenger production by the effector *(117)*. Co-translational N-terminal myristoylation is a common modification of eukaryotic cellular and viral proteins, serving to either stabilize the protein or increase its association with membranes *(118)*. Myristoylation of the src tyrosine kinase is required for oncogenicity *(119)*. In a number of signal transducing proteins, such as recoverin, which prevents phosphorylation of rhodopsin in the retina in response to a rise of intracellular calcium, the exposure of the lipid group and thus protein activity is controlled by a signal-linked conformational change. The structural aspects are discussed in Myristoyl-Switch Proteins.

Palmitoylation is closely linked with membrane association as established by a variety of mutation experiments. Attachment of the C16:0 fatty acid potentiates Gβγ subunit binding, which subsequently protects the palmitoyl residue from cellular esterases *(120)*. Despite their importance in membrane localization and coupling to receptors and effectors, acquiring the X-ray structural information required removal of the lipid-bearing N-terminus of Gt and Gαi; consequently, we have no information as to the structural role of the fatty acyl chains.

Gβγ Subunit-Membrane Targeting Subunit

The jumble of subtypes continues with 5 Gβ and 13 Gγ subtypes defined at this point. The dimer structure is stabilized through N-terminal coiled-coil interactions between the β and γ subunits. Crosslinking and chimera studies have established that significant interactions occur between the N-terminal half of Gγ1 and residues 210–293 *(121)* of the C-terminal portion of Gβ1. The native subunit complex is remarkably compact and is composed of two proteolytically stable domains. Trypsin cleaves at R129 in the β subunit *(122)*, and Lys-C protease cleaves at K32 in the γ subunit *(123)*, yielding fragments that remain associated until the proteins are denatured. The C-terminal 11-amino acids of Gγ1 have been shown to interact with activated rhodopsin when the Gβ1γ1 is in complex with Gαt by peptide competition and by mutational analysis *(124)*. This part of the protein was not accessible to antibody in the Gβ1γ1 or in Gαtβ1γ1 heterotrimer but readily reacted as the free Gγ1 subunit, prompting the postulate of a conformational change to induce interaction of this peptide sequence with the receptor. Coexpression of different pairs of Gβ and Gγ subtypes in mammalian or insect cells produced heterodimers with highly similar receptor and effector coupling properties. An exception is the Gβ1γ1 combination, the retinal isoform, which was only efficient with its complement of retinal proteins, rhodopsin/GRK1 (rhodopsin kinase)/Gαt. Clear subtype selectivity between receptors and effectors has been demonstrated only with antisense nucleotides to Gβ *(125)* and Gγ subtypes *(126)*.

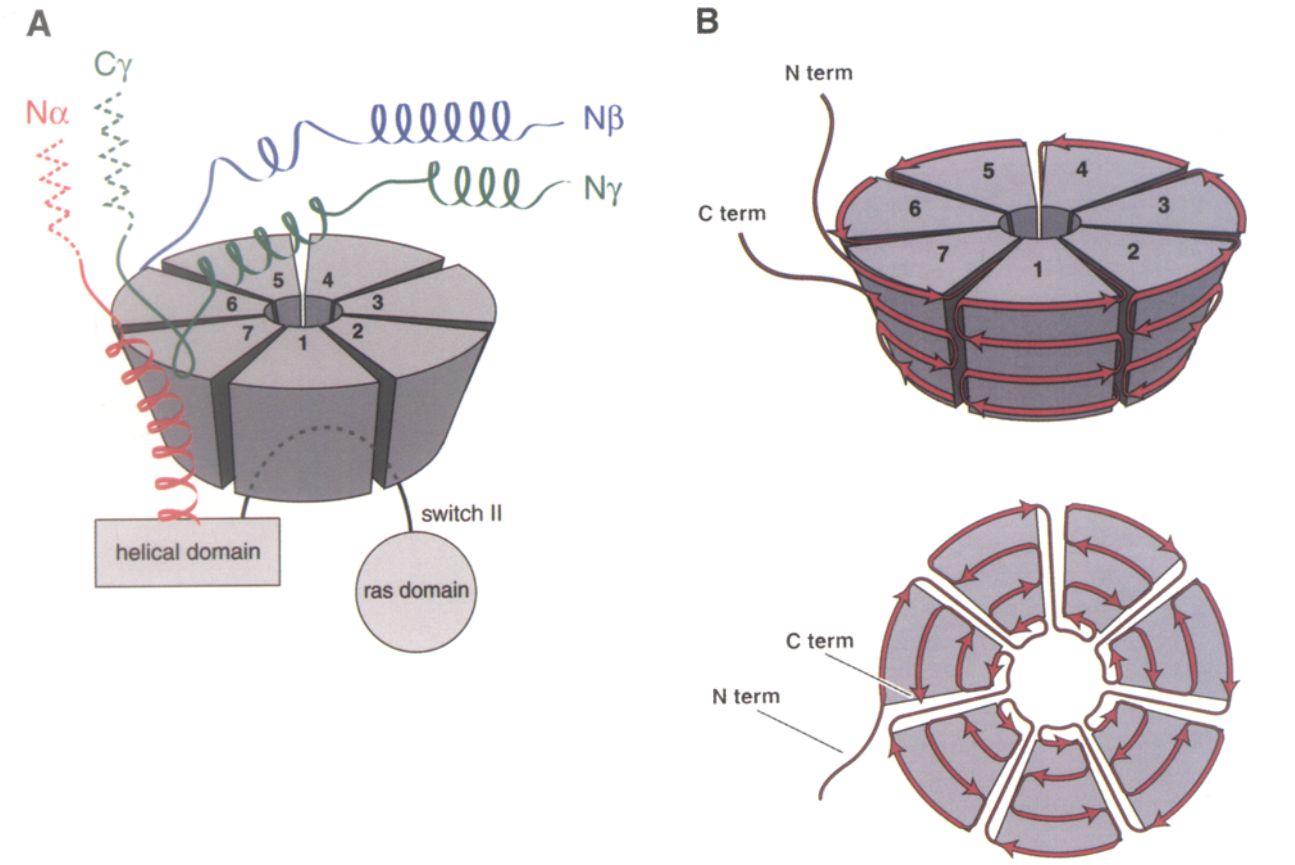

Fig. 2. Structure of heterotrimeric G proteins. **(A)** Gαβγ heterotrimer (schematic). N-terminal helical domain of Gα, Nα, N-terminus of Gα, *red;* switch II, mobile region of Gα whose conformation is determined by the nucleotide bound (GDP vs GTP); Gα ras domain, ras-homology domain bearing the GTPase activity; toroidal seven-bladed propeller motif of Gβ. Numbers 1–7, WD40 domain repeats; Nβ, N-terminus of Gβ, *blue;* Nγ, N-terminus of Gγ, *green;* zig-zag lines, attached lipid alkyl chains, dotted, presumed location because they are not present in the crystallized protein. **(B)** Gβ subunit, a seven-bladed propeller of WD40 repeat domains. *Arrows,* orientation of the β strands within each blade; N-term, N-terminus; C-term, C-terminus. *Top,* side view. *Bottom,* top view (from wide end of torus).

The structure of the complex between the Gα and Gβγ subunits has been determined for Gαt: Gβ1γ1 *(127)* and for Gαi:Gβ1γ2 *(128)* by X-ray diffraction. A schematic version is presented in Figure 2A. Gβγ forms a tapered toroidal β-propeller domain with the Gα largely contacting the top of the narrow end of the toroid and extending an N-terminal helix parallel to the toroidal axis along the edge of blade 1. Gα structure is largely unperturbed by the formation of the complex. The switch II region (residues 199–216) whose conformation is sensitive to the nature of the guanyl nucleotide bound is involved in the contact, as is the N-terminal α-helix of Gα. Dissociation of Gα from Gβγ is readily explained by the conformational changes in the switch II region induced by the replacement of GDP with GTP.

Interaction of domains of Gβ with receptors and effectors begins to make sense in light of the hepta-bladed propeller configuration of that subunit. Yeast two-hybrid studies impli-

cate the N-terminal 100 residues (blades 1 and 7) of Gβ interacting with type II adenylate cyclase (AC II) and the muscarinic acetylcholine receptor-gated atrial inwardly rectifying potassium channel G-protein-regulated inwardly rectifying K^+ channel (GIRK1) *(129)*. This site on Gβ may be shared among AC I, AC II, GIRK1, phospholipase Cβ3, and βARK, as a peptide encoding AC II residues (956–982) interferes with the interaction of Gβ with these molecules *(130)*. Molecular modeling of AC II (956–982) onto Gβ led to the experimental demonstration that Gβ residues 84–143, and particularly Gβ (86–105), would mimic Gβγ effector activity by inhibiting Ca^{2+}-calmodulin-stimulated AC I and blocking inhibition of AC II by Gβγ *(131)*. Gβ residues M101 and Y124 were critical for the biochemical activity of the peptides. The proposed area of contact is independent of the Gα binding surface on Gβ. By contrast, a peptide from the third intracellular loop of the α2-adrenergic receptor could be photochemically crosslinked to a 6-kDa fragment within the C-terminal 60 amino acids of Gβ1, encompassing the seventh, and possibly the sixth, WD40 domain of Gβ *(132)*.

The characteristic repeat domain structure of Gβ has been the subject of much conjecture. The repeating unit of 40 amino acids, usually ending in trp-asp (WD), was first characterized in Gβ. Homology searching defined a pattern

$$\{X_{6\text{–}94}\text{——}[GH\text{-}X_{23\text{–}41}\text{——}WD]\}^{N_{4\text{–}8}}$$

where X is any amino acid, G is glycine, H is histidine, W is tryptophan, and D is aspartate, which has since been found in numerous eukaryotic proteins involved in signal transduction, RNA processing, gene regulation and development, vesicular trafficking, and cytoskeletal and cell cycle regulation *(133)*. The prediction of a four-stranded β-sheet fold for the WD40 domain was largely confirmed with the solution of the X-ray structure of Gβ (schematic in Fig. 2B). The tertiary structure of Gβ places it in the family of β-propeller proteins, containing at this time mostly enzymes; four-bladed and six-bladed bacterial and viral sialidases, seven-bladed fungal and prokaryotic glyoxal and fungal galactose oxidases and protein GO; and eight-bladed methanol and methylamine dehydrogenases. Each blade consists of four antiparallel β strands. As with other propeller folds, the repeating primary sequences overlap between successive blades, the last, outer strand of one blade, and the first three, inner strands of the next blade (Fig. 2). The N-terminal ligand-binding domain of integrin α subunits containing seven 60-amino acid (phe-gly and gly-ala-pro FG-GAP) repeats has been predicted to form a seven-bladed propeller *(134)*. If this is verified by direct structural analysis, this may have implications for potential common signaling mechanisms shared by the Gαβγ and integrin αβ complexes, which consist of multiple subtypes and could determine their binding specificity for extracellular matrix proteins. The X-ray structure of an insert within the third FG-GAP repeat in the integrins Mac-1 and LFA-1 has been solved *(135)*. The I domain contains a metal ion-dependent adhesion site with an overall fold similar to that of nucleotide-binding enzymes, including the small GTP-binding proteins and the large G-protein α subunits. This site is thought to be involved in ligand binding and integrin activation. Several in vitro synthesized WD40 proteins whose functions are unknown or unassayable (sec13, RACK1, coronin, LIS1, and TUP) folded to yield hydrodynamically compact, trypsin-resistant structures while βTrcp formed an aggregate. The aggregation was interpreted as a requirement for a folding partner for this protein, in analogy with the Gβ requirement for Gγ to fold properly *(136)*.

Although the X-ray structure of the heterotrimeric G-protein complex located the Gα at the narrow end of the tapered doughnut, it did not delineate how this complex is oriented with respect to the plane of the membrane. The normally lipid-modified portions of Gα (N-terminus) and Gγ (not modified in the crystal) were not visible, and the peptide chain was disordered in the crystal. The modified regions are, however, on the same side of the molecule; if inserted into the membrane, they could con-

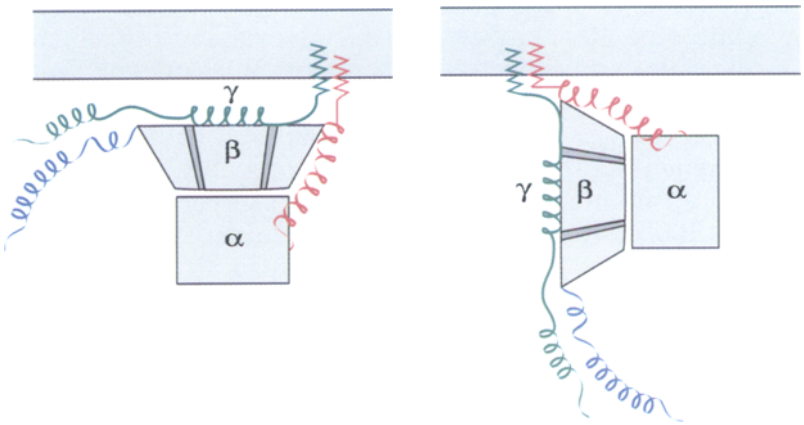

Fig. 3. Orthogonal orientations of Gαβγ with respect to the lipid bilayer. α, β, and γ are the subunits of the heterotrimeric G protein. Helices are the N-terminal α-helices of each of the three subunits. *red,* Gα; *blue,* Gβ; *green,* Gγ.

ceivably enter the bilayer near the same site. There are two possible orthogonal arrangements (Fig. 3) with implications for complex formation with other proteins in the signal transduction process. The binding of a modulatory protein, phosducin, to the wide end of the Gβγ torus, blocks association of the Gβ1γ1 (Gβγt) subunit with the membrane, translocating the G protein subunits into the cytosol *(137).* This finding suggests, but does not prove, that orientation B (Fig. 3) in which the wide end of the torus faces the bilayer is an appropriate arrangement. The translocation by phosducin of other than transducin Gβγ subunits has not been documented. It is possible that both configurations of the complex exist under different conditions or with different protein-binding partners. Measurements of fluorescence energy transfer between labeled membrane bilayer and complementarily labeled Gα, β, and γ subunits are consistent with the Gα subunit residing farthest from the membrane in reconstituted liposomal systems *(138).* A membrane-localized probe photolabels Gα and Gβ, but not Gγ. Gβ is modified by this reagent at several sites scattered throughout its sequence *(132).* Additional experiments will be required to distinguish between the two arrangements depicted in Fig. 3.

Lipid Modification

The Gγ subunit contains a prenyl (isoprenoid) group in thioether linkage with the C-terminal cysteine. Other proteins including *ras*-related GTP binding proteins and nuclear lamins are similarly modified by cellular prenyl transferases, resulting in increased membrane association. The characteristic -CAAX sequence is proteolytically processed to remove the –AAX tripeptide and the C-terminal cysteine is subsequently carboxymethylated by an *S*-adenosyl-methionine-dependent pathway, (X=S,M, or Q=Gγ1=farnesyl C15; X=L; Gγ2 and others = geranylgeranyl C_{20}), (reviewed in *(139,140).* Carboxymethylation of Gγ2 in isolated neutrophil membranes is stimulated by GTPγS alone and is potentiated by the proinflammatory formyl peptide receptor agonist fMet-leu-phe *(141).* Prenylation is not required for Gβγ assembly, but proteolytic processing to remove the -AAX moiety C-terminal to the modified cysteine occurs subsequent to dimer formation. The prenyl groups may serve functions in addition to membrane association by promoting activated receptor–G protein interactions. S-prenylated cysteine analogs applied to granulocytes *(142)* or platelets *(143)* exert effects on G-protein activation by receptors unrelated to their potency as prenyltransferase

or methyltransferase inhibitors. Again, in order to crystallize Gβγ and the heterotrimer the lipid modification sites were removed from both Gα and Gβγ, so no information is available on the structural role of the fatty acyl chains.

Modulators of GPCR Function

Thus far, we have discussed the signal recognition (receptor) and the coupling/amplifier (GTP binding protein) components of GPCR transmembrane signaling systems. We have alluded to other proteins that are involved in regulation of the signaling. These proteins regulate the number of receptors available for stimulation on the cell surface, as well as how long they remain in an activated state in response to changing conditions in both the intracellular milieu and extracellular medium. Less attention has been paid to these molecules, partly because they are not in the direct line of ligand-induced signal generation and partly because of the complexity of the reconstituted systems used to study their function. Nevertheless, the phrase "timing is everthing" holds in cell biology as well as in many human endeavors, and these modulatory influences are crucial for the temporal adaptation of intercellular responses to changes in physiology and to integrate the many signal inputs to which a cell is subject. These proteins are extrinsic intracellular membrane proteins in that they are associated with membrane systems in an activity-dependent fashion, but a substantial fraction can remain as a pool of free modulator that can be recruited to the membrane by activation of GPCRs. An increasing amount of physical structural information on GPCR modulator proteins is becoming available as their importance is recognized and as these soluble proteins are being expressed for reconstitution studies.

G-Protein Receptor Kinases

Numerous GPCR signaling systems have been observed to desensitize in response to the continuous presence of agonist. This loss of signaling has turned out to be a cell type-dependent combination of individual processes designed to turn off signaling over a range of time spans. After dissecting receptor synthesis, receptor degradation, sequestration, and protein kinase uncoupling of receptors, it became apparent that rapid responses were heavily influenced by agonist-activated phosphorylation. Heterologous desensitization of different receptors by second messenger-dependent protein kinases such as cyclic adenosine monophosphate (cAMP)-dependent protein kinase, protein kinase C, and calmodulin-dependent protein kinase II occurred at low agonist concentrations. This resulted in phosphorylation of susceptible receptors and uncoupling of the receptors from G-proteins regardless of whether the receptors were agonist-occupied at the time. These phosphorylation sites are present in the receptor intracellular loops (Fig. 1), as noted earlier.

Another type of protein kinase-dependent desensitization was revealed when kin^- mutant S49 lymphoma cells lacking cyclic AMP-dependent protein kinase continued to phosphorylate the β_2-adrenergic receptor and to desensitize the cellular response to isoproterenol *(144)*. This kinase, termed β-adrenergic receptor kinase (βARK), proved later by homology cloning to be a member of a family of G-protein receptor kinases (GRKs), which included rhodopsin kinase (Table 1). These kinases phosphorylate many GPCRs on multiple serine and/or threonine residues flanked by acidic residues often present in the C-terminal tail of the receptor *(84)*. Agonist-occupied receptor is the substrate for the kinases yielding a homologous type of desensitization requiring full receptor occupancy and saturating concentrations of agonist rarely found in bulk tissue fluids. This requirement fits the biology and anatomical distribution of GRKs 1–3 and provides a rapid pathway for a receptor regulation in circumstances such as in the retina, synaptic cleft, and olfactory cilia, which would be chronically desensitized by a heterologous mechanism. Studies with cardiac over-

Table 1
G-Protein Receptor Kinases

GRK	Common name	Size aa	Size kDa	Membrane localization domain
1	Rhodopsin kinase	561	63	Farnesylation
2	βARK1	689	80	PH domain, PIP
3	βARK2	688	80	PH domain, PIP
4	IT11 kinase	Multiply spliced 500–578	61–66	Palmitoylation, C-terminal basic region in some isoforms
5	GRK5	590	68	C-terminal basic region
6	GRK6	576	66	Palmitoylation

expression of GRK5 and GRK2 (βARK1) in transgenic mice *(145)* and βARK1 knockout effects on cardiac function *(146)* showed that the GRK family of kinases can have an impact upon physiology in the intact organism.

The GRKs reversibly associate with the membrane by different mechanisms as indicated in Table 1 (*see also* ref. 84.) Seminal studies with βARK1 showed that the agonist ligand-activated GPCR *(147)*, Gβγ subunits *(148–150)*, and phosphoinositide lipids *(151,152)* were required for expression of maximal enzyme activity, but see Deb Burman et al. *(153)*. βARK1 and βARK2 can be divided into the three structural and functional regions illustrated in Fig. 4A. An N-terminal region, also found in all the GRKs, (residues 51–171) is homologous to the Regulator-of-G-protein-signaling (RGS) gene family *(154)* which include Gα-binding proteins *(155)* and the yeast pheremone response regulator Sst2p *(156)*. It remains to be seen whether the GRK N-terminus bears any structural homology to RGS domains and what implications this might have for the receptor-effector coupling cycle. A central highly conserved kinase catalytic domain of about 240 residues shared by the family members allowed for the homology cloning of the other family members from βARK1 (GRK2), except for rhodopsin kinase-GRK1). The C-terminal 253 amino acids of GRK2 and GRK3 include a 125-amino acid region homologous to a domain of the platelet protein, pleckstrin, found also in a number of other proteins important in signal transduction *(157,158)*. PH domains are a conserved secondary structure motif lacking extensive amino acid homology *(159)*. This pleckstrin homology (PH) domain in βARK1 and βARK2 is one site of interaction of the kinases with Gβγ, serving to translocate the kinase to the membrane *(148–150,160)* and activating its catalytic domain, possibly in conjunction with phosphoinositides *(151)*. Noncatalytic PH-domain-containing constructs of βARK1 and 2 act as dominant negative regulators of Gβγ function in both biochemical and cellular systems *in vitro (161,162)* and transgenically in vivo *(163)*.

Interactions of the N-Terminus and the Catalytic Domain

βARK1 interacts at multiple sites with GPCRs. The kinase N-terminus is implicated in the recognition of the receptor substrate, which is distinct from the substrate peptide phosphorylated by the enzyme, and hence interacts with the kinase catalytic domain *(147)*. Rhodopsin with the substrate C-terminal peptide removed by Asp-N protease treatment directs light-dependent activation of βARK1 phosphorylation of a synthetic peptide *(164)*. Synthetic peptides derived from the loop regions of the β_2-adrenergic receptor, in partic-

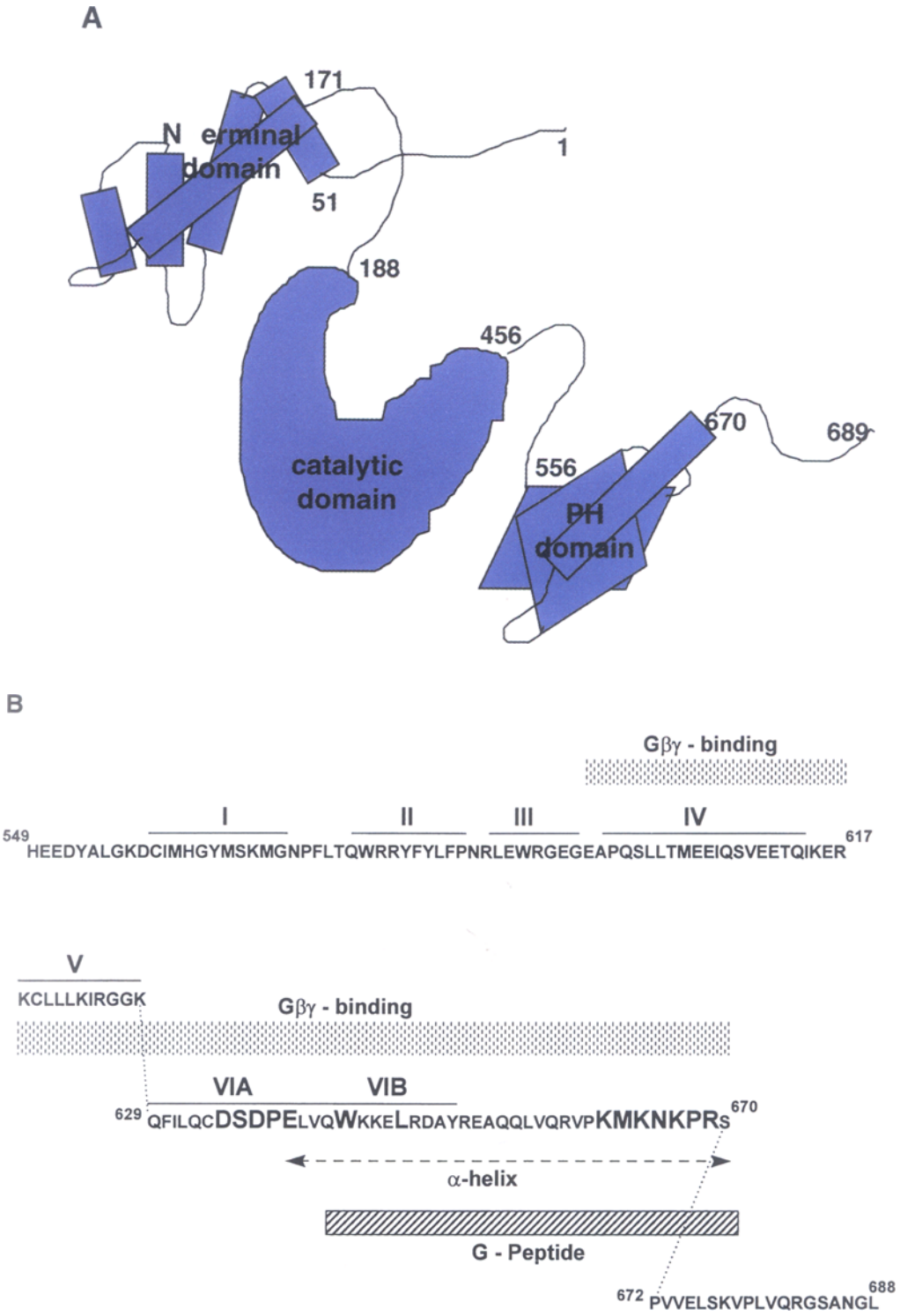

Fig. 4. Functional regions of the β-adrenergic receptor kinase (GRK 2,3) *(A)* Holoenzyme. N-terminal, catalytic, and PH domains of GRK 2,3. Numbers are amino acid residue numbers in GRK2. *(B)* PH domain substructure C-terminal portion of human GRK2 residues 549–688. I–VI represent subdomains of PH domain defined in *(167);* Gβγ-binding, regions determined by deletion analysis *(150);* α-helix, predicted helical region of PH domain; G-peptide (cross-hatched), synthetic peptide residues 639–669 that blocks PH domain: Gβγ association; residues in large bold type shown by mutation to be required for PH domain binding reactions *(167).*

ular residues *(56–74)* in ICI and *(219–243)* in the N-terminal region of IC3, inhibit phosphorylation of liposome-reconstituted receptor but are not themselves substrates *(63)*. Mutations in IC2 and IC3 of rhodopsin block binding of rhodopsin kinase to the light-activated receptor, although the phosphorylation sites are in the C-terminal tail of rhodopsin *(165)*. Preferred sites of βARK1 phosphorylation are serine or threonine residues flanked N-terminally by multiple acidic residues as opposed to the preference of rhodopsin kinase for C-terminal acidic residues *(166)* despite the high homology of the catalytic domains of all of the GRKs.

Interactions of the PH Domain: Gβγ Subunit Binding

The C-terminal PH domain of βARK1 has been studied intensively because its interactions with Gβγ are the best characterized function of this domain. The substructure of this region is shown in Fig. 4B. Whereas its physiological function is expressed as part of βARK, the fragment appears to be able to fold and function in its protein and lipid binding capacity as an autonomous domain. Truncation and mutagenesis studies have delineated regions of the C-terminal tail of βARK responsible for membrane association and binding to Gβγ subunits. They include the homologous PH domain and extend a number of residues C-terminally *(150,160)*. Recombinant PH domains from a variety of proteins involved in signal transduction (Ras-GRF, Ras-GAP, Phospholipase-Cγ, Atk, OSBP, IRS-1, β-spectrin, Racβ, mSOS) bound to Gβγ subunits (except for mSOS *(123)* with similar affinities and competed for Gβγ-dependent βARK translocation to membranes *(123,160)*. Mutational analysis of PIP2 and Gβγ binding to the βARK PH domain revealed that the PH domain-conserved W643 (Fig. 4B) was crucial to binding of both ligands *(167)*. The N-terminal portion of domain VI (VIA) contains determinants (D635-E639) for PIP2 binding while the C-terminal portion (VIB) with W643 and L647 and the basic region (K663–R669) controlled binding to Gβγ. Overall structural effects of the mutations are thought to account for the lipid binding observations. The lipid binding site is presumed by analogy to known structures (pleckstrin, β-spectrin, and phospholipase Cδ1 PH domains, see next section) to be quite distant from the mutated residues, in the N-terminus of the PH domain. The NMR solution structure of the βARK1 PH domain plus a C-terminal extension (residues 556–670) *(168)* suggested that subdomains VIA and VIB were important in determining the packing of the secondary structures in the PH domain. The effects of the mutations could be explained by tertiary structural alterations, rather than solely on the basis of primary sequence specificity.

Three-dimensional structures of several PH domains have been determined by X-ray scattering or NMR, or both. The overall fold was very similar among the different proteins which differed significantly in primary sequence (Fig. 5). Insertion of sequences in exposed loop regions at the edges of the core anti-parallel β-sandwich may account for specific biological properties of the different molecules, as indicated in the figure. The protein tyrosine phosphatase (PTB) phosphotyrosine binding domain exhibited a similar overall fold which may give a clue as to the multiplicity of binding specificities that may be present in different proteins *(169)*.

Interactions of the PH Domain: Phospholipid Binding

A high proportion of signaling proteins associated with membranes are lipid modified *(115)*. Membrane-bound proteins bearing PH domains lack lipid modification, suggesting that at least some might contain lipid binding sites. Some evidence has been obtained for association with inositol phospholipids by perturbation of the NMR signals of the lipid and of the C-terminal PH domain of pleckstrin *(170)*. Protein resonances were shifted in the N-terminal part of the domain distant from the C-terminal α-helical portion. Mutation of three conserved lysine residues in the pleckstrin PH domain demonstrated that they were key to PtdIns(1,4,5)P2 binding *(171)*. The binding site

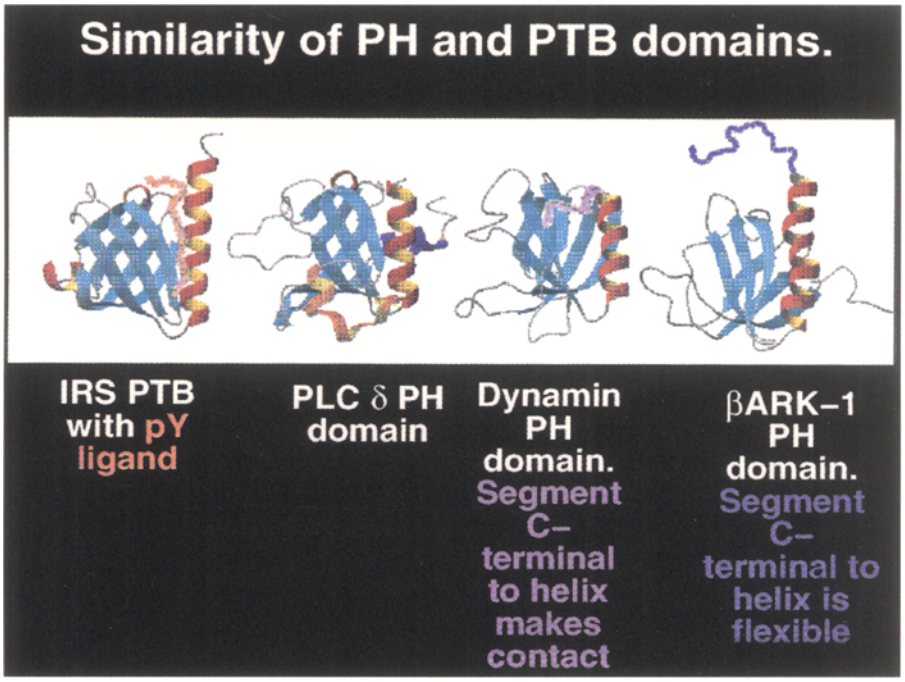

Fig. 5. Similarity of PH and PTB domains. Structural differences in several PH and PTP domains and their protein ligand binding site involve the C-terminal α-helix. Courtesy of Dr. David Cowburn, Rockefeller University.

for I(1,4,5)P3 on the β-spectrin PH domain was determined by X-ray scattering *(172)*, confirming a previous NMR structure of that PH domain and effects of PtdIns(1,4,5)P2 *(170)*. The binding requires the 4,5-bisphosphate on the inositol ring, while the inositol ring makes no contact with the protein and the 1-phosphate is fully solvent exposed. The positively charged binding site on the β-spectrin PH domain is located between the two loops connecting β strands 1–2 and 5–6, the same position as suggested for PtdIns(1,4,5)P2 on the pleckstrin PH domain *(170)*. The X-ray structure of Ins(1,4,5)P3 complexed with the phospholipase C-δ1 PH domain depicts a similar scenario with the inositol phosphate bound to a cationic patch between the loops of β strands 1–2 and 3–4 *(173)*. Different PH domains can have distinct specificities for inositol phospholipid. Dynamin GTPase activity is regulated by PtdIns(4,5)P2 and binds to liposomes containing that lipid while Bruton's tyrosine kinase (Btk) binds to PtdIns(3,4,5)P3 and its kinase activity is stimulated by that lipid *(174)*. Disease-causing mutations occur in analagous positions to two amino acid residues contacting the PtdIns(3,4,5)P3 in the PH domain of Btk which impair the inositol polyphosphate-binding capacity of that protein *(175)*. Btk and another PH domain-containing tyrosine kinase, Tsk, can be activated by Gβγ subunits in vitro and in cotransfected cells *(176)*.

The atomic resolution structure of a PH domain in a complex with a physiological ligand other than the inositol phosphates has not been reported. Because the relevance of βARK binding to Gβγ subunits is established, it is important to solve this complex, despite the

size of the protein ligand. Design of small molecule inhibitors of this interaction on the basis of the structure of the interface will allow pharmacological manipulation of βARK and perhaps other PH domain function. Selective small molecule and cell-penetrant inhibitors will be essential probes for studying protein–protein interactions involving PH domains.

Phosducin

Phosducin is a 28- to 33-kDa protein originally isolated from retina as a cytosolic complex with Gβ1γ1 *(177)* that prevented reassociation of Gαt with Gβ1γ1, depending on its phosphorylation state *(178)*. The retinal cGMP cascade is potently regulated by phosducin *(179)*. Phosducin and its homologs are found in many tissues and are particularly abundant in brain. The GTPase activity of Gαs from Gs, Gi, and Go are all inhibited by phosducin *(180)*, probably through its interaction with Gβγ. Association of the Gβγ subunit with phosducin is through the N-terminal 63 *(181)* or 105 *(182)* residues of phosducin. These results must be reconciled with the observation that a peptide, (residues 213–232), exhibits submicromolar inhibitory effects on Go GTPase activity *(183)*. Binding to Gβγ is itself unaffected by phosphorylation *(178)* and is nonselective with respect to Gβ or Gγ subtype *(184)*. Phosphorylation does affect the ability of the phosducin to interfere with the Gβγ interactions with other proteins such as Gα subunits and βARK. Phosducin also acts as a dominant negative regulator of βARK-mediated desensitization of the endogenous β2-adrenergic receptor response in transfected A431 cells that is potentiated by protein kinase A inhibitors *(185)*. It is present in the sensory cilia in the olfactory system and in reconstituted cilial systems blocks βARK2-mediated signal termination in a protein kinase A phosphorylation-sensitive fashion *(186)*. An S73A mutation blocked phosducin phosphorylation and signal termination, whereas a phosphorylation mimic, S73D, potentiated recruitment of βARK2 to the membrane and signal termination. Several phosducin-immunoreactive proteins have been purified from bovine liver; a 94-kDa species associates with Gβγ and disrupts GTP binding to Gα *(187)*. A phosducin-like (PhLP) sequence-related protein induced by chronic ethanol exposure was identified *(188)* that also inhibited Gβγ functions *(189)*. This protein lacked the N-terminal phosducin Gβγ-binding domain. A truncated 28-amino acid segment (V168-E195)-glutathione-*S*-transferase fusion protein derived from the C-terminus of PhLP bound to Gβγ-subunits and interfered with Gα interactions and the enhancement of rhodopsin phosphorylation by Gβγ subunits *(190)*. This binding site appears not to be related to PH domains or to the N-terminus of phosducin and may indicate another mode of interaction with Gβγ subunits.

The binding site for Gβγ on phosducin was assigned by recombinant methods to a polypeptide containing residues 1–63 of phosducin. Binding and functional properties were severely impaired by a W29V mutation *(181)*. The N-terminal 90 amino acids of phosducin are 46% homologous to residues 603–689 of βARK, encompassing the C-terminus of the PH domain and the C-terminal extended helix, although phosducin does not contain a PH domain *(182)*.

The X-ray structure of the phosducin/Gtβγ complex *(191)* showed that, unlike the interaction of Gtβγ with Gtα, which bound to the narrow face of the tapered propeller and along blades 4 and 5 on the side of the propeller, there was an extensive interface between phosducin and the β subunit along the wide end of the tapered propeller (schematically depicted in Fig. 6). Studies with truncated recombinant phosducin indicated that residues 1–63, which included only the first helical domain, were sufficient to confer stable association with Gtβγ *(181)*. The conformation of three loops in the β subunit were altered by their contacts with the third helix, (residues 87–105), and the C-terminal thioredoxin-like domain of phosducin. The N-terminal 105 residues of phosducin were arrayed in three helices contacting all of the

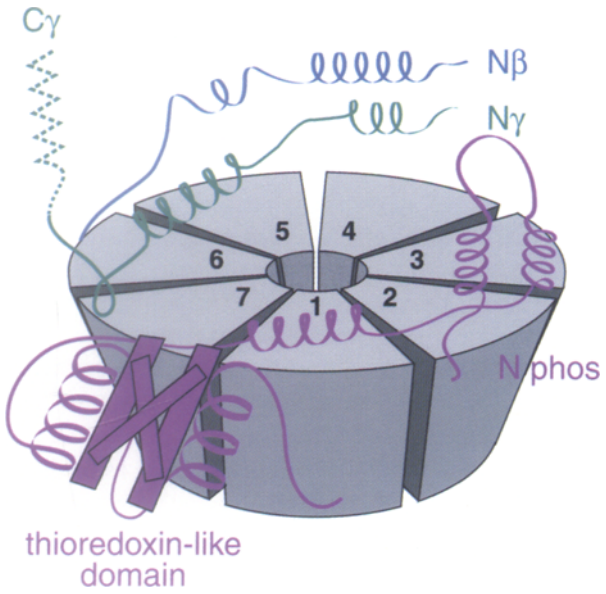

Fig. 6. Schematic of phosducin:–Gβγ complex from crystal structure. *Purple,* phosducin; *blue,* Gβ subunit; *green,* Gγ subunit; labeling as in legend to Figure 2, Nphos, N-terminus of phosducin.

interblade loops across the top of the doughnut-shaped seven-bladed propeller fold of Gβ and interacting with three residues in each blade. This occluded the binding of Gαt·GDP, which interacts through the switch II region in this area, accounting for the observed competition between Gα and phosducin binding. Helix 1 makes close contacts with Gβ and covers the hole in the doughnut. The sensitivity of W29 in helix 1 to mutation was attributed to its role in stabilizing a small hydrophobic core structure of the N-terminal domain and in contacting two residues of Gβ.

The C-terminal domain of phosducin is arranged in a thioredoxin-like folding pattern of five β strands capped by two helices. This motif contacts the side of the propeller at blades 7 and 1, on the opposite side of the structure from the Gβ:Gγ coiled coil. The placement of the C-terminal domain adjacent to the mutated Gγ prenylation site is unresolved but is well suited to alter the orientation of the complex with respect to the membrane as well as accessibility of the Gβγ subunit to receptors or effectors (Fig. 3).

RGS Proteins

The GTPase activity of heterotrimeric G proteins can be stimulated by a class of proteins termed regulators of G-protein signaling (RGS) that act by a different mechanism than other GTPase regulators *(154).* Instead of increasing the rate-limiting step of GDP dissociation as do some other factors, RGS proteins such as RGS4 interact preferentially with Gαi family (Gαo, Gαz, Gαq) alpha subunits bound to GTP in a distorted transition state *(192).* RG54 has been shown to speed the release of the inorganic phosphate from transduction and hence act as a single turnover catalyst. This mechanism has yet to be confirmed for other RGS proteins. No RGS proteins are known for the Gαs or Gα 12 families. RGS proteins have a high affinity for the Gα·GDP·AlF4 complex (a transition state analog), less for the Gα·GTP form, and low affinity for the Gα·GDP complex. A conserved of ca.130 amino acid domain is found in these proteins that is interupted by variable-length linker regions. The X-ray structure of RGS4 in complex with Gαil·Mg^{2+}·GDP·$AlF4^{-}$ revealed that the RGS core domain containing nine α helices of various lengths, is organized into two subdomains *(193).* A terminal domain containing five helices formed from both the N- and C-termini of the RGS protein contacts the Gα subunit only superficially, probably serving to orient a larger bundle domain composed of a classic right-handed, antiparallel four-helix bundle. This bundle subdomain interacts with all three switch regions of Gαi1, suggesting that it stabilizes the pentavalent transition state, although there remains the possibility that it could also help orient the attacking water nucleophile. In this position, RGS4 would block the interaction of the switch regions with downstream effectors normally coupled to Gαi1. The RGS/Gα system thus stands in contrast to the Ras family of small G proteins ($p21^{ras}$), which are weak GTPases activated by GTPase activating proteins (GAPs)

(194). Proteins such as rhoGAP or p120GAP fold into a topologically different type of four-helix bundle in which there is structural evidence for direct participation of GAP residues in constituting an active site for nucleotide hydrolysis (195).

Arrestin

Maximal GRK-dependent desensitization of rhodopsin and GPCRs like the well-studied β2-adrenergic receptor requires the participation of another protein, arrestin, which binds to the activated, GRK-phosphorylated receptor, allowing the system to return to its original quiescent state (196). Arrestin effects are most prominent for short-term homologous desensitization mediated through GRKs. βARK-phosphorylated β2-adrenergic receptor, but not receptor phosphorylated by protein kinase A, binds β-arrestin (197). Recent studies have highlighted the role of arrestin in promoting GRK-phosphorylated receptor sequestration and resensitization (90). Four families of arrestin have been defined by cloning—the S-antigens (rod arrestins), the β-arrestins (1 and 2), the invertebrate arrestins, and the cone arrestins—sharing some 52–84% overall homology (198). Point mutants of β-arrestin-1 V53D and β-arrestin-2 V54D suppress sequestration of β2-adrenergic receptors in HEK-293 cells, interfering with the binding of wild-type arrestin to phosphorylated receptor (199). Desensitization-induced internalization is mediated by a separate pathway involving secretory systems marked by dynamin (200). β-Arrestin and arrestin-3 function as adaptin subunits of the coated pit protein clathrin in the binding and internalization of β2-adrenergic receptors while visual arrestin does not (89). The discrepancy with visual arrestin is explained by the fact that rhodopsin is specially compartmentalized in the rod outer segment and is not desensitized by uptake into an internal vesicular system unlike most other GPCRs. Adaptins are responsible for determining the specificity of the interaction of membrane-bound proteins for coated pits. Among the properties the arrestins share with adaptins is the ability to bind inositol polyphosphates (201) that antagonize arrestin (202) and adaptin AP-2 (203) and AP-3 (204) function. A dominantly negative-acting fragment of the clathrin binding domain, (residues 319–418), attenuates β2-adrenergic receptor agonist-promoted internalization when expressed in COS-1 cells containing the receptor (205). A full-length β-arrestin V53D mutant binds to clathrin but only poorly to phosphorylated rhodopsin or to phosphorylated muscarinic m2-acetylcholine receptor when compared to the wild type β-arrestin.

Seven variable domains and four conserved domains have been identified in the arrestin family based on sequence homology (198). Figure 7 places these domains with respect to biochemically defined properties of the molecule. An extensive series of truncation and deletion mutants were in vitro translated and subjected to a series of assays to define the function of various regions (206), and references therein). Sequential, multisite binding of arrestin domains to the phosphorylated, activated rhodopsin molecule accounts for the selectivity of the interaction. A polybasic phosphate recognition region is located C-terminal to a tripartite activation recognition domain, both of which interact with the parts of rhodopsin that are conformationally altered by light activation. A hydrophobic secondary binding domain under control of a C-terminal regulatory region is exposed on arrestin upon binding of arrestin to activated, phosphorylated rhodopsin, producing a tight complex of the two molecules. An N-terminal regulatory domain interacts with the acidic C-terminus, stabilizing the N-terminal portion of the molecule, and may play a role in receptor selectivity (207). Although the major sequence differences between the different arrestin subtypes, arrestin I and II, β-arrestin, and β-arrestin-3, are in the C-terminal regulatory region, comparison of specificity for rhodopsin, β2-adrenergic receptors, and m2 muscarinic acetylcholine receptors in reconstituted systems indicated that the central phosphoryla-

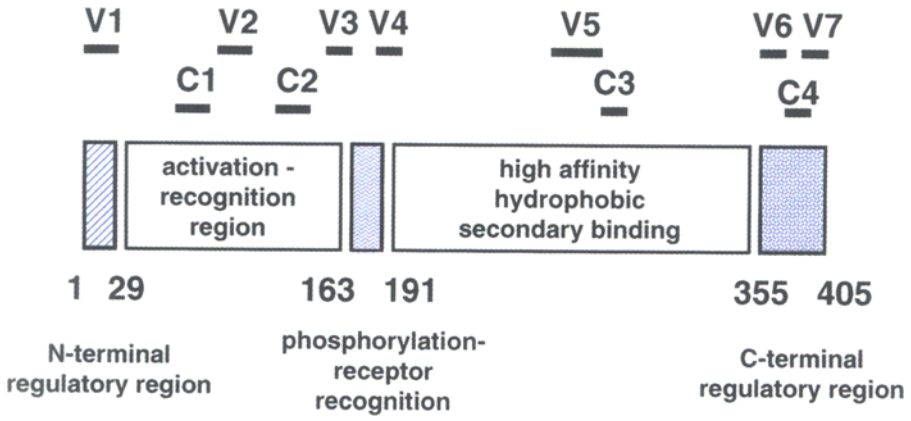

Fig. 7. Arrestin domain structure. Vn, variable regions; Cn, constant regions.

tion-receptor recognition region is important in determining receptor selectivity. Visual arrestin is the most discriminating, being highly selective for rhodopsin, whereas the other arrestins are only modestly selective *(208)*. This apparent lack of selectivity could be a function of the reconstitution, much as was observed for the Gα subunit subtypes. The third IC loop of rhodopsin, but not the other loops, competes for binding of arrestin to activated, phosphorylated rhodopsin, suggesting that arrestin could occlude G-protein access to activated receptor, thereby directly interrupting the signaling cascade.

The three-dimensional structure of arrestin remains to be determined, although diffraction quality crystals have been obtained *(209)*. Biophysical analysis suggests a content of β sheet of ≤ 40%, and little to no α-helix by CD spectroscopy down to 175 nm. Fourier transform infrared spectroscopy reaches a similar conclusion *(210)*. Heparin, which induces a conformational change similar to that upon binding to activated phosphorylated receptor detected by proteolysis *(211)* and SH-group modification does not affect the CD spectrum *(212)*. The interpretation is that the arrestin secondary structure is not affected by binding and may involve only side chain perturbations *(213)*.

Myristoyl-Switch Proteins

Membrane recruitment engendered by signal-dependent exposure of a co- or posttranslationally incorporated hydrophobic myristoyl chain is observed in a number of eukaryotic intracellular biochemical systems *(214)*. The signal can vary from calcium (recoverins, frequenin, GCAP, YCP) to phosphorylation (HIV-1 p17, MARCKS, src), and nucleotide binding (ARFs, transducin). Structural information is available for recoverin and for p17 *(215)*.

Recoverin and its more than 10 sequence homologs, such as neurocalcin *(216)*. and hippocalcin *(217)*, inhibit the phosphorylation of rhodopsin by rhodopsin kinase in a Ca^{2+}-sensitive fashion. Recoverin binds to rhodopsin kinase *(218)* and therefore presumably to other GRKs potentially influencing other GPCRs. X-ray diffraction of the Ca^{2+}-free and Ca^{2+}-replete states of unmyristoylated recoverin *(219)* show that Ca^{2+} dramatically influences the conformation of the N-terminal region of the protein, which contains four EF hand motifs. Ca^{2+} binds to EF-2 and EF-3, but not to EF-1 and EF-4 in recoverin. NMR studies with Ca^{2+}-free myristoylated protein show the fatty acyl chain in an extended conformation sequestered within an aromatic hydrophobic pocket formed from five flanking α-helices of

the protein *(220)*. Four helices contributed by three EF hands form a box perpendicular to the axis of the fatty acid, whereas a single amphipathic helix formed from residues 4–16 of recoverin lies like a cap over the length of the acyl chain with the G2 and N3 residues in a tight hairpin turn with the myristate attached to the G2 α-NH_2 group. Studies with recoverin bearing ^{13}C-labeled myristate show that the myristoyl residue is extruded into solvent in the presence of Ca^{2+}, where it could interact with a lipid bilayer or a hydrophobic region of another protein *(221)*.

Recoverin is the only protein for which detailed structural information is available for signal-mediated fatty acyl exposure. There is little evidence for modulation of fatty acyl exposure in the lipid-modified Gαs. No convenient nearby hydrophobic pocket is obvious in the structures of Gαt and Gαi to provide for a GTP-dependent exposure of myristate by Gα. Direct evidence is lacking as the lipid modifications were removed to facilitate crystallization. Extrapolation of this translocation mechanism to recoverin homologs in other systems involving vesicle fusion or phosphorylation requires further study.

Effectors Coupled to GPCRs

GPCRs regulate numerous effectors mediated through their interactions with Gα and G$\beta\gamma$ subunits. The effectors vary from ion channels and nucleotide cyclases to protein kinases and phospholipases. For a variety of reasons, ranging from their abundance to integral membrane association and their large size, these molecules have evaded structural determination. Functional resolution has been established for members of effector families through the study of cloned subtypes. Recombinant expression of domains of these proteins will allow the eventual solution of molecular structures. As was indicated in the Introduction, piecing together structures from smaller conserved domains will be useful for the moment in coming to grips with the mosaic of motifs that have evolved in GPCR signaling systems.

A Phospholipase C

An example of the utility of this approach is the phospholipase C isozyme δ1 whose structure, like the other eukaryotic phospholipase classes β and γ, is a mosaic of domains with specialized functions. The various functional domains of the phospholipases are keyed to the response elements of the different signaling systems coupled to them (described in Table 2). These domains include multiple Ca^{2+}-and non-Ca^{2+}-binding EF hand motifs, a catalytic domain with a conserved C2 domain, and an N-terminal PH domain. Additional functions are provided with the inclusion of two SH2 and one SH3 phosphotyrosine interaction modules, within a second split PH domain in the γ subtypes. An extended C-terminal region endows the β subtypes with interactions with Gα, but not G$\beta\gamma$. In response to GPCR stimulation these enzymes, which cleave the phosphoinositide headgroup from the phospholipid, are activated releasing IP3 and diacyl glycerol effecting Ca^{2+} and protein kinase C, respectively. Unlike the ion channels and adenylate cyclases, effector phospholipases are classified as peripheral membrane proteins in that they associate with either the membrane surface or with integral, or embedded proteins. Thus, soluble forms can be prepared and studied by classical physical techniques. An X-ray crystal structure is available for recombinant PLC-δ 1 with the N-terminal PH domain removed *(222)*. The X-ray structure for the PLC-δ 1 PH domain *(173)* was discussed in Modulators of GPCR Function, G-Protein Receptor Kinase with the other PH domains. Comparison with structures for individual domains *(223)* indicate that the individual domains fold separately and assemble as modules. These additions extend interactions of a common catalytic domain with a variety of protein and membrane components that localize and modulate that entity within the cell.

Table 2
Mammalian Phospholipase C Families

PLC class	No. of aa	PH domain (aa)	EF hands (aa) X/Y	Catalytic Region			C2 domain (aa)	C-terminus (aa)
				X (aa)	Y (aa)	Other		
δ(1–4)	756	120	(4) 140	407	46	—	130	—
β(1–4)	1216	124	(4) 159	334	67	—	124	420 aa
γ(1,2)	1290	119	(4) 160	744	483	split PH SH2(2), SH3	128	—

Adenylate Cyclase

The prototypical effector for GPCRs is adenylate (adenylyl) cyclase (AC), the enzyme responsible for cyclizing ATP to produce the second messenger, 3′,5′-cAMP and releasing pyrophosphate. This enzyme is found ubiquitously in bacteria, fungi, and multicellular organisms. In the former two groups of organisms, the ACs are primarily peripheral membrane proteins, although *Dictylostelium* also contains an AC form with a single TMH domain. Higher eukaryotic organisms have evolved a family of at least nine large integral membrane protein ACs *(224)*. These enzymes are regulated in a complex fashion by the diterpene forskolin, Gα, Gβγ, Ca^{2+}/calmodulin, protein kinase C (PKC), and other modulators *(225)*. They consist of a short N-terminal region, and two low homology six-TMH domains flanked by two ca.40-kDa cytoplasmic domains of 50–90% sequence homology depicted schematically in Fig. 8A.

The sequence similarity of portions of the eukaryotic cytosolic domains to prokaryotic ACs suggested that they formed the catalytic portion of the enzyme. Cloning and expression studies established that both 40-kDa domains were necessary for catalytic activity *(226)* and that the N-terminal portions of the cytoplasmic domains, C1a and C2a, were sufficient to produce forskolin and Gαs responses. A ca.60-kDa soluble AC consisting of the AC I-Cla (ICl) and AC II-C2a (IIC2) regions of the type I and type II ACs, respectively, connected by an added short polyglycine linker peptide, could also be inhibited by Gβγ subunits *(227)*. Regulated activity could also be obtained by expressing C1 and C2 regions separately and then mixing them in solution *(228)*. Catalytic domain reconstitution with nearly native enzyme-specific activity was achieved with VC1 and IIC2 domains expressed separately in *Escherichia coli* and then mixed forming a 1:1:1 complex with Gαs-GTPγS *(229)*.

X-Ray Structure of the AC Catalytic Core

The successful complementation of soluble truncated cytoplasmic domains retaining many of the regulatory features of the wild type membrane-bound enzyme expedited the crystallization and X-ray structure determination of AC II-C2 domains (residues 871–1090 of rat AC II) bearing the same regulatory features but much reduced catalytic activity *(230)*. Although not exactly reproducing the C1–C2 interactions of the native AC, the structure gives many indications that asymmetric interactions take place in the wild-type molecule. In the crystal containing forskolin, two C2-regions interact extensively, probably corresponding to the catalytic intramolecular dimer, which then interacts more weakly with two more monomers, producing a wreath-like arrangement. Whether such intermolecular tetramers form in the wild-type molecule is unknown. Each monomer comprises a three-layer α/β sandwich containing a central largely antiparallel β sheet and external α helices. The active site of the enzyme is

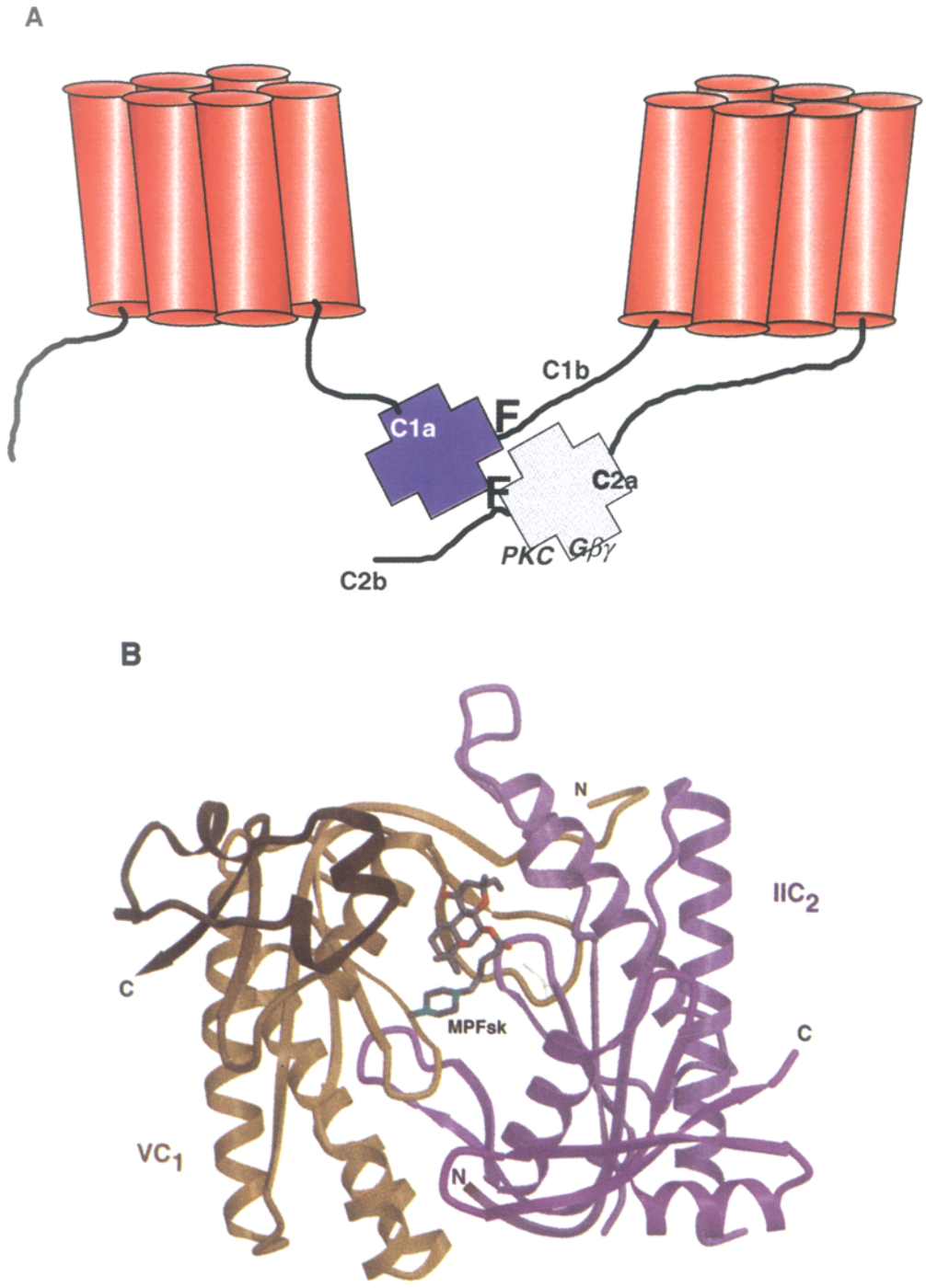

Fig. 8. Mammalian adenylate cyclase. **(A)** Schematic representation of native adenylate cyclase. NAC, N-terminus of adenylate cyclase; C1a, C1b, C2a, C2b, catalytic site domains; F, forskolin (activator); PKC, protein kinase C regulatory site; Gβγ, Gβγ-interacting region. **(B)** Ribbon Diagram of the VCI–IIC2 catalytic dimer *(113).* View along the pseudo-twofold axis toward the ventral surface of VC1 *(mauve)*–IIC2 *(khaki).* N and C are the last ordered residues in the crystal. MPFsk, 7-deacetyl-7-(*O*-*N*-methylpiperazino)-—butyryl forskolin. **(C)** Ribbon diagram of the VCI–IIC2: Gαs-GTPγS complex *(113).* Coloring of VC1–IIC2 as in (B). *Left,* Oriented with the wide interdomain clef (ventral) facing the bottom of the page. Dorsal surface presumably faces the membrane. The ras-like domain is charcoal, the switch II segment is *red,* and the helical domain *ash gray. Right,* The complex is rotated 70° around a horizontal axis from that in the *left* swinging the Gαs helical domain forward, permitting a view of the ventral surface of the complex. Reprinted with permission from ref. *13.* (Figure continues.)

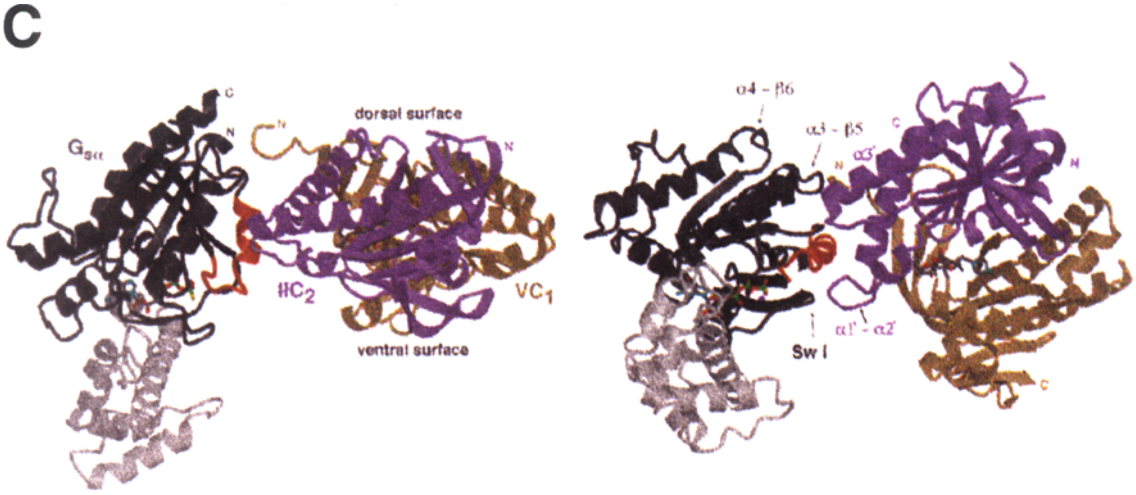

Fig. 8 (Continued)

formed jointly by the monomer interfaces in the dimer, with forskolin stabilizing dimerization through hydrophobic interactions. This ventral cavity in the enzyme is the site of the catalytically important N1025 and R1029 and a putative ATP binding pocket. The binding of ATP may stabilize a protein fold such that 8-azido-ATP can photolabel a peptide (residues 1057–1090) in the disordered C-terminus. The nearby regulatory region for PKC (residues 1034–1068), which does not contain a phosphorylation site, is also partially disordered in the crystal. The N-terminal part of the Gβγ subunit binding region (residues 956–982) *(130)* is also disordered. Stabilization of these conformationally labile regions by regulators is a potential activation mechanism for the enzyme.

The crystal structure of the heterodimer of VC1 and IIC2 in complex with Gαs elucidates the interaction of the G protein with the effector and the changes induced by the binding of adenylate cyclase regulators *(113)*. Figure 8B shows a closeup of the Cα backbone of the adenylate cyclase catalytic domains in which the assembled active site can be viewed. Other perspectives of the enzyme in this paper show the P-site nucleotide inhibitor 2′-deoxy-3′-AMP bound in the lower half of the active site. Two views of the VC1–IIC2–Gαs complex in Figure 8C indicate the contact between the adenylate cyclase and the G protein, which buries some 1800 Å^2 of surface that is most obvious in the right side view.

Supramolecular Organization of GPCR System Components

The localization of signaling components to the two-dimensional surface of the lipid bilayer provides both increased bimolecular reaction rates because of the reduced dimensionality (3-D→2-D) of the system as well as improved orientational selectivity at the membrane interface. Further organization of signaling components by interactions with the cellular cytoskeleton control trafficking and access of the various proteins and can either increase or decrease intrinsic reaction rates *(55)*. The structural information now available for individual molecules and subunit complexes provides the opportunity to model their interactions with the additional constraint of membrane localization. Still missing for the most part, however, is detailed information on how the folded protein structures are oriented in space, whether they are perpendicular or parallel to the membrane surface. This supramolecular organization represents the next level of understanding that will require either more powerful technologies to deter-

mine directly or inference from the application of available indirect approaches.

The "divide-and-conquer" approach to structural determination of large complex molecules such as AC and PLC has yielded considerable detail, if not the final structure, of the intact molecule. These lessons may assist in understanding the structural basis for interactions among other intrinsically folding cytoplasmic or extracellular domains of other membrane-embedded molecules such as ion channels. The appropriate biochemical, pharmacological, and cell biological experiments will need to be performed to establish the validity of any new models proposed from these structures.

Conclusion

Molecular structure determinations of receptor, coupler, effector, and modulatory components have confirmed the self-assembling capability of GPCR-linked transmembrane signaling systems. The fidelity of the reconstruction had been hinted at by the reconstitution experiments carried out with cellular and subcellular adenylate cyclase-coupled systems during the 1970s and early 1980s. Molecular biology has identified the basis for subtype specificities that may govern the exquisite selectivity among coupling pathways that seem to exist in intact cells. The technology is now poised to put these observations onto firm physical grounds. Such selective interactions may delineate potential sites for pharmacological intervention to selectively modulate processes in cells bearing common receptors or effectors.

Still, the supramolecular structure of GPCR-coupled signaling processes remains largely undetermined. Questions as basic as the arrangement of all the components with respect to each other, as well as to the bilayer surface, and the lifetimes of the molecular complexes remain unanswered. The autonomy of the folding of the separate domains of effector molecules such as phospholipase C and adenylate cyclase facilitated their structural elucidation, but the overall structure of the full-length molecules remains a puzzle. The adenylate cyclase catalytic domain structure revealed possible mechanisms of enzyme activation by small molecule activators or inhibitors but remained disordered in the region of the Gβγ binding site. The role of lipid moieties in stabilizing the structure of the lipid-modified proteins Gα and Gγ, or in orienting the complexes on the membrane is also unclear because the modified proteins did not crystallize and these functional groups had to be removed.

The first steps have been taken in understanding GPCR-linked transmembrane signaling at the structural level. Further progress will require the application of new approaches or technologies to deal with macromolecular structures at atomic resolution, or perhaps some novel experimental systems.

The crystal structure for visual arrestin has now been determined. The N-terminal domain is a β-sheet stack backed by a short alpha helix that can be docked successfully with a theoretical model of activated, phosphorylated metarhodopsin II. The C-terminal half of the molecule is another β-sheet bundle connected through a flexible linker region to the receptor-binding portion of arrestin *(231)*. The crystal structure of the 55 kDa N-terminal globular fragment and linker from the 190-kDa clathrin heavy chain that makes up the end of the triskelion "leg" reveals a seven-bladed propeller structure similar to the Gβ propeller. This feature helps to provide an explanation for the interaction of this structure with multiple binding partners, such as the AP-1 and AP-2 sorting adaptins and with the nonvisual arrestins *(232)*.

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