

BEYOND BINDING: MOLECULAR AND CELL BIOLOGICAL APPROACHES TO STUDYING G-PROTEIN-COUPLED RECEPTORS

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The origins of the modern concept of receptors can be traced to the beginnings of the 20th century (1). Almost two decades passed until the first neurotransmitter, acetylcholine, was identified from classic physiologic studies on the vagus nerve performed by Otto Loewi in 1921. Since these seminal discoveries the pace of advance has increased enormously. A revolution in the field began in the 1950s, with the discovery that neurotransmitter receptors are targets of clinically relevant psychotropic drugs and the development of radioligand binding techniques (2,3). Radioligand binding methodologies remain a mainstay of modern neuropsychopharmacology, and have facilitated the identification of receptor subtypes as well as the discovery of novel receptors that mediate the actions of important drugs.

The application of recombinant DNA methodologies sparked a second revolution in neuropsychopharmacology. These methods facilitated the cloning of complementary DNAs (cDNAs) encoding distinct receptors, the identification of large families of homologous receptors, and unprecedented insight into subtype diversity within individual receptor families (4,5).

Important families of receptors include steroid hormone receptors, receptor tyrosine kinases, ligand-gated ion channels, and G-protein-coupled receptors (GPCRs). GPCRs comprise the largest class of signal-transducing receptors, with well over 1,000 members identified in humans. In some organisms, genes encoding GPCRs comprise 1% of the genome (6). GPCRs mediate the actions of the majority of neurotransmitters and neuromodulators, as well as other important biological ligands. These receptors are also criti-

cally important drug targets. Indeed, the majority of psychopharmaceuticals presently in use either bind directly to specific GPCRs (e.g., antipsychotics) or indirectly influence GPCR function by modulating the availability of endogenous ligands (e.g., selective serotonin reuptake inhibitors, SSRIs). Therefore elucidating mechanisms of GPCR function and regulation is of central importance to understanding the actions of clinically relevant drugs.

During the past several years there has been a great deal of progress in elucidating specific mechanisms of GPCR function and regulation. Much of this progress can be attributed to the application of newer molecular and cell biological techniques, which have complemented previously developed pharmacologic approaches for probing receptor function. This chapter discusses some of these molecular and cell biological approaches for isolating and studying cloned receptors, focusing specifically on GPCRs expressed in a variety of systems. Although we restrict our scope in this chapter to representative approaches applied to GPCRs, these methods have broad potential application and have been used to study other important receptor families.

ISOLATION AND IDENTIFICATION OF RECEPTORS

The identification of GPCRs by biochemical purification is a challenging task because of the generally low abundance of these proteins in cells and tissues, and because GPCRs are highly hydrophobic molecules that are easily denatured when solubilized in detergent solutions. Molecular cloning techniques have greatly facilitated the identification of GPCRs. Molecular cloning takes advantage of the ability to generate and screen “libraries” containing cDNAs corresponding to the messenger RNAs (mRNAs) that encode cellular proteins.

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A detailed discussion of molecular cloning techniques is beyond the scope of the present review and has been described elsewhere (7). In general, a cDNA library is generated from a specific tissue and animal source (such as rat brain) by purifying mRNA from the tissue, using the enzyme reverse transcriptase to generate a strand of DNA complementary to each mRNA present in this mixture, and then using a DNA polymerase to generate double-stranded DNA from this sequence that is suitable for insertion into an appropriate plasmid or phage vector that facilitates faithful replication of the sequences and allows selection of individual clones corresponding to a single cDNA. The main challenge in receptor cloning is to isolate or “screen” for the appropriate receptor-encoding cDNA from a library. Several different approaches to library screening have been used successfully for cloning cDNAs encoding GPCRs.

Receptor Cloning from Protein Sequence

Early isolation and cloning of receptors relied on purifying sufficient quantities of receptor and then microsequencing peptide fragments. The hamster β_2 -adrenergic receptor was cloned using partial sequence information derived from protein purified from hamster lung (8). The sequence of amino acids present in a GPCR fragment allows one to predict the sequence of the corresponding region of the mRNA sequence by “back-translation” from the genetic code. This nucleic acid sequence can be searched for in the cDNA library by virtue of the ability of complementary strands of DNA to anneal together with extremely high specificity. Because of the degeneracy of the genetic code, many amino acids can be encoded by more than one nucleic acid codon. Therefore, nucleic acid “probes” used to screen cDNA libraries often contain mixtures of sequences representing multiple potential “spellings” of the known peptide fragment. cDNA clones encoding the sequence of interest can be isolated by hybridization of a labeled probe or by polymerase chain reaction (PCR)-mediated amplification from a cDNA mixture.

Receptor Cloning by Sequence Homology

Sequence homology within the GPCR superfamily of receptors enables the use of DNA sequence from a previously cloned receptor to probe libraries for other related receptors. Cloning of the dopamine D2 receptor is an example of where sequence homology was used to clone other members of the GPCR superfamily of receptors. Olivier Civelli's group (9) used the hamster β_2 -adrenergic receptor gene as a hybridization probe to isolate cDNA encoding the rat D2 dopamine receptor. The authors used two major criteria to determine that the cDNA isolated encoded a functionally relevant dopamine receptor. First, they demonstrated that mRNA corresponding to their cDNA sequence was expressed in tissues that had been previously shown by radioli-

gand binding to express functional D2 receptors. They accomplished this by Northern blotting, a procedure by which RNAs isolated from cells or tissues is resolved by gel electrophoresis and the specific RNAs homologous to a particular sequence is detected by hybridization of a specifically labeled probe. Second, the authors demonstrated that the cDNA isolated from their library encoded a functional D2-class dopamine receptor. This was accomplished by applying conventional radioligand binding and receptor signaling assays to detect functional D2 receptor activity in fibroblast cells that do not normally express dopamine receptors and that were transfected with the cDNA isolated from library screening.

Receptor Cloning by Functional Expression

GPCRs can also be cloned based on their functional properties. The cloning of the serotonin (5-hydroxytryptamine) 5-HT_{1C} receptor used this approach (10). Taking advantage of the high expression level of the 5-HT_{1C} receptor in the choroid plexus, the authors isolated mRNA from this source and injected this preparation into *Xenopus* oocytes, which allows both injected mRNA and cDNA to be translated and expressed. The activation of the 5-HT_{1C} receptor by its ligand leads to an increase in intracellular calcium through the inositol phosphate signaling pathway. This rise in intracellular calcium leads to the opening of Ca²⁺-dependent chloride channels that was detected by means of highly sensitive electrophysiologic techniques. Similar results could be obtained by injecting cDNAs produced from the mRNAs. Pools containing progressively smaller numbers of cDNAs were then analyzed until a single cDNA encoding a functional serotonin receptor was isolated.

The cloning of the delta opioid receptor (11,12) was also achieved by a functional assay. A cDNA library prepared from cells that endogenously express the delta opioid receptor was expressed in cultured fibroblast-like cells that do not normally express opioid receptors. Cells expressing opioid receptors were identified by binding of a radiolabeled opioid peptide. cDNA isolated from the selected cell was isolated and retransfected, to allow multiple rounds of purification until a single cDNA encoding the delta opioid receptor was isolated. The isolated cDNA was confirmed to encode a delta opioid receptor by analysis of radioligand binding properties and ligand-induced signal transduction in transfected cells.

Identification of Receptor Subtypes (Fig. 22.1)

A general principle in molecular pharmacology is that multiple receptors exist that can recognize the same ligand. This “one ligand, multiple receptors” principle has led to great interest in the identification of multiple receptor subtypes.

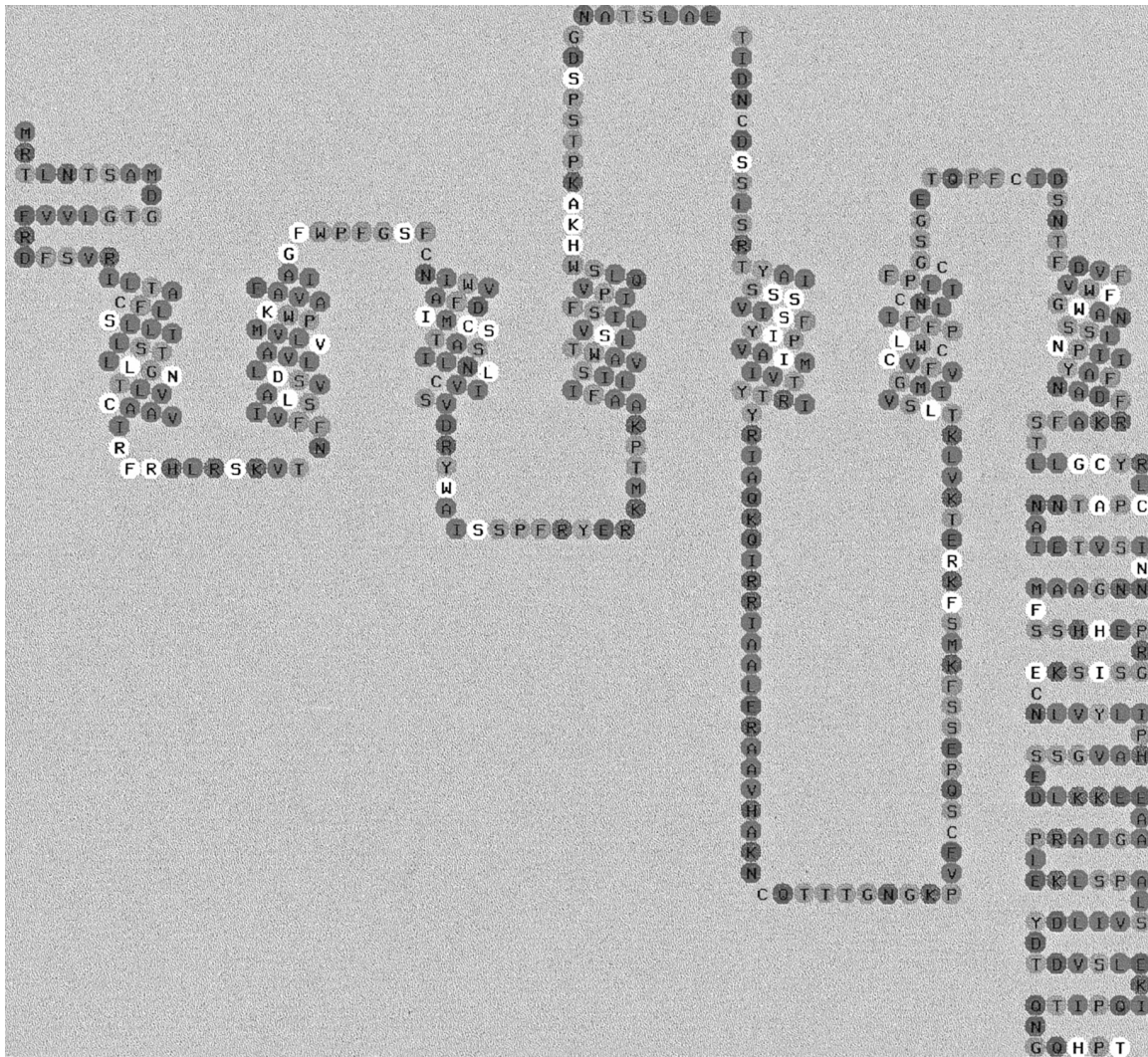


FIGURE 22.1. Dopamine D1 receptor two-dimensional (2D) snake diagram downloaded from the G-protein–coupled receptor database (GCRDb), <http://gcrdb.uthscsa.edu/index.html>, which is a very useful site for researchers working on GPCRs (13,14). This diagram shows the seven-transmembrane structure of GPCRs and the long carboxy terminus tail characteristic of D1-like dopamine receptors. In the GCRDb, amino acid mutations found in the receptor are listed in *white* in the 2D snake diagram and hyperlinked to the GRAP mutant database. [<http://tinygrap.uit.no/>]. (Used by permission of the GCRDb.) See color version of figure.

For example, radioligand binding techniques and assays of receptor-mediated signal transduction originally defined two classes of dopamine receptor: D1 receptors that stimulate adenylyl cyclase, and D2 receptors that inhibit this enzyme. Molecular biological techniques confirmed that these receptors represent distinct gene products and led to the discovery of additional, structurally homologous subtypes of receptor protein. These subtypes of dopamine receptors would have been impossible to identify definitively using classic pharmacologic approaches, as their pharmacologic properties are quite similar and some subtypes are expressed at very low levels in native tissues. For example, the D4 dopamine receptor is a member of the “D2-class” of dopa-

mine receptors that was cloned by sequence homology to the cloned D2 receptor (15). D4 receptors are of great interest because they bind the atypical antipsychotic clozapine with approximately 10-fold higher affinity than cloned D2 receptors (16).

Molecular biological methods also led to the discovery of additional diversity among closely related GPCRs encoded by genetic variants or by modification of the receptor gene after transcription. For example, multiple variants of D4 receptor, which differ only in the structure of the third cytoplasmic loop of the receptor protein, are encoded by closely related genes that are inherited in a mendelian manner ((17)). Two variants of the D2 dopamine receptor are

generated from the same gene by alternative splicing, which occurs during posttranscriptional processing of the RNA. Another interesting example of such receptor diversity is the 5-HT_{2C} receptor, which exists in variant forms determined by a posttranscriptional process called RNA editing (18). In many cases the functional significance of such variation among GPCRs is not known. However in some cases, such as RNA editing of the 5-HT_{2C} receptor, individual receptor variants have significantly different functional properties.

As a further extension of the “one ligand, multiple receptors” concept, molecular biological methods led to the discovery that a specific neurotransmitter can often bind to more than one class of receptor protein. The first example of this can be found in the acetylcholine receptors (AChRs). The idea that distinct muscarinic and nicotinic AChRs exist was first indicated by pharmacologic studies. Molecular cloning of the corresponding receptor cDNAs confirmed this idea and revealed very precisely the differences between these classes of AChR: muscarinic-type AChRs are GPCRs, whereas nicotinic-type AChRs are members of the structurally distinct family of ligand-gated ion channels (LGICs). There are now several examples of this type of diversity, including the existence of distinct GPCRs and LGICs for serotonin, glutamate, and γ -aminobutyric acid (GABA).

Discovery of Orphan Receptors

Orphanin FQ and Its Receptor

In addition to identification of receptor subtypes, the cloning approaches discussed above have also enabled the isolation of GPCRs before the identification of any known ligands. Putative receptors for which a ligand is not known, called orphan receptors, hinge on sequence similarity with other GPCRs. The cloning of an opioid-like GPCR, known as ORL1 or the orphanin receptor, was accomplished by several groups looking for additional members of the opioid receptor family by cloning structurally homologous cDNAs (19,20). Expression of the ORL1 cDNA revealed that this putative receptor did not bind any of the typical mu, delta, or kappa ligands with high affinity, despite having particularly high sequence homology to the kappa receptor. In addition, there are large differences in anatomic distribution of ORL1 mRNA compared to the known distribution of opioid receptors. Later, elegant studies led to the isolation of an endogenously expressed peptide ligand for this receptor, nociceptin or orphanin FQ, allowing the ORL1 gene product to be clearly established as a *bona fide* GPCR (21).

Orexins and Their Receptors

Many other cDNAs encoding candidate orphan receptors have been identified by DNA sequence analysis. Current estimates suggest that there are ~100–200 such orphan

GPCRs encoded in the human genome (not including predicted olfactory receptors). The isolation of so many candidate orphan receptors has led some groups to attempt systematic approaches to identifying their endogenous ligands. Identification of orexin and the orexin/hypocretin receptor was accomplished by using a cell-based detection system using cells transfected with a large number of candidate orphan receptor cDNAs (22). A cDNA (HFGAN72) was identified that was capable of causing an elevation in cytoplasmic free calcium in response to a crude peptide-containing extract prepared from brain. A specific peptide ligand was isolated from this mixture according to its ability to activate this orphan GPCR. This peptide is expressed highly in the lateral hypothalamus and influenced feeding behavior when introduced into rat brain, and is called orexin (from the Greek *orexis* meaning appetite). The receptor encoded by HFGAN72 was named the orexin receptor. Recently, a canine narcolepsy gene was identified by positional cloning as belonging to a subtype of orexin receptor (23). Thus the identification of orphan GPCRs can lead to powerful new insights relevant to diverse areas of neuropsychopharmacology.

EXPRESSION AND PURIFICATION OF CLONED GPCRS

Expression

The ability to express cloned cDNAs in various cell types has provided powerful tools for studying the functional properties of defined GPCRs. In many cases, receptors expressed in heterologous cell systems have remarkably similar functional properties to those in their native tissue of origin, although this is not always the case. For example, D2 dopamine receptors differ in their properties in pituitary GH4C1 cells and Ltk-fibroblasts. In the pituitary cells, D2 receptors fail to elicit phosphoinositide hydrolysis and induce a decrease of intracellular calcium. In contrast, in the fibroblast cells, the D2 receptor induced a rapid stimulation of inositol 1,4,5-trisphosphate and an increase of intracellular Ca²⁺ (24). Therefore it is important to compare results obtained from studies of cloned receptors in heterologous systems to the properties of receptors in native tissues.

In addition to facilitating functional studies, heterologous expression can also be used to produce large amounts of receptor protein, which is necessary for certain biophysical and structural studies. Mammalian cells are typically used for functional studies of expressed GPCRs; however, it is sometimes preferable to use nonmammalian cells (such as insect cells or yeast cells) for large-scale expression of GPCRs because these cells can be grown economically in very large amount. Prokaryotic expression systems (such as *Escherichia coli*) have potential advantages for large-scale production but have not been used widely in GPCR research because, in general, it has been difficult to obtain functional activity of GPCRs using these systems.

Purification

The ability to express cloned receptors makes it possible to modify the receptor protein to facilitate purification and detection. There are many strategies that have developed from this capability. For example, one useful approach is to insert an antigenic epitope into the GPCR, which can facilitate detection and purification by standard immunochemical procedures using well-characterized antibodies (see ref. 25 for review). This approach obviates the sometimes laborious path of generating antibodies that recognize the native receptor. Epitope tags are typically short sequences (~10 residues) that bind tightly to a highly specific antibody. In many cases, epitopes chosen for this purpose are either synthetic (not derived from any known biological sequence) or derived from nonmammalian sources, in an attempt to reduce the probability of cross-reaction with endogenous antigens. Popular epitopes include a region from the hemagglutinin molecule of influenza virus (“HA” epitope) or the “Flag” epitope, a synthetic sequence recognized by a series of well-characterized antibodies. The use of such epitopes allows the purification of tagged proteins using commercially available reagents. Tagged receptors can be isolated from extracts prepared from cultured cells by immunoaffinity chromatography. A column can be made by immobilizing an antibody onto a solid support that will bind to tagged receptors as the extracts are passed over the column. Elution of the specifically bound receptor can be accomplished by changing either the salt concentration or the pH to disrupt the antigen-antibody interaction. An example of this approach is found in the high-level production of the human β -adrenergic receptor (26).

MECHANISMS OF LIGAND BINDING AND ACTIVATION

Use of Site-Directed Mutagenesis

The availability of cDNAs encoding specific GPCRs and the development of various cellular expression systems provide powerful tools for examining the specificity of ligand binding to receptors. In particular, site-directed mutagenesis can be used to alter residues in the GPCR structure, and then the functional consequences of these modifications on ligand binding can be examined. Many techniques have been developed for introducing specific mutations into the receptor cDNA, as discussed in detail elsewhere (27). Selected examples of the use of these techniques for understanding the structural basis of ligand binding and receptor activation are outlined below.

Point Mutagenesis

Point mutations refer to modifications of the cDNA that result in the substitution of a single amino acid residue in

the expressed receptor protein with another amino acid. Point mutagenesis can be used to dramatically change the physicochemical properties of a specific residue (e.g., substitution of a basic residue with an acidic one); or cause a subtle change in residue structure (such as substitution of a serine residue with cysteine, which results in a change of a single atom in the protein structure). Point mutations are extremely useful because they often do not cause global perturbations of receptor structure and therefore allow highly specific analysis of the function of defined receptor residues. For example, point mutations have been used to define important determinants of receptor-ligand interaction with considerable precision (28).

Point mutations can identify essential features in the receptor structure. Mutation of a single aspartic acid residue present in the predicted third transmembrane domain of catecholamine receptors abrogates high-affinity binding of catecholamines to the β_2 -adrenergic receptor. Since an aspartic acid residue is present at the corresponding position in other catecholamine receptors as well, this residue is said to be “conserved” in the structure of multiple receptors. Point mutagenesis applied to these receptors indicates that this aspartic acid is required for ligand binding to a number of receptors, apparently by serving a conserved function as a counter-ion for the positively charged amine moiety of catecholamine ligands (28,29). Like the aspartic acid residue discussed above, other amino acids that serve a specific function in the receptor generally are found to be conserved between receptors with similar properties.

Point mutations can identify nonconserved residues that determine the specificity with which drugs bind to structurally homologous receptors. Nonconserved residues present in the receptor sequence may play a pharmacologically important role in determining unique properties of individual receptor subtypes. Nonconserved residues can be essential for determining the specificity with which a drug binds to closely related subtypes of G-protein–coupled receptors. Point mutation analysis combined with appropriate pharmacologic assays can be used to identify such divergent receptor residues that are critical for drug binding, thus providing insight into the structural basis of ligand binding specificity that is useful for drug design.

Point mutations can provide insight into species- and population-specific differences in receptor pharmacology. For the very reason that nonconserved residues are often not essential for basic receptor function, these residues are often not conserved across species. Thus the pharmacology of many subtype-specific drugs can be highly dependent on the species of animal studied. For example, three homologous genes encoding distinct subtypes of α_2 -adrenergic receptor are expressed in various mammalian species. However, the pharmacology with which subtype-selective drugs bind to receptor subtypes encoded by mouse or rat receptor genes can differ substantially from the pharmacology characteristic of the corresponding human receptor (30). This

has led to considerable confusion in the correspondence between pharmacologic and molecular biological definitions of specific receptor subtypes across species, and has important implications for the use of animal models for the development of subtype-specific drugs for humans. Furthermore, nonconserved residues involved in subtype-specific drug binding can also differ within the human population, as a result of random mutation and genetic drift. This concept has not yet been extensively explored but may be an important direction for the use of pharmacogenomics in clinical medicine.

Deletion Mutagenesis

Another mutational approach useful for probing receptor structure and function is removal of certain residues from the receptor structure entirely. Deletions of multiple residues in certain parts of the receptor protein (e.g., transmembrane helices) can be difficult to interpret because they often lead to massive disruption of receptor structure. However, deletion of limited regions in extracellular or cytoplasmic domains are often well tolerated and have been quite informative. For example, deletion of residues located in the amino-terminal extracellular domain of polypeptide receptors [such as the follicle-stimulating hormone (FSH) receptor] and the calcium receptor implicate this domain in ligand interaction. Deletion of residues located in the third cytoplasmic loop of various receptors, such as the muscarinic acetylcholine receptors, implicated this domain in functional coupling to heterotrimeric G proteins (27).

Substitution or Chimeric Mutagenesis

A very powerful approach to site-directed mutagenesis is to substitute entire series of residues from one receptor with the corresponding residues of another. This approach is based on the idea that receptors are composed of modular structural domains, and takes advantage of the fact that receptor domains that mediate similar functions often have conserved amino acid sequence. Chimeric substitutions are often less disruptive than deletions to the overall structure of the receptor protein. For example, chimeric mutagenesis has been useful for defining transmembrane residues that mediate subtype-specific and species-specific differences in ligand binding to adrenergic receptors. Receptor chimeras between α_2 - and β_2 -adrenergic receptors defined multiple cytoplasmic domains that contribute to the specificity of receptor interaction with their cognate heterotrimeric G proteins (4).

Use of Random Mutagenesis

In contrast to site-directed mutagenesis, random mutagenesis is an unbiased approach that can examine a much broader range of modifications of the receptor protein. Random

mutagenesis, therefore, has the potential to reveal unanticipated features of receptor structure and function. Random mutagenesis typically requires functional assay of a much larger number of mutant receptors than analyzed using site-directed mutation. The relatively low throughput inherent to traditional methods of receptor characterization have limited the practical utility of random mutagenesis of mammalian GPCRs. This limitation has become less significant with the recent development of higher throughput functional assays and the successful expression of mammalian GPCRs in more genetically tractable organisms. For example, the budding yeast *Saccharomyces cerevisiae* has been used recently for studying the functional properties of a large number of mutant chemokine receptors in which selected regions of the receptor protein were mutagenized in a non-biased manner. Analysis of these data identified residues in the receptor protein essential for ligand binding and activation. In addition, this nonbiased screening approach yielded unanticipated information, including the identification of mutations that constitutively activate receptors and the identification of functional mutant receptors predicted to contain fewer than seven transmembrane domains (31).

Use of Biophysical Approaches

Biophysical techniques are essential for detailed examination of protein structure and conformational change. One reason these methodologies have had limited application in the study of GPCRs is that they typically require milligram quantities of receptor, a quantity difficult to acquire from native tissue sources. For many years rhodopsin, purified from retina, was the only GPCR that could be generated in sufficient quantity for biophysical study. Indeed, much of what we know about GPCR structure and conformational change has been elucidated from elegant biophysical studies of rhodopsin. Recently, the development of improved expression and purification strategies have made it possible to obtain other GPCRs in sufficient quantity and purity for biophysical study. Thus it is likely that biophysical approaches will play an increasingly important role in future studies of GPCR structure and activation.

Structural Studies of Rhodopsin

High-resolution structural information can be provided by x-ray diffraction methodologies applied to ordered three-dimensional crystals of pure protein. Rhodopsin, a GPCR mediating phototransduction in the retina, has been a favorite for such studies because it can be purified in sufficient amount and purity to facilitate crystallization. Previous studies using electron diffraction of two-dimensional crystals of rhodopsin obtained structural information to a resolution of approximately 7.5 Å (angstroms; 1 angstrom = 10^{-10} m), revealing the relative orientation of the transmembrane helices in the lipid bilayer (32). Recently x-ray diffraction has

been used to solve the structure of three dimensional crystals of rhodopsin to a resolution of 2.8 Å. This accomplishment is truly a major milestone in the field, revealing for the first time the atomic structure of any GPCR and providing detailed information about specific interactions between this GPCR and retinal, its physiological ligand (33).

Molecular Modeling of GPCR Structure

As the hydrophobic domains predicted to form transmembrane helices are extensively conserved among all GPCRs, it is believed that the general features of rhodopsin's transmembrane structure are relevant to other GPCRs. This has motivated the use of rhodopsin's structure as a "template" on which to predict the structure of other GPCRs, via computational approaches that identify homologous residues and infer thermodynamically stable conformations of extracellular and cytoplasmic loops. It remains to be determined the degree to which specific features of diverse GPCRs are actually conserved at the level of atomic resolution. Indeed, based on well established differences in the pharmacology of individual GPCRs, one might expect there to be significant limitations of such homology-based predictive methods, at least with respect to structural features involved in drug binding. Nevertheless, the available experimental data leave little doubt that this approach is an important starting point for mechanistic studies and for rational drug development (34).

Biophysical Studies of Conformational Dynamics Involved in GPCR Activation

While crystallographic methods have the potential to provide detailed information regarding the relative positions of all residues of the receptor protein, these methods are inherently limited to reporting on a static structure. Thus additional methods are required to examine dynamic conformational transitions that mediate ligand-dependent signal transduction via GPCRs. Several biophysical approaches have been utilized for this purpose. Classic studies of rhodopsin measured the optical absorbance properties of this photoprotein that are highly sensitive to changes in protein conformation. Sophisticated studies using optical spectroscopy indicate that rhodopsin cycles rapidly through a series of distinct conformational states following photon-induced activation. Many other types of biophysical techniques have been applied to examine specific features of light-induced conformational changes of rhodopsin, as well as to examine ligand-induced conformational changes of other GPCRs (35). Specific residues in the receptor protein can be labeled with a chemical probe, typically using a combination of site-directed mutagenesis and organic chemistry techniques. Spectroscopic methods can then be used to detect conformational changes involving the labeled residue, by measuring changes in the local environment or mobility of the

chemical probe. Approaches of this type have been applied to several GPCRs, and have begun to yield interesting new information about the dynamic effects of clinically relevant drugs on GPCR structure (29).

Potential for Rational Drug Design

The availability of increasingly detailed mutational and biophysical data and the development of sophisticated molecular models suggest that it may be possible in the future to design new classes of therapeutically useful drugs based on this information. A precedent for such an approach is the structure-based design of the angiotensin-converting enzyme inhibitor captopril, the first drug on the market that was designed based on its interactions with its target (36). Inferences about the structure of GPCR-ligand interaction are currently used in a limited manner to guide the modification of existing drugs. However, an important goal is to design completely new drugs *de novo* based on the structural basis of GPCR activation. A clue that this may be possible comes from recent studies of mutant GPCRs, in which histidine residues have been introduced at defined positions in the receptor structure that can be coordinated by certain metal ions. Addition of the metal ion to the receptor, by coordinating histidine residues introduced within specific transmembrane helices, influences the receptor conformation to either activate or inactivate the receptor (37). Thus the metal ion can serve either as an "engineered" agonist or antagonist for certain mutant receptors. While it is unlikely that this strategy will directly yield clinically useful drugs, these exciting studies serve as a proof of the principle motivating further studies of GPCR structure and conformational change.

REGULATION OF RECEPTOR SIGNALING

Methods to Examine Regulation of Receptors by Posttranslational Modification: GPCR Phosphorylation

Many different types of posttranslational modification have been implicated in the regulation of GPCR function, localization or stability. A detailed discussion of this large area of research is beyond the scope of this chapter. Instead, we illustrate the use of specific methods by discussing some aspects of protein phosphorylation, the most extensively characterized type of posttranslational modification that regulates GPCRs.

Work by Edwin G. Krebs and his collaborators in the 1950s demonstrated that enzyme-catalyzed protein phosphorylation and dephosphorylation reactions were involved in the regulation of glycogen phosphorylase and suggested the notion of the phosphate group as a "covalently bound allosteric ligand" (38). Since these seminal studies, phosphorylation has been shown to play a critical role in the

regulation of a wide variety of cellular proteins, including many GPCRs. Phosphorylation of mammalian proteins typically occurs on serine, threonine, or tyrosine residues. Serine/threonine phosphorylation is widely recognized to regulate GPCRs. Tyrosine phosphorylation, a more recently discovered modification that is well established to mediate signaling via non-GPCR growth factor receptors (39), may also play a role in regulating certain GPCRs (40).

A family of enzymes called G-protein-coupled receptor kinases (GRKs) are well known to attenuate GPCR signal transduction and promote the endocytosis of certain GPCRs by clathrin-coated pits. Other kinases, such as the 3',5'-cyclic adenosine monophosphate (cAMP)-dependent protein kinase (PKA) and protein kinase C can also regulate GPCRs by phosphorylating distinct cytoplasmic serine/threonine residues (41–44). Certain kinases (such as PKA) typically phosphorylate residues located within a well-defined “consensus sequence,” making it possible to predict potential sites of regulatory phosphorylation simply by examination of the primary structure (polypeptide sequence) of the cytoplasmic domains of the receptor. Residues phosphorylated by other kinases, such as GRKs, are more difficult to predict because they do not conform to a rigidly defined consensus sequence. However, even in the case of enzymes with relatively well-understood substrate specificity *in vitro*, there are major limitations to the use of sequence analysis for predicting phosphorylation sites *in vivo*. Residues conforming to a specific consensus sequence are not always phosphorylated under physiologic conditions, and, conversely, in some cases residues that do not conform to a well-defined consensus sequence can be phosphorylated in the intact cell. Thus it is important to determine the phosphorylation of GPCRs when expressed in the appropriate mammalian cells.

Analysis and Identification of Phosphorylated Proteins *In Vivo*

There are many ways of detecting phosphorylated proteins. A starting point for many of these methods is resolution of phosphorylated proteins by electrophoresis in sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE). In SDS-PAGE, proteins dissolved in SDS are loaded onto one end of a porous gel and exposed to an electric field, which causes the SDS-coated proteins to move as “bands” in the gel according to differences in relative molecular mass. By using appropriate radiolabeled compounds (such as inorganic phosphate added to the culture medium), it is possible to apply the technique of autoradiography to specifically detect radioactive, phosphorylated proteins resolved by SDS-PAGE. It is also possible to use gel electrophoresis to separate proteins according to relative charge, a property that is modified predictably by certain modifications such as phosphorylation. These types of separation can be combined in the use of two-dimensional gel electrophoresis,

which allows high resolution of proteins as “spots” differing in relative size and charge.

Proteins resolved by gel electrophoresis can be transferred to a membrane composed of nitrocellulose or polyvinylidene difluoride (PVDF). This allows many manipulations to be performed, such as detection of a specific protein from a complex mixture by the ability of the protein to be bound by a specific antibody. This procedure, called immunoblotting or “Western” blotting, can be used with commercially available antibodies recognizing phosphorylated peptide sequences or phosphorylated amino acids.

GPCRs resolved by gel electrophoresis can also be analyzed by chemical sequencing, typically by a process called Edman degradation, which sequentially cleaves residues from the amino-terminal end of the protein. Phosphorylated residues can be distinguished from their nonphosphorylated counterparts by chromatography or by incorporation of radioactive phosphate, allowing the identification of specific phosphorylated residues in a polypeptide sequence by the order of appearance in the eluate collected after multiple cycles of Edman degradation. A very powerful method for determining amino acid sequence and detecting posttranslational modifications of proteins is via mass spectrometric analysis. For example, with tandem mass spectrometry it is possible to measure the mass of specific protein fragments with an accuracy of one part in 10,000 up to 12,000 daltons and one part in 1,000 up to 25 kd (45). The impressive accuracy of this method makes it possible to detect phosphorylation as well as many other posttranslational modifications, even those that cause subtle changes in the protein size or charge.

Chromatography, which refers to any separation based on differential behavior of a molecule between a stationary phase and a moving phase, offers many ways of identifying protein modifications. High-performance liquid chromatography (HPLC) using reverse-phase (e.g., C18) columns allows the precise separation of peptides derived from proteolytic or chemical fragmentation of GPCRs. By comparing the pattern of peptide fragments derived from the native protein and the modified protein, one can identify specific polypeptide fragments containing the modification of interest. Subsequently, these fragments can be isolated and further analyzed by methods such as Edman degradation or mass spectrometry.

Methods to Examine Regulation of Receptors by Localization and Trafficking

It has been appreciated for many years that a critical parameter that can regulate the strength of functional signal transduction via GPCRs is the actual number of receptors present in target tissues and, in particular, the number of receptors present in the plasma membrane of individual cells. Indeed, disturbances in the regulation of receptor number and/or distribution may be of primary importance in the patho-

physiology of certain neuropsychiatric disorders. For example, long-term administration of dopamine receptor antagonists can induce upregulation of specific receptors, which may contribute to the apparent supersensitivity of dopamine receptors associated with tardive dyskinesia (46). In contrast, prolonged stimulation of certain GPCRs with agonist ligands can lead to a decrease in the number of binding sites available on the cell surface. This phenomenon is termed receptor “down-regulation” and may contribute to the effects of certain antidepressant drugs (47). Studies using radioligand binding and subcellular fractionation techniques provided early evidence that multiple mechanisms are capable of mediating changes in the number of GPCRs present at the cell surface (48). More recently developed molecular and cell biological approaches provide powerful tools for directly visualizing the subcellular localization of GPCRs and for performing biochemical studies of specific receptor trafficking mechanisms.

Immunochemical Methods to Visualize the Subcellular Localization of Receptors

GPCRs can be detected *in situ* in cell or tissue preparations using immunochemical techniques and receptor-specific antibodies. Antibodies that recognize the native receptor protein can be used to examine the localization of endogenously expressed receptors, whereas epitope-tagging methods (see above) can be used to detect mutated versions of the receptor protein or as a means to detect recombinant receptors for

which antibodies recognizing the native receptor are not available. In either case the general scheme is as follows: Cells or tissues expressing the receptor of interest are fixed using standard histologic methods. The fixed cells or tissue can be “permeabilized” with a nonionic detergent, to facilitate biochemical access to receptors situated in intracellular membranes, and then specimens are incubated with antibodies recognizing the receptor of interest. After sufficient time has elapsed to allow antibodies to bind to their respective epitopes in the specimen (typically several hours), the specimens are washed extensively to remove nonspecifically associated antibodies. Antibodies bound to the receptor are then detected by incubation of specimens with a “secondary” antibody that binds specifically to the “primary” antibody (the antibody bound initially to the receptor). The secondary antibody is typically coupled to a fluorochrome (such as fluorescein), a recognizable particle (such as colloidal gold), or an enzyme that can produce a localized reaction product (such as horseradish peroxidase) to facilitate direct visualization of the receptor-containing immune complex using various light or electron microscopic techniques (Fig. 22.2).

Biochemical Methods to Assay Specific Receptor Trafficking Processes

Whereas microscopic imaging can readily provide a great deal of qualitative information about GPCR localization and trafficking, it can be quite challenging to quantitate

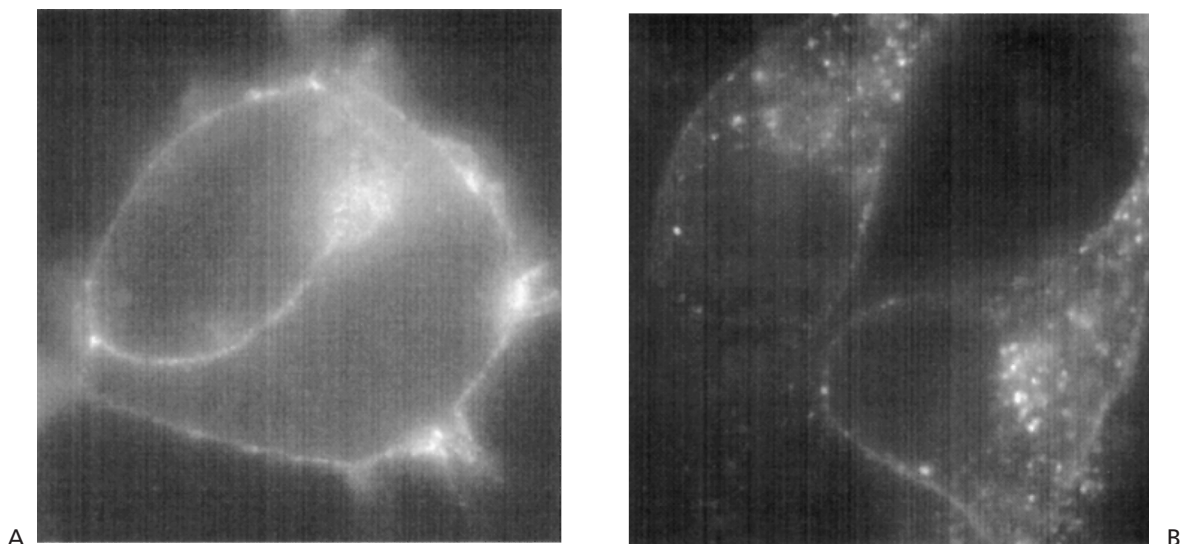


FIGURE 22.2. Visualization of HA epitope-tagged dopamine D1 receptors in transfected cells, using a fluorochrome-labeled secondary antibody and fluorescence microscopy. The ability of this receptor to undergo regulated internalization is indicated by the dopamine-induced redistribution of immunoreactive receptors from the plasma membrane (visualized as linear staining at the cell periphery) to endocytic vesicles (visualized as punctate structures located throughout the cytoplasm). **A:** Untreated cells (no ligand). **B:** Treated with 10 μ M dopamine. (Photograph courtesy of Gabriel Vargas.)

from these data the precise amount of receptor present in a specific subcellular localization or to measure accurately the rate or extent of specific trafficking processes. The importance of these processes has motivated the development of biochemical methods for examining GPCR trafficking. In addition to their utility for receptor localization, antibodies specifically recognizing GPCRs facilitate biochemical studies of GPCR trafficking using techniques adapted from other areas of cell and molecular biology. For example, one method that has been extremely useful for quantitative studies of GPCR endocytosis is cell-surface biotinylation coupled with immunoprecipitation of receptors. Proteins present in the plasma membrane of cells can be specifically labeled by incubating intact cells in the presence of biotin coupled to an activated ester, which is membrane-impermeant and therefore forms a covalent bond only with exposed amine moieties present in plasma membrane proteins. In general, biotinylation in this manner does not adversely affect GPCR function, allowing biotinylation to be used as a chemical tag for surface receptors. The biotin moiety is extremely useful for subsequent detection or purification of surface-tagged proteins because it binds with extremely high affinity ($K_a \sim 10^{15} \text{ M}^{-1}$) to the proteins avidin or streptavidin. Using variations of this basic biochemistry, it is possible to measure a wide variety of membrane trafficking processes. For example, internalization of GPCRs has been measured by the inaccessibility of biotinylated receptors to a membrane-impermeant reducing agent that “cleaves” the biotin moiety away from tagged proteins (49), and surface biotinylation has been used to measure the rate and extent of proteolytic degradation of receptors after endocytosis (50, 51).

Methods for Examining Specific Protein Interactions Involved in GPCR Function and Regulation

A salient lesson emerging from recent cell biological studies is that GPCR signal transduction can be viewed, in essence, as a dynamically regulated network of protein–protein interactions that occur in specific subcellular locations. Therefore, an important goal of current and future research is to define these critical protein interactions and elucidate their temporal and spatial regulation in intact cells and tissues. A great deal of effort is presently going into developing and applying novel methods for the study of protein–protein interactions both *in vitro* and *in vivo*, as illustrated by the following examples.

Coimmunoprecipitation Techniques to Examine Defined Protein Interactions with GPCRs in Intact Cells

As discussed above, it is possible to rapidly purify GPCRs from cell or tissue extracts using receptor-specific antibodies

or epitope tagging methods. In addition to being extremely useful for examining posttranslational modifications of GPCRs, in some cases it is possible to use these techniques to isolate receptor-containing complexes that presumably reflect protein interactions occurring in the intact cell. The basic idea is to immunopurify a specific GPCR from cell or tissue extracts (or from a partially purified subcellular fraction prepared from a cell or tissue lysate) using an antibody recognizing the native receptor or an engineered epitope tag, and then to analyze proteins specifically associated with this complex using a different antibody. In general, this is accomplished by immunoprecipitation of the receptor followed by analysis of associated proteins in the complex by immunoblotting with the appropriate additional antibody. In some cases, the protein complexes are sufficiently stable that they remain associated through the initial immunopurification of the receptor. In other cases this is not true, and the complexes dissociate before the receptor can be purified from the extract. In this case, various chemical agents can be added prior to cell lysis to physically “cross-link” closely associated proteins with one another by forming covalent bonds that prevent dissociation of the complex. Coimmunoprecipitation has been used to assay GPCR interaction with heterotrimeric G proteins (52) and with β -arrestins (53), and to examine the regulation of these protein interactions by ligand-induced activation of the receptor.

Use of Coimmunoprecipitation to Examine Oligomerization of GPCRs

The idea that GPCRs may function *in vivo* as higher-order molecular complexes has been suspected for many years. Recent studies provide strong support for this idea and, specifically, provide evidence for homo- and heterodimerization of individual GPCRs *in vivo*. This principle is perhaps best established for receptor tyrosine kinases, where it is well established that oligomerization of receptors is required for appropriate ligand-dependent signal transduction (54). A relatively early hint that GPCRs may also undergo oligomerization came from studies of the β_2 -adrenergic receptor using epitope-tagging techniques, where it was observed that receptors tagged with one epitope could specifically coimmunoprecipitate receptors tagged with a distinct epitope (55). Early evidence suggesting a functional role of oligomerization in GPCR signaling came from mutational studies in which structural domains present in distinct, functionally inactive mutant receptors could “complement” one another when coexpressed in cells, suggesting the formation of a functional “hybrid” oligomeric receptor protein (56). More recently, evidence for oligomerization of many GPCRs has been reported. A particularly compelling example of this is the recent observation that distinct subtypes of GABA-B receptor hetero-oligomerize in cells, and that oligomerization is essential for the formation of recombinant receptors possessing the functional properties charac-

teristic of native GABA-B receptors observed *in vivo* (57,58). Recent studies using epitope-tagging and coimmunoprecipitation have demonstrated the formation of homo- and heterodimers of opioid receptors, and suggest that receptor oligomerization may contribute to the remarkable diversity of pharmacologic properties observed in natively expressed opioid receptors (59). There is also emerging evidence that certain GPCRs may associate *in vivo* with completely different classes of receptor protein, such as the dopamine D5 receptor (a GPCR) and the GABA-A receptor (a ligand-gated ion channel). In a recently published study (60), glutathione S-transferase (GST)-fusion proteins encoding the C-terminal tail of the D5 receptor were shown to interact with the GABA-A receptor present in rat hippocampal extracts. Additionally, using an antibody recognizing the dopamine D5 receptor, it was possible to coimmunoprecipitate the GABA-A receptor from cell extracts. Interestingly, this coimmunoprecipitation was detected only when both receptors were stimulated by their respective ligands, suggesting that this heterotypic interaction is regulated in a ligand-dependent manner.

Identification of Novel Protein Interactions with GPCRs

In addition to known proteins that mediate and regulate GPCR signaling (heterotrimeric G proteins, GRKs, arrestins), which were originally identified by functional assays using biochemical purification, cDNA cloning methods have facilitated the identification of additional protein interactions with GPCRs that were completely unanticipated (61). These novel protein interactions, while their functional relevance remains unclear in many cases, are of great interest and potential therapeutic importance as drug targets.

Of the many techniques for identifying novel protein–protein interactions developed over the last 10 years, interaction cloning methods such as the yeast two-hybrid system (62) have been particularly useful for studies of GPCRs. In the yeast two-hybrid system, protein interactions are detected by their ability to reconstitute the activity of a “split” transcriptional activator complex. A transcription factor such as GAL4 can be divided into two domains: a DNA binding domain and a transcriptional activation domain. For the transcription factor to be active, these two domains must be in close proximity to one another, so that the DNA binding domain can bind the promoter sequence in a “reporter” gene and the activation domain can promote gene transcription. A polypeptide sequence for which one wishes to identify putative interacting proteins (such as a sequence derived from a cytoplasmic domain of a GPCR) is cloned into a vector coding for the isolated DNA binding domain from the GAL4 transcription factor, thereby producing a fusion protein containing the GPCR-derived sequence as “bait” with which to search for potential protein

interactions. A cDNA library prepared from a tissue of interest is cloned into a cDNA encoding an isolated transcriptional activation domain, producing a large number of fusion proteins containing tissue-derived polypeptide sequences as potential “prey” for protein interaction with the GPCR-derived fusion protein. Both the bait and prey plasmids are transformed into a strain of yeast harboring a “reporter” gene that can be transcribed only in the presence of an “intact” GAL4 transcription factor. Either “half” of the transcription factor is not sufficient to promote efficient transcription of the reporter gene. However, if the fused bait and prey polypeptides form a sufficiently stable protein–protein interaction, they bring their corresponding DNA binding and transcriptional activation domains into close proximity, thus reconstituting transcriptional activation of the reporter gene. Transformed yeast cells containing plasmids encoding the corresponding interacting protein domains can be identified by screening techniques based on GAL4-dependent transcription of reporter genes conferring antibiotic resistance or other selectable metabolic activities, or encoding enzymes that can be detected using colorimetric assays. Assays using the *E. coli*-derived *lacZ* gene, for example, can be used to screen for a characteristic blue reaction product when exposed to 5-bromo-4-chloro-3-indolyl α -D-galactoside.

Protein interactions suggested to occur by the yeast two-hybrid system can be examined using various *in vitro* biochemical techniques, such as affinity chromatography facilitated by GST-fusion proteins. In addition to serving as an independent assay for previously defined candidate interacting proteins, this method can be used to identify novel protein interactions with GPCRs *de novo* (63). In this method a DNA encoding a polypeptide sequence of interest is fused to GST using standard cDNA cloning techniques and expressed as a recombinant protein in *E. coli*. The GST portion of the fusion protein allows the efficient immobilization of the protein by binding to agarose beads covalently derivatized with glutathione. Proteins from a cell or tissue extract that bind to the fusion protein then can be isolated as an immobilized protein complex by affinity chromatography. As an example of the use of these methods, it was shown recently (64) that the third cytoplasmic loop of the dopamine D2 receptor binds specifically to spinophilin, a large cytoskeleton-associated protein that also binds to protein phosphatase-1. This binding was initially identified through use of the yeast two-hybrid system, and then the identification of the specific domains that mediate this protein interaction was accomplished by affinity chromatography using GST-fusion proteins.

Methods for Examining Candidate Protein Interactions in Intact Cells

A major question regarding novel protein interactions with GPCRs, such as those identified using interaction cloning

or protein affinity chromatography, is whether or not they actually occur in an intact cell. Immunocytochemical techniques can provide some insight into this question by determining whether candidate interacting proteins “colocalize” in cells with the appropriate GPCR, as expected if the proteins physically interact *in vivo*. However, even in the event that extensive colocalization is observed, immunocytochemical techniques of this sort do not provide direct evidence for a physical interaction between candidate proteins. Coimmunoprecipitation techniques, as discussed above, provide a useful method for addressing this question. However, demonstrating that a specific protein association can occur *in vivo* is only the first step in the process of assessing the potential physiologic relevance of a novel protein interaction, as this method generally does not provide any information regarding the possible functional activity of a candidate protein interaction. Addressing this question can be a challenging task that involves creative application of diverse techniques and functional assays. Examples of novel protein interactions with GPCRs for which compelling functional data exist include the aforementioned interaction of the D2 dopamine receptor with ABP280 (65) and interaction of the β_2 -adrenergic receptor with NHERF/EBP50-family proteins (51,63).

EMERGING HORIZONS

Unexpected Signaling, Cross-Talk, and Transactivation Involving GPCRs (Fig. 22.3)

Another line of evidence suggesting the existence of functionally relevant, novel protein interactions involving GPCRs comes from recent work by several labs suggesting that unanticipated functional interactions can occur between GPCRs and receptor tyrosine kinases (RTKs), a distinct family of single-transmembrane receptors involved in growth, differentiation, and oncogenesis (66). The RTK family includes the epidermal growth factor receptor (EGFR), the first receptor shown to have intrinsic tyrosine kinase activity (67,68). Whereas the classic pathway for RTK-mediated signaling is initiated by binding of polypeptide growth factors (such as EGF) to the extracellular domain of the RTK, it has been observed recently that certain GPCRs can initiate signaling cascades traditionally thought to be controlled by RTKs. In this situation, the primary signal appears to be through the GPCR, which in turn “transactivates” the RTK. For example, several GPCRs can mediate transactivation of coexpressed EGFRs, thus stimulating mitogenesis by a similar downstream pathway as that initiated by binding of EGF directly to the EGFR (69). One mechanism of GPCR-mediated transactivation involves the activation of a membrane-associated metalloproteinase, which cleaves the EGF precursor protein to generate increased amounts of ligand for the EGFR (70). Another

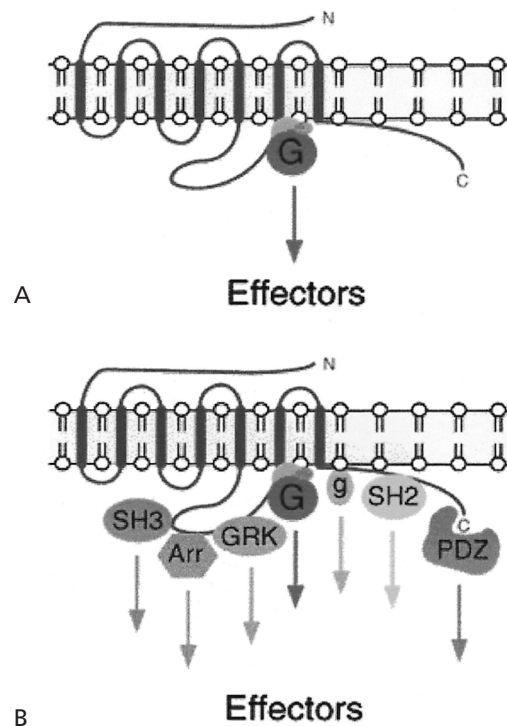


FIGURE 22.3. Schematic diagram of G-protein–coupled receptor (GPCR) signaling. **A:** The G-protein paradigm. Following agonist binding, GPCRs activate heterotrimeric G proteins (G), which then regulate the activity of specific cellular effectors. **B:** Beyond the G-protein paradigm. Following agonist binding, GPCRs can associate with members of diverse families of intracellular proteins, including heterotrimeric G proteins (G), polyproline-binding proteins such as those containing SH3 domains (SH3), arrestins (Arr), G-protein–coupled receptor kinases (GRK), small guanosine triphosphate (GTP)-binding proteins (g), SH2 domain–containing proteins (SH2), and PDZ domain–containing proteins (PDZ). These interactions allow GPCRs to initiate multiple intracellular signaling pathways, with each subtype of receptor likely coupled to a relatively unique set of effectors. (From Hall RA, Premont RT, Lefkowitz RJ. Heptahelical receptor signaling: beyond the G protein paradigm. *J Cell Biol* 1999;145:927–932, with permission.) See color version of figure.

mechanism of cross-talk involves the formation of heteromeric signaling complexes, which include components of both “classical” GPCR and RTK signaling cascades. For example, recent studies suggest that the nonreceptor tyrosine kinase c-Src can associate with the β_2 -adrenergic receptor and the β -arrestin in endocytic membranes, thus mediating mitogenic kinase activation either by a c-Src-mediated phosphorylation of downstream effectors (71) or by c-Src-mediated phosphorylation of co-endocytosed EGFR (72).

Visualization of Protein Localization and Interaction in Living Cells

As discussed above, immunochemical methods are useful for examining the localization of proteins in intact cells.

However, these methods are typically applied to fixed cells because they require disruption of the cell membrane and prolonged incubation of specimens with antibodies used to detect the receptor of interest. The discovery of proteins from certain marine animals that have high levels of intrinsic fluorescence has fostered a revolution in the ability to localize proteins in living cells. These proteins, such as the green fluorescent protein (GFP) isolated from the jellyfish *Aequorea victoria*, are brightly fluorescent molecules that can fold properly in many environments and do not require any additional chromophore for their fluorescence (73,74). This allows them to be used to “tag” GPCRs and other important signaling proteins in intact cells. This is accomplished by using site-directed mutagenesis to create a fusion between the GPCR polypeptide sequence and the sequence encoding the GFP tag, analogous to the introduction of an antigenic epitope tag. The localization of the fusion protein can be examined in intact cells using fluorescence microscopy. Examples of this methodology include the visualization of ligand-induced endocytosis of a GFP-tagged β_2 -adrenergic receptor in living cells and visualizing the dynamic recruitment of GFP-tagged β -arrestin from the cytoplasm to the plasma membrane induced by activation of various GPCRs (75,76).

While GFP has facilitated the localization of proteins in living cells, localization by itself does not necessarily indicate the occurrence of a physical interaction of a GPCR with a specific protein. The development of mutant versions of GFP, which differ in their excitation and emission spectra, has made it feasible to examine *in vivo* protein interactions using the process of fluorescence resonance energy transfer (FRET) (77). FRET occurs when two suitable fluorophores are present in extremely close proximity so that light produced from one fluorophore can be “transferred” efficiently into exciting the other. FRET can be detected in living cells using sophisticated microscopy, making it possible in principle to detect specific protein interactions with GPCRs and study their localization in real time. FRET imaging has not yet been used extensively for GPCR research but holds great promise for future study of the spatial and temporal dynamics of protein interactions with GPCRs in intact cells and tissues.

SUMMARY AND CONCLUSIONS

We have discussed a subset of experimental approaches that have provided powerful new tools for studying GPCR function and regulation. These approaches are responsible, in large part, for the vast explosion of new information about specific mechanisms of GPCR biology that has emerged over the past several years. In many cases these developments have extended directly from seminal observations made originally through classic pharmacologic approaches, which remain of central importance to understanding GPCR func-

tion and regulation. Indeed, we view newer molecular and cell biological approaches as complementing, rather than replacing, the sophisticated pharmacologic methods that have been developed over the years since the discovery of receptors as important drug targets.

Molecular cloning techniques have allowed the isolation of cDNAs encoding many G-protein–coupled receptors. The isolation of receptor cDNAs has provided insight into the remarkable structural homology among GPCRs, revealed an unanticipated level of molecular diversity in the GPCR superfamily, allowed functional characterization of defined receptor subtypes in heterologous systems, and made it practical to produce large amounts of receptor protein for pharmacologic, biochemical and biophysical studies.

Structural, biophysical, and molecular modeling approaches hold great promise for ultimately defining the precise atomic determinants of receptor–ligand interaction and for understanding protein conformational changes involved in receptor activation and regulation. Continued progress in this important area may lead to entirely new concepts and methods relevant to therapeutic drug design. Site-directed mutagenesis techniques complement structural and biophysical approaches and have enabled, in the absence of precise structural information, the empirical identification of residues and receptor domains important for ligand binding and activation. Cell biological methods have elucidated mechanisms of signal transduction and regulation in impressive detail, and have revealed a previously unanticipated level of specificity and complexity of crosstalk between signal transduction systems. Emerging technologies for detecting protein interactions in intact cells are suggesting new insights into cell biological mechanisms of GPCR function and regulation, and are beginning to allow real-time examination of the temporal and spatial dynamics of defined protein interactions in living cells.

Based on the new methodologies available today and current pace of progress in using these methods for elucidating GPCR function and regulation, we anticipate that the next several years will see even greater progress in our understanding of the fundamental biology of GPCRs. Indeed, the field of GPCR research is rapidly moving away from a focus on any one set of experimental techniques and has become a vanguard area of integrative structural, molecular, and cell biology. Further developments of these experimental methods, combined with new *in vivo* imaging and genomics approaches that have appeared on the horizon, are likely to fuel continued rapid progress in the field. This exciting progress is fundamentally and directly relevant to the main mission of neuropsychopharmacology: to develop and provide effective therapies for the complex neuropsychiatric disorders that affect our patients.

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