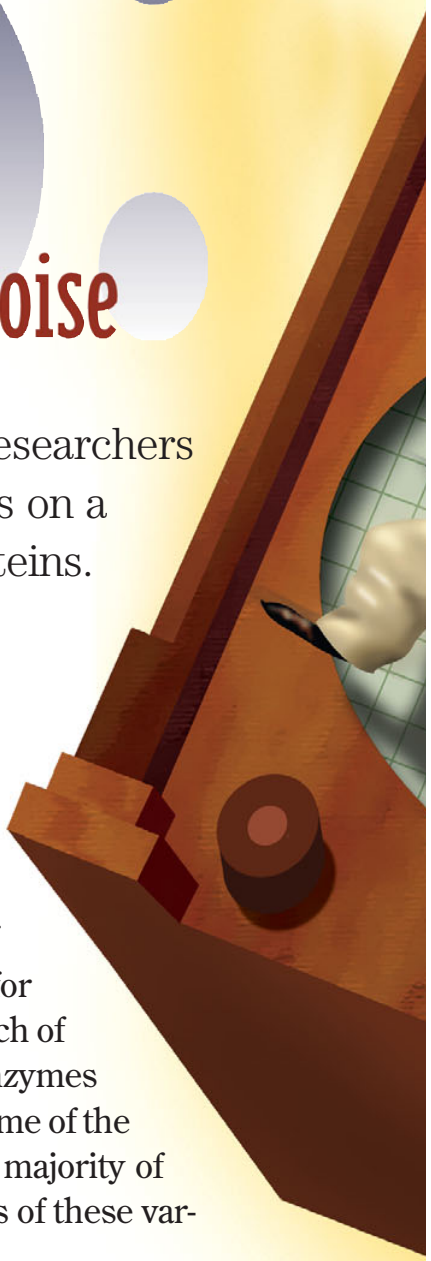
HTS:

Seeking signals through the noise

Facing an overabundance of potential targets, researchers are using high-throughput methods to focus on a promising group—the GPCR signaling proteins.

BY MARK S. LESNEY



In the cacophony of the physiological complexity of cells, tissues, organs, and bodies, the quest for drug targets has found a few sources of clarity—promising signals heard above the noise of the many thousands of genes, proteins, membranes, and metabolites. In the world of proteomics, signal transduction proteins, responsible for most intercellular communication, have been the source of much of this promise. They help regulate everything from individual enzymes to cell differentiation to cancerous growth. As such, they are some of the most exciting potential drug targets. As history indicates, the majority of modern pharmaceuticals turn out to be agonists or antagonists of these various (usually membrane) proteins.

But historical precedent will not serve in these harsh economic times. Researchers in their individual laboratories cannot continue to examine potential targets one by one, hoping to pick a single signal out of the noise of the bioinformatics explosion. Automation and high-throughput screening (HTS)

ILLUSTRATION: MICHELLE BARBERA

methods are required. These methods are being developed to investigate the hundreds of signaling and membrane protein candidates available in a quest for answers to innumerable diseases.

All in the (GPCR) family

Among the most studied of these signaling proteins are the GPCRs (G-protein coupled receptors). They consist of a superfamily of protein receptors in the plasma membrane and include a number of the most important drug targets already known (see Table 1). In fact, more than 50% of the drugs on the market target GPCRs. These receptors contain seven transmembrane-spanning domains that have been labeled "serpentine" because of their resemblance to a coiled snake. They also contain an extracellular ligand binding site, critical to their differential activation, as well as an intracellular domain for coupling to a G-protein (a heterotrimeric GTP-binding or hydrolyzing protein) that gives them their name. Binding of a ligand, such as a hormone, causes a conformational change in the receptor that activates and releases components of the bound G-protein, which subsequently performs one or more predefined cellular activities, thereby effecting transmission of signaled commands from outside to inside the cell (see Figure 1). Like an enzyme, the GPCR subsequently reconfigures to its original state once the ligand dissipates or is removed.

GPCRs can be categorized by their extracellular ligand, by their G-protein partner, or both. The extracellular ligand can be any of a huge number of triggers, including lipids, nucleotides, odorants, photons, peptides, and proteins. Evolutionarily, they can be traced back to the origins of eukaryotic life, with tremendous proliferation throughout the expansion of the animal kingdom. GPCRs not only control metabolism, they also modulate our senses, allowing us to see, smell, and taste (*1*).

The eyes have it. The visual photoreceptors, such as rhodopsin, are actually a class of GPCRs. Rhodopsin, which contains a photosensitive visual pigment as its triggering mechanism, is the best studied of all GPCRs, and its crystal structure has served as the basis for most modeling efforts in the field. (Many of the GPCRs, because of their membrane-bound nature, have been difficult to crystallize, and structural information is often sketchy or based on models rather than actual observations.)

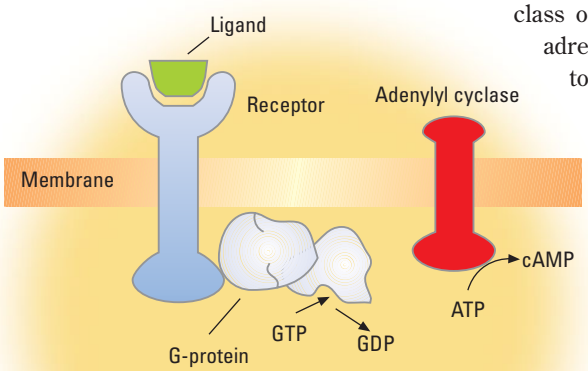


Figure 1. Pass the signal. With the binding of its ligand, a cell receptor promotes the release of GDP and the uptake of GTP by the G-protein, thus activating it. Among the proteins then activated by the GTP-binding G-protein is adenylyl cyclase, which converts ATP to cyclic AMP (cAMP), a second messenger.

cAMPing it up. One particularly important class of GPCRs (including the human β-adrenergic, glucagon, and odorant receptors) produces the cascade increase or decrease of cyclic adenosine monophosphate (cAMP) through the activation or inhibition of plasma membrane-bound adenylate cyclase by the coupled G-protein complex (see Figure 1). Because cAMP is one of the most important secondary messengers for controlling the myriad cellular metabolic pathways, a variety of assays have been developed for HT monitoring of cAMP production in the study of GPCR activation. These assays include a version of the Hit-Hunter Enzyme Fragment Complementation assay by Applied Biosystems, which uses reintegration of a fragmented, engineered β-galactosidase as a luminescent tag.

Chemokine receptors. A class of GPCRs that bind a variety of growth and chemotactic factors known as chemokines, chemokine receptors regulate the biological activity and movement of leukocytes and thus are an intrinsic component of the immune response, participating in disease systems from inflammatory disorders to AIDS and cancer. The tremendous interest in developing chemokine agonists and antagonists makes the screening of chemokine receptors a prominent area for HTS research (*2*).

Many other kinds and classes of GPCRs exist, some more specific to plants and fungi than animals, others more ubiquitous. The development of such complex signaling mechanisms as the GPCRs was undoubtedly one of the enabling factors for the evolution of the vast diversity of eukaryotic life present in the world today. The evolutionary relatedness of GPCRs in form and function has enabled researchers to gather much of the information on the nature and behavior of these proteins from studies on *Drosophila* and other model organisms. A now famous mutant GPCR called Methuselah has been shown to modulate stress responses in *Drosophila*, greatly prolonging the fly's life span. (Unfortunately, human homologues of this particular gene have not yet surfaced.)

The best place to find GPCRs electronically is at the G Protein-

Table 1

Some GPCR-related drugs

Disease	GPCR	Drug
Allergies	Histamine (H1)	Claritin
Ulcers	Histamine (H2)	Tagamet, Zantac
Ulcers	Prostaglandin	Cytotec
Hypertension	Angiotensin	Cozaar, Teveten
Hypertension	Adrenergic (β1)	Tenormin
Glaucoma	Adrenergic (β-mixed)	Inderal, Timoptol
Asthma	Adrenergic (β2)	Cerevent, Ventolin
Enlarged prostate	Adrenergic (α)	Cardura
Congestive heart failure	Adrenergic (β1/β2/α1)	Coreg
Parkinson's disease	Dopamine	Requip

Advertisers

Coupled Receptor Data Base (*www.gpcr.org/7tm/*). Sponsored by the European Union, the website provides the most extensive GPCR database system. It contains sequences, mutant data, and ligand binding constants for the majority of known GPCR receptors.

Hitchhiker's guide to the GPCRs

Because of the ubiquity of transmembrane receptors in the plasma membrane and their highly conserved nature, it is not surprising that pathogens can take advantage of them as docking stations for binding and entry into cells. Perhaps of most critical interest today is the fact that HIV takes advantage of two particular chemokine receptors, CCR5 and CXCR4, to achieve entry into target cells. Researchers are attempting to develop therapeutics based on blocking these receptors.

In a reverse example, host genes for GPCRs can be captured and used by viral genomes. It appears that the herpes virus associated with Kaposi's sarcoma in individuals with compromised immune systems has a tag-along viral-coded GPCR that integrates into the host cell membrane, shifting metabolic regulation to a state more favorable to the virus than the cell (*3*). The importance of understanding and controlling the chemokine receptors is demonstrated not only by the normal role this class of proteins plays in controlling the immune system in the fight against infectious diseases and cancer but also in this adventitious hitchhiking and co-option of receptors.

"There is [a] large number of orphan GPCRs for which the ligands are unknown."

Genomes and orphans

With the success of the Human Genome Project and the growth of bioinformatics analysis technology, GPCRs have further gained the limelight. According to Robert S. Ames of GlaxoSmithKline, "While there are over 150 different subtypes of GPCRs with known ligands, there is an equally large number of orphan GPCRs for which the ligands are unknown" (*6*). DNA analysis has identified these orphan receptors among the host of human genes, recognizing them as GPCRs by their consensus sequences. This has led to a multimillion-dollar race in Big Pharma and among many start-up biotech companies to adopt and raise orphan GPCRs with the hope that they will turn out to be successful drug target candidates for important diseases. Much of modern HTS in this area, especially using FLIPR technology (see box, "They call it FLIPR"), has been involved in the attempt to find ligands for these orphans. Since 1998, reports in the literature have paired more than 20 of the 200-odd orphan GPCRs with their natural ligands (*6*).

In a presentation given at the 7th G-Protein Coupled Receptors Conference in San Diego last month, Joseph Dillon from the University of Iowa reported a particularly exciting linkage of an orphan GPCR to a DHEA—an adrenal steroid with hitherto no known cellular receptor or mechanism of effect. He discovered that activation of the receptor with DHEA leads to production of the potent vasodilator nitric oxide. Similarly, researchers at Stanford University identified two orphan GPCRs as receptors for the peptide hormone relaxin, which appears to function not only in pregnancy but also in the regulation of heart contractions, making it of tremendous interest for cardiac medicine (*7*). Once such receptor–ligand pairs are identified, the gamut of HTS technologies can be focused on finding potential therapeutics for these new targets. The patentability of these various orphans is, of course, one of the major driving forces for cutting up these slices of the GPCR pie.

G-protein regulator proteins

Recently, a hidden layer of secondary regulation that further modulates signal transduction behavior has been revealed in many of the GPCRs. This additional regulatory system involves the interaction of activated G-protein components intracellularly with a class of proteins known as regulators of G-protein signaling (RGSs). After GPCR cascade initiation, these RGSs modulate the effects of the activated G-proteins and their subsequent reconstitution into the inactive GPCR complex. This provides

They call it FLIPR

The FLIPR (fluorescent imaging plate reader) system developed by Molecular Devices is one of the technologies that researchers at several major pharmaceutical companies are directly turning to the HTS of GPCRs. The FLIPR system monitors the response of cell populations in well plates to potential drug candidates designed to target a variety of receptors, including GPCRs. Researchers at the 6th International Drug Discovery Product Users Meeting reported on the use of FLIPR to search libraries of small molecules for agonists and antagonists to a variety of chemokine receptors (*4*).

Similarly, other HT fluorescent techniques have been developed. For example, Amersham Biosciences combines its CyDye labeling system with its FARCyte Fluorescence Plate Reader to follow fluorescence polarization as a means of detecting the binding of tagged ligands to a variety of membrane signaling receptors, including GPCRs such as the muscarinic acetylcholine receptors, which are important in controlling the function of neurons and are implicated in several neurodegenerative diseases (*5*).

an added ability to fine-tune the effects of signals from outside the cell based on internal cellular cues and the residual effects of other extracellular signaling events. Many believe that the RGS proteins may provide a host of therapeutic drug target possibilities related to but beyond the GPCRs (*8*).

It is unlikely that the current interest in GPCRs as drug targets will diminish in the foreseeable future.

CHO et al., meet GPCR

One of the most important breakthroughs that permitted the development of HT assays for GPCRs was the ability to mass-produce these proteins through recombinant DNA techniques. Human GPCRs can be cloned into Chinese hamster ovary (CHO) cells and used to produce assay-quality reagents. For example, Packard BioSciences (now a component of PerkinElmer Life Sciences) produces a variety of ScreenReady Targets that contain immobilized GPCRs, such as the motilin receptor, on isolated transformed CHO cell membranes bound to well plates. Radiolabeled ligands are screened for binding by an automated microplate scintillation and luminescence counter.

Similarly, Euroscreen, a spin-off from Brussels University, was found to "de-orphanize" GPCRs by advancing studies in cloned GPCRs in more than 70 recombinant CHO cell lines. For use in these cell lines, they developed an HTS method for measuring increases in intracellular calcium, often stimulated when ligands bind to GPCR. Aequorin is a photoprotein from a jellyfish. Upon binding to calcium, aequorin oxidizes an added substrate, coelenterazine, to produce photons. The emitted light can be detected with a luminometer. The system is capable of HTS of GPCRs when used with appropriate fluorescent instrumentation, such as the Hamamatsu FDSS 6000 screening system.

Similarly, the company Heptagen is attempting to develop a yeast model system for the study of recombinant GPCRs. Yeast is an especially viable candidate for several reasons: It is easy to grow and transform; its genome has been completely sequenced; and perhaps more important, it has only a limited set of endogenous G-proteins and only one native GPCR. As an increasing variety of such transgenic systems and assays develop, the identification of orphan GPCRs as well as the further elucidation of the physiological behavior of known GPCR–ligand combinations become inevitable.

It is unlikely that the current interest in GPCRs as drug targets will diminish in the foreseeable future. Their involvement in such a multitude of cell processes in development and metabolism, from growth (healthy and cancerous) to apoptosis, makes them undeniably important. Further enhancements in microarray and automation technologies, including new methods in assays and tagging for studying these proteins, are developing rapidly (*9*). This can only quicken the pace of discovery. If historical precedent is predictive, then the value of studying these proteins—both in terms of potential new biomedicines and increased metabolic understanding—is incalculable.

Some GPCR-HTS-related companies

Advanced Bioconcept	www.bioconcept.com
Agilent	www.agilent.com
Amersham Biosciences	www.amershambiosciences.com
Applied Biosystems	www.appliedbiosystems.com
Axon Instruments	www.axon.com
Beckman Coulter	www.beckmancoulter.com
Biacore	www.biacore.com
BioMol Research Products	www.biomol.com
BioSource International	www.biosource.com
Calbiochem	www.calbiochem.com
Cellomics	www.cellomics.com
Cerep	www.cerep.com
Corning, Inc.	www.corning.com/lifesciences
DiscoveRx	www.discoverx.com
Euroscreen	www.euroscreen.be
Hamamatsu	www.hamamatsu.com
Heptagen	www.businessplans.org/Heptagen/Hepta00.html
Molecular Devices	www.moleculardevices.com
Norak Biosciences	www.norakbio.com
PanVera	www.panvera.com
PerkinElmer Life Sciences	http://lifesciences.perkinelmer.com
Pierce Biotechnology	www.piercenet.com
Roche Biochemicals	www.roche.com
Tecan	www.tecan.com
Tripos	www.tripos.com
Universal Imaging Corp	www.image1.com
Upstate	www.upstatebiotech.com

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