Annu. Rev. Pharmacol. Toxicol. 2004. 44:43–66
doi: 10.1146/annurev.pharmtox.44.101802.121419

First published online as a Review in Advance on August 18, 2003

THE IDENTIFICATION OF LIGANDS AT ORPHAN G-PROTEIN COUPLED RECEPTORS

Alan Wise,[1] Steven C. Jupe,[2] and Stephen Rees[3]
[1]7TMR Systems Research Europe, [2]Bioinformatics, and [3]Molecular Screening Department, GlaxoSmithKline, Gunnels Wood Road, Stevenage, Herts SG1 2NY, United Kingdom; email: alan.x.wise@gsk.com, steven.jupe-1@gsk.com, steve.x.rees@gsk.com

Key Words orphan, GPCR, 7-transmembrane receptor

■ **Abstract** The completion of the human genome sequencing project has identified approximately 720 genes that belong to the G-protein coupled receptor (GPCR) superfamily. Approximately half of these genes are thought to encode sensory receptors. Of the remaining 360 receptors, the natural ligand has been identified for approximately 210 receptors, leaving 150 so-called orphan GPCRs with no known ligand or function. The identification of ligands active at orphan GPCRs has been achieved through the development of a number of experimental approaches, including the screening of putative small molecule and peptide ligands, reverse pharmacology, and the use of bioinformatics to predict candidate ligands. In this review, we discuss the methodologies developed for the identification of ligands at orphan GPCRs and include examples of their successful application.

INTRODUCTION

Drugs active at G-protein coupled receptors (GPCRs) have therapeutic benefit across a broad spectrum of human diseases as diverse as pain, cognitive dysfunction, hypertension, peptic ulcers, rhinitis, and asthma. Of the approximately 500 clinically marketed drugs, greater than 30% are modulators of GPCR function, representing approximately 9% of global pharmaceutical sales, making GPCRs the most successful of any target class in terms of drug discovery (1). These drugs exert their activity at approximately 30 well-characterized GPCRs. The completion of the human genome sequencing project has identified approximately 720 genes that belong to the GPCR superfamily (2, 3). Each of these genes is characterized by the possession of seven membrane-spanning domains, a putative extracellular ligand binding domain, and an intracellular domain responsible for interaction with G-proteins or other intracellular signaling proteins. Approximately half of these genes are thought to encode sensory receptors. Of the remaining 360 receptors, the natural ligand has been identified for approximately 210 receptors, leaving 150 so-called orphan GPCRs with no known ligand or function (Figure 1).

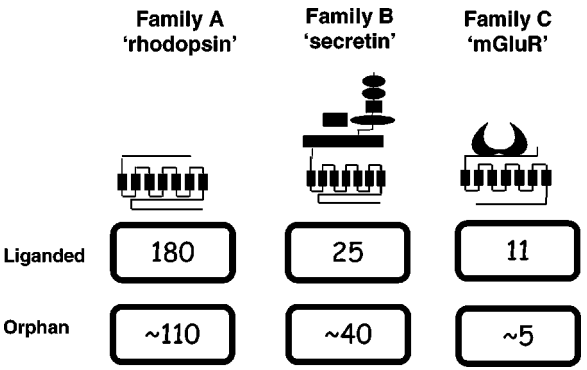

Figure 1 Schematic representation of the number and classification of liganded and orphan GPCRs.

Considerable effort has resulted in the identification of either natural or surrogate ligands for many orphan GPCRs (Table 1). These studies have revealed the existence of additional receptor subtypes for well-defined pharmacological ligands, such as the identification of the histamine H4 receptor (4). Further studies have identified the receptor targets for well-defined pharmacological ligands for which the cognate receptor was unknown. Examples of this include the identification of the GABA-B receptor (5), the calcitonin gene related peptide receptor (6), the amylin receptor (7), and the nicotinic acid receptor (8, 9). However, perhaps the most exciting studies are those that have resulted in the identification of novel GPCR ligands followed by the identification of the receptors for those ligands. Examples of this include the identification of a family of trace amine receptors (10), the identification of the prokineticin receptors (11–13), and the identification of two receptors for Neuromedin U (14, 15). In these examples, both novel ligands and novel receptors have been characterized, resulting in the identification of novel receptor signaling systems and providing new insights into cell biology and disease processes. The characterization of the physiological role of these receptors now represents a major challenge.

STRATEGY FOR THE IDENTIFICATION OF LIGANDS FOR ORPHAN GPCRs

A number of authors have reviewed the general strategy in use for the identification of ligands for orphan GPCRs (16–19). In such studies, the GPCR is first expressed in a recombinant assay system. Following expression, candidate ligands are screened against the receptor to identify molecules capable of specific regulation of that receptor. Such ligands can include tissue extracts, expressed or purified proteins or small peptides, natural and synthetic small molecules, and lipids. Once

TABLE 1 Ligand-orphan 7TMR pairings identified using reverse pharmacology strategy (modified from Reference 22)

Orphan receptor	Assay used	Ligand	Ligand source	Ligand properties	Year found	Reference
ORL-1	cAMP	Nociceptin/ Orphanin FQ	Brain extract	Novel	1995	(31)
Orexin-1 and -2	$[Ca^{2+}]_I$	Orexin-A and -B	Brain extract	Novel	1998	(69, 70)
GPR10	Arachidonic acid	Prolactin-releasing peptide	Brain extract	Novel	1998	(33)
APJ	ext. pH	Apelin	Stomach extract	Novel	1998	(71)
GHS-R	$[Ca^{2+}]_I$	Ghrelin	Stomach extract	Novel	1999	(72)
GPR14	$[Ca^{2+}]_I$	Urotensin II	Brain extract, synthetic ligand	Novel	1999	(34, 73)
KIAA0001	Yeast	UDP-glucose	Synthetic ligand	Novel	2000	(47)
AXOR12 (GPR54)	$[Ca^{2+}]_I$	KiSS-1	Synthetic ligand, placental extract	Novel	2001	(74)
G2A	$[Ca^{2+}]_I$	Lysophosph-atidylcholine	Synthetic ligand	Novel	2001	(41)
GPR7 and 8	cAMP	Neuropeptides B and W	Brain extract, synthetic ligand	Novel	2002–2003	(75–77)
GPR40	$[Ca^{2+}]_I$	Medium-long-chain fatty acids	Synthetic ligand	Novel	2003	(42–44)
GPR41 and 43	Yeast	Short-chain fatty acids	Synthetic ligand	Novel	2003	(45)
CIRL	Radioligand binding	Latrotoxin	Synthetic ligand	Surrogate	1997	(78)

(*Continued*)

TABLE 1 (*Continued*)

Orphan receptor	Assay used	Ligand	Ligand source	Ligand properties	Year found	Reference
BRS-3	Oocytes	Bombesin	Synthetic ligand	Surrogate	1993	(79)
CRLR	Oocytes	CGRP	Synthetic ligand	Known	1998	(6)
AZ3B	$[Ca^{2+}]_I$, Oocytes	C3a	Synthetic ligand	Known	1996	(80)
EDG1, 3, 5, 6, and 8	$[Ca^{2+}]_I$, cAMP	S1P	Synthetic ligand	Known	1998–2000	(48–52)
EDG2, 4, and 7	$[Ca^{2+}]_I$, cAMP	LPA	Synthetic ligand	Known	1998–2000	(53–55)
HG57 (Cys-LT1R)	$[Ca^{2+}]_I$	LTD4	Synthetic ligand	Known	1999	(81)
GPR38	$[Ca^{2+}]_I$	Motilin	Synthetic ligand	Known	1999	(82)
SLC-1 (MCH1)	$[Ca^{2+}]_I$	Melanin-concentrating hormone	Synthetic ligand, brain extract	Known	1999	(35, 83)
OGR-1	$[Ca^{2+}]_I$	Sphingosyl-phosphorylcholine	Synthetic ligand	Known	2000	(40)
PSECO146 (CysLT2R)	$[Ca^{2+}]_i$, Oocytes	LTC4 and D4	Synthetic ligand	Known	2000	(84, 85)
GPR16 (BLT2)	cAMP, radioligand binding	LTB4	Synthetic ligand	Known	2000	(32)
FM-3/4	$[Ca^{2+}]_I$	Neuromedin U	Synthetic ligand	Known	2000	(14, 15, 86, 87)
GPRv53 (H4-R)	cAMP, radioligand binding	Histamine	Synthetic ligand	Known	2000	(39, 88–91)

HLWAR77	$[Ca^{2+}]_I$	Neuropeptides FF and AF	Synthetic ligand	Known	2000	(92)
P2Y12	Oocytes	ADP	Synthetic ligand	Known	2001	(93)
CRTH2	$[Ca^{2+}]_I$	Prostaglandin D2	Synthetic ligand	Known	2000	(94)
MCH2	$[Ca^{2+}]_I$	Melanin-concentrating hormone	Synthetic ligand	Known	2001	(95)
TDAG-8	cAMP	Psychosine	Synthetic ligand	Known	2001	(36)
TA_1, TA_2	Oocytes	Trace amines (tyramine)	Synthetic ligand	Known	2001	(10)
GPR4	$[Ca^{2+}]_I$	Sphingosyl-phosphorylcholine, lysophosphati-dylcholine	Synthetic ligand	Known	2001	(37)
TG1019	TG1019-$G_{i1}\alpha$ fusion, GTPγS binding	Eicosanoids, unsaturated fatty acids	Synthetic ligand	Known	2002	(96, 97)
LGR7 and 8	cAMP	Relaxin	Synthetic ligand	Known	2002	(98)
BG37/TGR5	cAMP	Bile acids	Synthetic ligand	Known	2002–2003	(99, 100)
HM74A	GTPγS binding	Nicotinic acid	Synthetic ligand	Known	2003	(8, 9, 101)
C5L2 (GPR77)	Radioligand binding	C5a, C5a des Arg, C3a des Arg (acylation-stimulating protein)	Synthetic ligand	Known	2002–2003	(66, 67)

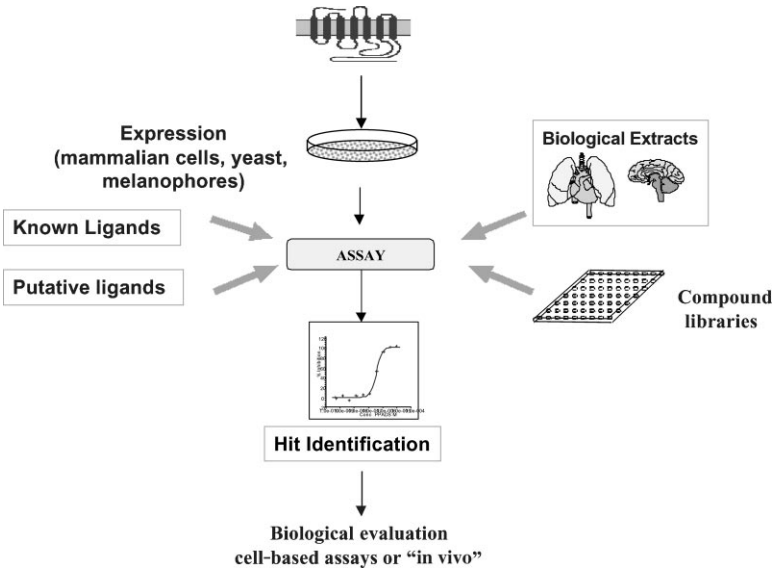

Figure 2 Strategy for the identification of ligands at orphan GPCRs. Orphan GPCRs are expressed in a recombinant expression system, such as mammalian cells, yeast, or *Xenopus* melanophores. Following expression, it is usual to generate an assay amenable to the screening of candidate ligands in 96 well or 384 well microtitre plate formats. Candidate ligands, including small molecules, peptides, proteins, lipids, or tissue extracts, are screened in the assay. The identification of an activating ligand is detected according to the activation of an intracellular signaling cascade (see text for details). An activating ligand, often termed a hit molecule, will be identified according to its ability to cause a concentration-dependent increase in the activity of a signaling cascade. Once identified, the ligand may be further characterized against other GPCRs to determine its activity and selecticity profile prior to being used in cell-based, tissue, and in some cases whole-animal experiments in order to study the physiological role of the newly liganded receptor.

a candidate ligand is identified, further studies are performed to confirm that the activity of the ligand is specific to the receptor (Figure 2).

RECOMBINANT EXPRESSION OF ORPHAN GPCRs

The first stage of any orphan GPCR screening experiment is to express the cloned receptor in a recombinant expression system that provides the necessary trafficking and G-protein signaling machinery to enable the successful identification of an activating ligand. A number of expression/assay systems have been adopted for orphan GPCR screening, including the fission yeast *Saccharomyces cerevisiae* (20),

immortalized mammalian cell lines, and *Xenopus* melanophores (21). Irrespective of the expression system, the success of a ligand screening experiment is entirely dependent upon the receptor being expressed at the cell surface and being able to couple to the signal transduction machinery of that cell to generate a readily detectable signal in the assay. A number of approaches have been adopted to demonstrate successful expression at both the RNA and protein level (22). Although the demonstration of the presence of mRNA in the cell is indicative of receptor expression, it does not always follow that receptor protein is expressed or is indeed at the cell surface. The generation of an antibody to the receptor facilitates the demonstration of the presence and site of receptor expression through the use of studies such as fluorescence activated cell sorting (FACS) or immunocytochemistry. However, the generation of GPCR-specific antibodies is a time-consuming procedure. Hence, to avoid this, many groups have generated receptors containing short epitope tags, such as FLAG, HA, or myc, at their N terminus, and used the corresponding antibodies to demonstrate receptor expression. An alternative approach has been to generate fusion proteins with the intrinsically fluorescent *Aequorea victorea* green fluorescent protein (GFP) and again use techniques such as FACS or confocal microscopy to confirm receptor expression (23). In such studies, GFP is typically fused directly to the C-terminal intracellular tail of the GPCR. In recent years, numerous examples of such fusion proteins have been generated to study the process of receptor trafficking and internalization. It is perhaps surprising that the generation of such a fusion protein has little or no effect upon the processes of receptor trafficking, receptor pharmacology, and receptor internalization and desensitization (23).

ORPHAN GPCR SCREENING ASSAYS

GPCRs couple to heterotrimeric G-proteins to regulate a variety of signal transduction events (22). There are 16 mammalian G-protein alpha subunits that fall into three broad families based on the primary signaling cascade regulated: The stimulatory G-proteins $G\alpha_s$ couple to adenylyl cyclase to cause an increase in the level of intracellular cAMP. In contrast, the five members of the $G\alpha_{i/o}$ family inhibit adenylyl cyclase to cause a decrease in the level of intracellular cAMP, and the $G\alpha_{q/11}$ family members activate phospholipase Cβ to cause an increase in intracellular calcium levels (24). Prior to ligand identification, it is difficult to predict the likely G-protein coupling of the receptor; hence, assay design for ligand screening has relied upon one or both of two strategies: First, the use of the phenomena of constitutive activity to predict the likely signaling mechanism of the receptor (25) and second, the use of promiscuous or chimeric G-protein coupling systems designed to facilitate receptor coupling (26). When expressed in recombinant systems, many GPCRs exhibit constitutive activity, a phenomena that can be defined as the activation of a signal transduction cascade in the absence of ligand. Constitutive signaling is typically demonstrated through transient

expression studies in which the transfection of increasing amounts of DNA correlates with an increase in the activity of a known GPCR signaling cascade. The phenomena of constitutive signaling has been used to predict receptor coupling in yeast, mammalian cells, and *Xenopus* melanophores [(25); S.J. Dowell & A.J. Brown, personal communication)]. In parallel, the promiscuous G-protein alpha subunits $G\alpha_{15}$ and $G\alpha_{16}$ have been used to establish calcium flux assays in recombinant mammalian cell lines based upon their demonstrated ability to couple to many, if not all, GPCRs (27). In an extension of these studies, many groups have generated so-called chimeric G-proteins to direct receptor activation through a specific signaling cascade. Such studies typically involve the replacement of the C-terminal five/six amino acids of one G-protein with those of another. In the most successful application of this approach, chimeric G-proteins have been generated in which the C-terminal five amino acids of $G\alpha_q$ have been replaced with the C-terminal five or six amino acids of $G\alpha_i$ or $G\alpha_s$. Such G-proteins are capable of interacting with normally $G\alpha_i$ or $G\alpha_s$ coupled receptors to activate the $G\alpha_q$ signaling cascade (28). Similar chimeric G-proteins have been developed for the yeast assay system (29).

Perhaps the most successful method used to identify ligands for GPCRs has been through the detection of changes in intracellular calcium concentration using the Fluorescence Imaging Plate Reader (FLIPR^{TM}; Molecular Devices, Sunnyvale, CA). This instrument allows the simultaneous compound addition and signal detection in every well of a 96- or 384-well assay plate. In these assays, the GPCR is usually transiently transfected along with a cocktail of chimeric or promiscuous G-proteins to facilitate signaling (30). Other methods used to identify ligands at orphan GPCRs following expression in mammalian cells include detection of changes in intracellular cAMP levels (4, 31, 32), the measurement of arachidonic acid release (33, 34), and receptor translocation studies (35–37).

The presence of endogenous GPCRs, particularly in mammalian expression systems, can result in so-called background responses due to ligand activation of endogenously expressed receptors. For this reason, in all mammalian cell assays, candidate ligands have to be screened against the transfected cell line and the untransfected "host" cell line to confirm that the signaling event is a consequence of the transfection of the orphan GPCR. The fission yeast *S. cerevisiae* contains a single GPCR (38). Hence, engineered yeast strains in which this GPCR has been deleted represent a null background for GPCR screening. Assay systems have been developed using a range of chimeric and transplant G-proteins to facilitate receptor coupling in which GPCR signaling results in the activation of a β-galactosidase reporter gene (20, 29).

Melanophore cells derived from the neural crest of *Xenopus laevis* offer a highly sensitive system for the screening for ligands at orphan GPCRs (21). These cells contain the pigment melanin within intracellular organelles termed melanosomes. The aggregation or dispersion of these organelles within the cell can be controlled by GPCR signaling. The activation of phospholipase C or adenylyl cyclase causes pigment dispersion to cause the cells to darken in color. The inhibition of adenylyl

cyclase causes pigment aggregation, which causes the cells to lighten. This has lead to the establishment of an orphan receptor screening assay in which compound activity is determined according to the dispersion of this pigment in the cells. This assay is particularly attractive for orphan GPCR screening, as receptors expressed in melanophores efficiently couple to melanophore G-proteins to regulate pigment dispersion. Following transient transfection of the receptor, constitutive activity can usually be observed allowing the elucidation of receptor coupling prior to screening (25). However, melanophores express a range of endogenous GPCRs, including receptors for serotonin, prostinoids, adenosine, acetylcholine, and adrenaline (C. Jayawickreme, personal communication). This can complicate data analysis and can lead to extensive further studies to confirm that any signaling event is specific to the transfected receptor. This technology may have application for the study of orphan GPCRs for which functional expression in mammalian cells is difficult to achieve.

The phenomena of constitutive activity has been used by Arena Pharmaceuticals (San Diego, CA) to develop a screening technology termed constitutive active receptor technology (CART), which relies upon the generation of receptor mutations that display constitutive signaling (25a). When transfected into mammalian cell lines or *Xenopus* melanophores, such mutant receptors display constitutive activity. Candidate ligands may be screened to identify agonist ligands that increase signaling and antagonist ligands that cause a decrease in constitutive activity. The advantage of this approach is that the ability of the receptor to regulate a signal transducton cascade is known ahead of the start of the ligand screening experiment. However, the success of this methodology for the identification of ligands at orphan receptors remains to be proven.

THE SELECTION OF CANDIDATE LIGANDS FOR ORPHAN GPCR SCREENING

Many pharmaceutical companies have compiled sets of known and putative GPCR ligands for screening. These sets typically contain known 7-transmembrane receptor (7TM) small-molecule, lipid, peptide, and protein ligands, as well as candidate ligand-like peptides or proteins, either expressed in other organisms or predicted from bioinformatics. Many biotechnology and pharmaceutical companies have generated small-molecule compound sets of thousands to hundreds of thousands in size to screen for so-called surrogate small-molecule ligands capable of regulating the orphan receptor. Small-molecule surrogate ligands are often of low potency and display poor selectivity for the receptor under study and may require a medicinal chemistry program to optimize these parameters before they can be used to characterize the biology of the orphan GPCR. The third approach for the identification of novel ligands relies upon the screening of tissue extracts for biological activity. Following the demonstration of activity in an extract, it is necessary to purify the active component, a process that can be experimentally very challenging.

Although this approach has been successful, there are many technical challenges that have to be addressed to ensure a successful conclusion.

USE OF SEQUENCE HOMOLOGY TO PREDICT CANDIDATE LIGANDS

The rapid progression of bioinformatics to help identify and classify novel genes has generated a wealth of genomic information on both orphan GPCRs and their potential ligands. The ability to harness this information in such a way that it can be used to help predict candidate ligands for orphan GPCRs may have a significant impact on the future of orphan GPCR screening strategies. Initial examples of this approach involved comparing sequences of novel receptors with those of their liganded counterparts and then predicting cognate ligands based on sequence homologies. Such an approach was used successfully to identify the histamine H4 receptor following the deposition of an expressed sequence tag (designated Axor 35) bearing significant homology to a number of biogenic amine receptors, including the H3 receptor (39; Figure 3). A further recent success of this strategy is highlighted by the identification of a cluster of four orphan GPCRs as receptors for bioactive lipids following the initial demonstration that OGR-1 could act as a high-affinity receptor for the lipid sphingosylphosphorylcholine (40). The related orphans TDAG8 (36), G2A (41), and GPR4 (37) were subsequently reported

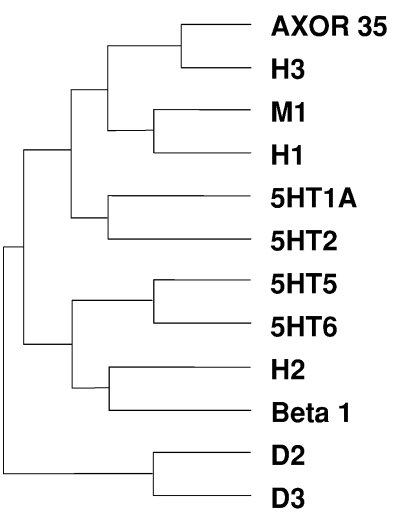

Figure 3 Nucleotide sequence homology of Family A biogenic amine GPCRs. The orphan receptor Axor 35 (39) shows the greatest nucleotide homology to the histamine H3 receptor. Axor 35 was thus characterized as the histamine H4 receptor.

as receptors for the lipids sphingosylphosphorylcholine, lysophasphatidylcholine, and psychosine within 18 months of the initial ligand:orphan GPCR pairing being published.

A further example of how the ligand binding properties of a cluster of related receptors can be unraveled following the identification of a "lead" ligand: GPCR pairing is highlighted by the recent reports of a family of receptors for fatty acids (42–45). The genes for four receptors, GPR40, -41, -42, and -43 are tandemly arranged on chromosome 19 (46). GPR40, the most divergent member of the cluster, was found to be activated by medium- and long-chain fatty acids (43, 44), whereas GPR41 and GPR43 were found to be activated by shorter-chain fatty acids, such as propionate (42, 45). GPR41 and GPR43 display 43% identity at the amino acid level and demonstrate differing specificity for length of carbon chain. However, pentanoate was found to be the most potent agonist at both receptors. The fourth family member, GPR42, differs from GPR41 by only six amino acids; however, it does not elicit responses to fatty acids following recombinant expression. This family member is likely to be a recent gene duplication of GPR41 and may be a pseudogene. These examples show how quickly receptors and their cognate ligands can be paired simply by using the nucleotide and protein sequence homology of orphan receptors to known liganded receptors to predict likely ligands; in effect, similar receptors are likely to have similar ligands.

An extension of the observation that homologous receptors can have similar ligands is the prediction of ligand type by phylogenetic classification of orphan GPCRs. This method has been used with considerable success. The orphan receptor FM3 (now known as NMU1R or GPR66) was investigated as a possible peptide receptor because it was found to group with other neuropeptide receptors, resulting in the identification of Neuromedin U as its ligand (15). The orphan receptor KIAA0001 (now known as GPR105 or P2Y14) was screened against a ligand collection, including nucleotide di- and triphosphate conjugates, owing to its classification with other nucleotide-liganded purinergic receptors, leading to the identification of UDP-glucose as the ligand (47). The identification of sphingosine-1-phosphate as the ligand for the EDG1 (48) receptor led to the prediction and subsequent confirmation that EDG3, EDG5, EDG6, and EDG8 have the same ligand (49–52). Phylogenetic classification of the EDG receptors also include EDG2, EDG4, and EDG7, which are receptors for the similar lipid lysophosphotidic acid (53–55).

Many orphan receptors fall readily into a phylogenetic classification with liganded receptors yet their ligands remain unidentified. This may be explained, in part, by the difficulties inherent in constructing a representative phylogeny for a functionally diverse group, such as GPCRs. Many different GPCR phylogenies exist, and the methods of their construction differ widely. Some authors remove the highly variable amino termini upstream of the first transmembrane (TM) domain and the carboxyl termini downstream of transmembrane domain seven (56), whereas some go further and remove the intracellular and extracellular loops also.

This simplifies construction of the sequence alignments upon which the phylogeny is based but also removes domains that may be involved in ligand recognition and signaling.

Many protein families have conserved sequence motifs that can be used to identify potential family members. A number of motifs representing GPCR subtypes or specific GPCRs exist in databases such as Interpro (http://www.ebi.ac.uk/interpro/). These are an aid to the classification of orphan receptors and as such, can offer a clue to the nature of the ligand. A method based on a statistical analysis of amino acid composition within functional subtypes was suggested as a rapid means to make a preliminary classification (57).

So-called virtual screening in silico methods for the identification of ligands for orphan receptors are hampered by the lack of three-dimensional (3-D) structures for orphan GPCRs, as the 3-D structure of the ligand is an important consideration when generating a 3-D model structure of the GPCR. However, in a recent paper, Bock & Gough (58) describe a method that estimates the free-binding energy between a ligand and GPCR without direct information about their 3-D structures. This method is suggested as a tool to identify a pool of likely ligand partners, rather than making a precise identification.

INFORMATION-BASED SCREENING TO IDENTIFY HM74A AS THE NICOTINIC ACID RECEPTOR

Ligand/receptor pairings can, in some cases, be predicted by a careful analysis of information, such as tissue distribution patterns of receptors or known ligands and knowledge of the sites of action of such ligands. For example, the expression pattern of an orphan GPCR, which was identified as a receptor for neuropeptide Y (NPY1R), perfectly matched that of the expression pattern of the ligand neuropeptide Y (59, 60). We have recently used such a similar "information-based" or targeted approach to identify receptors for nicotinic acid (8), part of the vitamin B_3 complex whose clinical use produces a desirable normalization of a range of cardiovascular risk factors, including a marked elevation of high-density lipoprotein and a reduction in mortality. The precise mechanism of action of nicotinic acid has remained unknown, although it was believed that activation of a $G\alpha_i$-linked G-protein coupled receptor could contribute to its clinical efficacy (61). Following a report that the pharmacological sites of action of nicotinic acid were localized largely to adipose and spleen tissue (62), we identified a shortlist of ten candidate orphan GPCRs that displayed relevant mRNA distribution patterns. These were then screened for responses to nicotinic acid in an assay for activation of $G\alpha_i$-G proteins. Using this strategy, HM74 was identified as a low-affinity receptor for nicotinic acid (Figure 4). Follow-up bioinformatics searches then identified two other receptors sharing varying degrees of homology with HM74, HM74A, and GPR81. HM74A was highly homologous, sharing

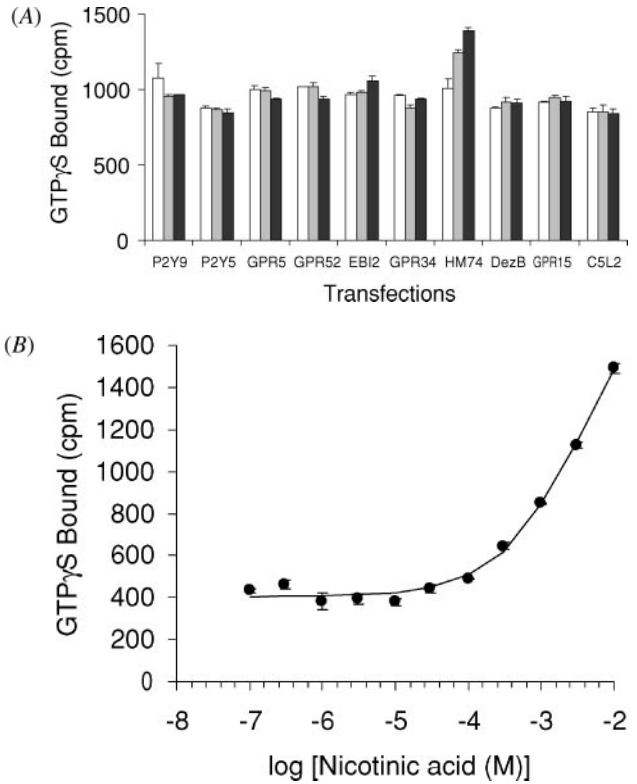

Figure 4 Characterization of HM74 as a G-protein coupled receptor that responds to nicotinic acid. (*A*) Application of 300 μM (*hashed columns*) and 1 mM (*filled columns*) nicotinic acid to membranes from HEK293/T cells expressing a variety of orphan G protein–coupled receptors was found to stimulate [^{35}S]-GTPγS binding in membranes from cells expressing HM74 (*open columns*, basal conditions). (*B*) Nicotinic acid stimulated a dose-dependent increase in [^{35}S]-GTPγS binding in cells expressing HM74 [modified from (8)].

96% identity with HM74 at the amino acid level. GPR81 displayed lower homology (57%). mRNA expression profiling of HM74 and HM74A showed that both exhibited similar distribution patterns, which were largely restricted to adipose tissue and spleen (Figure 5); that is, their expression patterns were commensurate with those of a nicotinic acid receptor. Interestingly, GPR81 appeared to be highly restricted to adipose tissue. HM74A was subsequently identified as a high-affinity receptor for nicotinic acid (Figure 6). Hence, this strategy demonstrates that it is possible to use both genomic and pharmacological information to help unmask novel "lead" ligand:orphan GPCR pairings. The beneficial cardiovascular

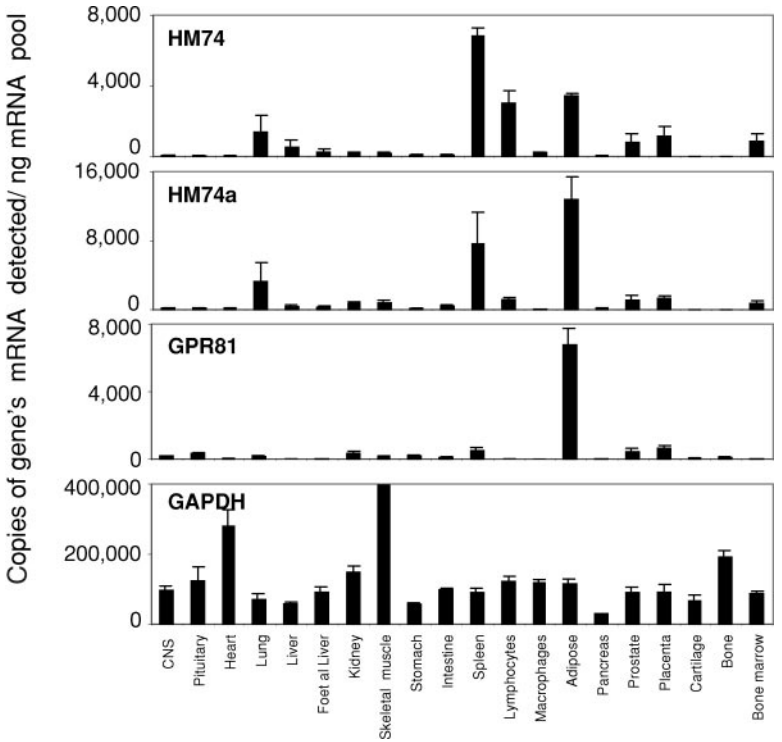

Figure 5 TaqMan quantitative reverse transcriptase-PCR analysis of mRNA levels in human tissues. The cDNA from the reverse transcription of 1 ng of poly $(A)^+$ RNA from multiple tissues for four different nondiseased individuals was subjected to TaqMan analysis to determine the tissue expression pattern of the HM74, HM74A, and GPR81 receptors. The expression pattern of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was assayed to control for the integrity of the cDNA [from (8)].

effects of nicotinic acid have been extensively described for over 40 years; therefore, the discovery of a high-affinity receptor may facilitate the discovery of superior drug molecules to treat dyslipidaemia. This example also shows how knowledge of the physiological role of the endogenous ligand can provide invaluable information regarding the potential role of a novel GPCR in a particular disease.

There are a number of other naturally occurring bioactive ligands whose sites of action have been well defined and that are thought to elicit their effects through binding to and activating cell surface GPCRs (Table 2). The G-protein coupling specificity of the unidentified receptors for some of these ligands have also been well defined using various second messenger assays and inhibitors of particular

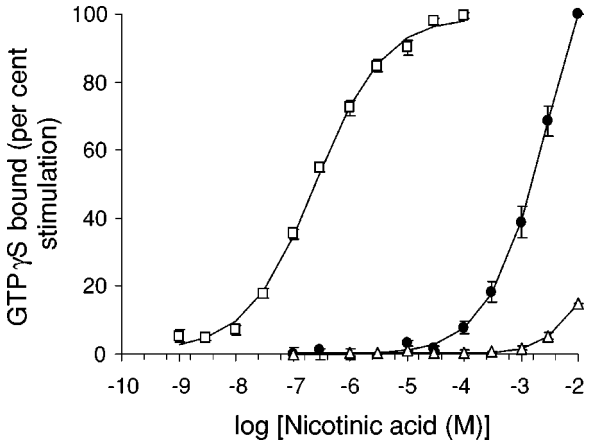

Figure 6 HM74A and HM74 are high- and low-affinity receptors for nicotinic acid. Stimulation by nicotinic acid of [^{35}S]-GTPγS binding in HM74- (*filled circles*), HM74A- (*squares*), and GPR81-expressing (*triangles*) membranes from HEK293T cells transiently coexpressing $G_{o1\alpha}$ and relevant receptor [from (8)].

signaling pathways. For example, a peptide neurotrophic factor, prosaposin, which has been shown to induce neural differentiation and protect against cell death, appears to activate pertussis toxin-sensitive $G\alpha_{i/o}$ proteins in neuroblastoma SH-SY-5Y cells (63). Given such information about the likely G-protein coupling specificity of a ligand-GPCR pairing, assays can then be employed that are tailored to measuring output from specific signaling pathways. It will be interesting to see whether a "knowledge-based" approach used to identify the nicotinic acid receptor can be employed to expedite identification of other novel ligand:orphan GPCR pairings.

TABLE 2 Examples of naturally occurring ligands that are likely to mediate their biological effects via GPCRs

Natural ligand	Sites of action	G protein–coupling profile	Reference
Kyotorphin	CNS	$G_{i/o}$	(102)
Nocistatin	Brain, spinal cord	$G_{i/o}$	(103)
Prosaposin	Brain, PC12 cells, Schwann cells, SHSY5Y cells	$G_{i/o}$	(63)
Chromostatin	Adrenal gland (chromaffin cells)	$G_{i/o}$	(104)
Pancreastatin	Heart, liver, adipose	$G_{q/11} > G_{i/o}$	(105, 106)

ORPHAN GPCRs AS ``LIGAND SINKS,'' ``LIGANDLESS'' RECEPTORS, AND NON-G-PROTEIN SIGNALING RECEPTORS

Although there are numerous examples of the identification of ligands for orphan GPCRs, there has been a decrease in the number of published orphan receptor/ligand pairings in the past two years. This suggests that traditional approaches for the identification of ligands for orphan GPCRs, largely based on the screening of putative GPCR ligands, will not be successful for the identification of ligands at the remaining 160 or so orphan GPCRs and alternative approaches are required. Indeed, this also raises the possibility that not all orphan GPCRs require a ligand, and some may play an alternative role in cell biology.

There is now extensive literature describing the phenomena of GPCR dimerization (63a). A number of techniques have been developed to demonstrate that GPCRs are capable of forming both heterodimers and homodimers. The functional relevence of this remains unclear and is the subject of intense speculation. It may be that GPCR heterodimers or homodimers are required for the processing or trafficking of the receptor. A well-characterized example of this is the GABA-B-R2 receptor, which appears to function as a trafficking protein to deliver the functional GABA-B-R1 receptor to the cell surface (5, 64, 65). It is possible that GPCR heterodimers may exhibit distinct pharmacologies. Although there is little published evidence for this, it remains possible that some orphan GPCRs may act as accessory proteins, which in combination with other orphan or liganded GPCRs, may modulate responses to known ligands or confer new ligand binding characteristics. This may be best addressed by taking a proteomics approach. Orphan GPCRs could be epitope tagged, transfected into cell lines, and immunoprecipitation experiments performed to isolate receptor complexes for study. Alternatively, receptor combinations could be studied in mammalian cell lines using techniques such as bioluminescence resonance energy transfer (BRET) or time-resolved fluorescence resonance energy transfer (TR-FRET) (65a). The challenge in such studies would be the prediction of which receptor pairs are likely to interact. The theoretical maximum number of possible combinations is 129,600!

Two recent reports have described the orphan GPCR C5L2, a receptor sharing homology with the anaphylatoxin C5a and C3a receptors, as having high-affinity binding sites for C5a and the desarginated forms of both C5a and C3a (C5adR[74] and C3adR[77], respectively) (66, 67). These findings were demonstrated using radioligand binding studies, and, interestingly, the authors reported that there was no functional correlate to this binding using standard G-protein–dependent second messenger pathways. Hence, C5L2 appears to function as a "ligand sink" whose physiological role as a novel anaphylatoxin binding protein has yet to be defined. An alternative explanation for this observation may be that anaphylatoxins are binding to C5L2 to activate non-G-protein-dependent signaling cascades. This raises the possibility of the existence of further examples of orphan GPCRs that either do not signal or use alternative non-G-protein-linked signaling

pathways to mediate the actions of their cognate ligands. In addition, it must be noted that the binding of these ligands to C5L2 would not have been identified using a conventional functional assay screening approach.

It has been assumed that all signaling events mediated by GPCRs occur as a consequence of G-protein activation. As a consequence, all orphan GPCR ligand screening experiments have relied upon the detection of the ability of candidate ligands to activate one of the classical heterotrimeric G-protein signaling pathways as described in this manuscript. There are now a number of examples of GPCRs that activate signal transduction pathways through G-protein-dependent and -independent signaling pathways. Perhaps the first example of this phenomena was the demonstration that in addition to classical G-protein-mediated signaling, the C-terminal tails of the β_2-adrenoceptor and the P2Y1 purinergic receptor interact with the Na^+/H^+ exchanger regulatory factor (NHERF) to directly regulate Na^+/H^+ exchange (67a). Further examples of G-protein-independent signaling include the coupling of metabotropic glutamate receptors directly to intracellular calcium stores through homer proteins and GPCR activation of phospholipase D as a consequence of activation of the small G-proteins Arf and RhoA (67b). This raises the possibility that there may be examples of the GPCR family that do not require G-proteins for signaling: C5L2 may be such an example; although, if this receptor does activate signal transduction, the mechanism remains to be defined (66, 67). A recent demonstration that this may be the case comes from the isolation of two receptors for the antiatherogenic adipokine adiponection, designated AdipoR1 and AdipoR2 (67c). These receptors, which are distantly related to the GPCR family, possess seven predicted TMs, and, in contrast to classical GPCRs, the N terminus of the receptor is intracellular and the C terminus of the receptor extracellular. These receptors, isolated by expression cloning, using a fluorescent-labeled adiponectin and FACS analysis, bind adiponectin with high affinity but do not regulate any of the classical GPCR signaling cascades. Rather, they regulate AMP kinase and PPARα activity, in a G-protein-independent manner, to cause fatty acid oxidation and glucose uptake (67c). This finding raises the possibility that some of the remaining orphan GPCRs also signal through G-protein-independent mechanisms. If so, novel screening approaches will have to be established in order to identify activating ligands. Indeed it may only be possible to identify receptors for ligands such as adiponectin through expression cloning strategies such as that used to identify this ligand. An alternative approach may be to take candidate ligands, add to these epitope tags, and then perform cross-linking experiments with cell types believed to respond to these ligands in order to isolate receptor/ligand complexes for protein sequencing.

It is possible that not all GPCRs are expressed at the cell surface; rather, they may be expressed on intracellular organelles and play a role in intracellular signaling. If so, classical ligand binding experiments that rely upon the activation of a receptor at the cell surface using exogenously applied ligand may not apply to these receptors. The intracellular localization of such receptors could be determined by the generation of fusion proteins with GFP (23). Receptors

expressed only on intracellular membranes would require alternative ligand screening strategies. Such studies would also demonstrate whether the receptor is processed to the cell surface or is retarded in the endoplasmic recticulum. Such receptors may require accessory proteins, perhaps other GPCRs as with the GABA-B receptor (65), to translocate to the cell surface. The identification of such accessory proteins would be required to generate a functional receptor at the cell surface prior to the initiation of a ligand screening experiment. It is possible that not all orphan GPCRs are functionally expressed, some may be pseudogenes, others may be expressed in very discrete tissues or for very short times, perhaps during development. If so such receptors may not possess ligands, or, if the ligands are also expressed in discrete tissues or for very short times, the ligand may not yet have been identified.

Finally, a number of receptors encoded by viruses, such as ORF74 encoded by Kaposi's sarcoma-associated herpesvirus, are highly constitutively active, appearing to function in the absence of added agonist (68). There may be other examples of orphan GPCRs that demonstrate similar "ligandless" mechanisms of action.

CONCLUDING REMARKS

In recent years, small-molecule, peptide, protein, and lipid ligands have been identified for many orphan GPCRs. However, approximately 160 orphan GPCRs remain for which there is no known ligand. Efforts continue to identify ligands for these receptors using the strategies outlined in this review. However, it is now necessary to investigate alternative strategies for the identification of ligands for these receptors. Such studies may involve the identification of new ligands using bioinformatics to predict likely peptide or protein ligands. It may be that the remaining orphan GPCRs are atypical in that they do not signal through heterotrimeric G-proteins. If this is the case, then the assays used to date, which all rely upon G-protein activation of a signal transduction cascade, cannot be used for these receptors. In support of this hypothesis, there is an emerging literature describing G-protein-independent signaling by GPCRs. The identification of ligands at such receptors depends on the development of new assays. It is also possible that not all of these receptors will have ligands; receptors have been identified that act as trafficking factors (5, 64, 65), are believed to act as ligand sinks (66, 67), and exhibit constitutive signaling in the absence of ligand (68). The challenge in the coming years is to identify the biological function of the remaining orphan GPCRs.

ACKNOWLEDGMENTS

The authors would like to express their thanks to the various members, past and present, of Systems Research, Screening Sciences, and Bioinformatics in GlaxoSmithKline, who have been involved in many of the successful orphan GPCR:ligand pairings outlined in this review.

The *Annual Review of Pharmacology and Toxicology* is online at http://pharmtox.annualreviews.org

LITERATURE CITED

1. Drews J. 2000. Drug discovery: a historical perspective. *Science* 287:1960–64
2. Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, et al. 2001. Initial sequencing and analysis of the human genome. *Nature* 409:860–921
3. Venter JC, Adams MD, Myers EW, Li PW, Mural RJ, et al. 2001. The sequence of the human genome. *Science* 291:1304–51
4. Oda T, Morikawa N, Saito Y, Masuho Y, Matsumoto S. 2000. Molecular characterization of a novel type of histamine receptor preferentially expressed in leukocytes. *J. Biol. Chem.* 275:36781–86
5. White JH, Wise A, Main MJ, Green A, Fraser NJ, et al. 1998. Heterodimerization is required for the formation of a functional GABAB receptor *Nature* 396:679–82
6. McLatchie LM, Fraser NJ, Main MJ, Wise A, Brown JD, et al. 1998. RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. *Nature* 393:333–39
7. Christopoulos G, Perry KJ, Morfis M, Tilakaratne N, Gao YY, et al. 1999. Multiple amylin receptors arise from receptor activity-modifying protein interaction with the calcitonin receptor gene product. *Mol. Pharmacol.* 56:235–42
8. Wise A, Foord SM, Fraser NJ, Barnes AA, Elshourbagy N, et al. 2003. Molecular identification of high and low affinity receptors for nicotinic acid. *J. Biol. Chem.* 278:9869–74
9. Tunaru S, Kero J, Schaub A, Wufka C, Blaukat A, et al. 2003. PUMA-G and HM74 are receptors for nicotinic acid and mediate its antilipolytic effect . *Nat. Med.* 9:352–55
10. Borowsky B, Adham N, Jones KA, Raddatz R, Artymyshyn R, et al. 2001. Trace amines: identification of a family of mammalian G protein-coupled receptors. *Proc. Natl. Acad. Sci. USA* 98:8966–71
11. Soga T, Matsumoto SI, Oda T, Saito T, Hiyama H, et al. 2002. Molecular cloning and characterization of prokineticin receptors. *Biochim. Biophys. Acta* 1579:173–79
12. Lin DCH, Bullock CM, Ehlert FJ, Chen JL, Tian H, Zhou QY. 2002. Identification and molecular characterization of two closely related G protein-coupled receptors activated by prokineticins/endocrine gland vascular endothelial growth factor. *J. Biol. Chem.* 277:19276–80
13. Masuda Y, Takatsu Y, Terao Y, Kumano S, Ishibashi Y, et al. 2002. Isolation and identification of EG-VEGF/prokineticins as cognate ligands for two orphan G-protein-coupled receptors. *Biochem. Biophys. Res. Commun.* 293:396–402
14. Howard AD, Wang RP, Pong SS, Mellin TN, Strack A, et al. 2000. Identification of receptors for neuromedin U and its role in feeding. *Nature* 406:70–74
15. Szekeres PG, Muir AI, Spinage LD, Miller JE, Butler SI, et al. 2000. Neuromedin U is a potent agonist at the orphan G protein-coupled receptor FM3. *J. Biol. Chem.* 275:20247–50
16. Civelli O, Nothacker HP, Saito Y, Wang ZW, Lin SHS, Reinscheid RK. 2001. Novel neurotransmitters as natural ligands of orphan G-protein-coupled receptors. *Trends Neurosci.* 24:230–37
17. Hinuma S, Onda H, Fujino M. 1999. The quest for novel bioactive peptides utilizing orphan seven-transmembrane-domain receptors. *J. Mol. Med.* 77:495–504
18. Stadel JM, Wilson S, Bergsma DJ. 1997. Orphan G protein-coupled receptors—a neglected opportunity for pioneer

drug discovery. *Trends Pharmacol. Sci.* 18:430–37
19. Wilson S, Bergsma DJ, Chambers JK, Muir AI, Fantom KG, et al. 1998. Orphan G-protein-coupled receptors: the next generation of drug targets? *B. J. Pharmacol.* 125:1387–92
20. Broach JR, Thorner J. 1996. High-throughput screening for drug discovery. *Nature* 384:14–16
21. Lerner MR. 1994. Tools for investigating functional interactions between ligands and G-protein coupled receptors. *Trends Neurosci.* 17:142–46
22. Wise A, Gearing K, Rees S. 2002. Target validation of G protein-coupled receptors. *Drug Discov. Today* 7:235–46
23. Kallal L, Benovic JL. 2000. Using green fluorescent proteins to study G-protein-coupled receptor localization and trafficking. *Trends Pharmacol. Sci.* 21:175–78
24. Hepler JR, Gilman AG. 1992. G proteins. *Trends Biochem. Sci.* 17:383–87
25. Chen G, Way J, Armour S, Watson C, Queen K, et al. 2000. Use of constitutive G protein-coupled receptor activity for drug discovery. *Mol. Pharmacol.* 57:125–34
25a. Chalmers DT, Behan DP. 2002. The use of constitutively active GPCRs in drug discovery and functional genomics. *Nature Rev. Drug Disc.* 1:599–608
26. Conklin BR, Farfel Z, Lustig KD, Julius D, Bourne HR. 1993. Substitution of three amino acids switches receptor specificity of Gq alpha to that of Gi alpha. *Nature* 363:274–76
27. Offermanns S, Simon MI. 1995. Ga15 and Gα16 couple a wide variety of receptors to phospholipase C. *J. Biol. Chem.* 270:15175–80
28. Conklin BR, Herzmark P, Ishida S, Voyno-Yasenetskaya TA, Sun Y, et al. 1996. Carboxyl-terminal mutation of Gqα and Gsα that alter the fidelity of receptor activation. *Mol. Pharmacol.* 50:885–90
29. Klein C, Paul JI, Sauve K, Schmidt MM, Arcangeli L, et al. 1998. Identification of surrogate agonists for the human FPRL-1 receptor by autocrine selection in yeast. *Nat. Biotechnol.* 16:1334–37
30. Szekeres PG. 2002. Functional assays for identifying ligands at orphan G protein-coupled receptors. *Recept. Channels* 8:297–308
31. Meunier JC, Mollereau C, Toll L, Suaudeau C, Moisand C, et al. 1995. Isolation and structure of the endogenous agonist of opioid receptor-like ORL1 receptor. *Nature* 377:532–35
32. Kamohara M, Takasaki J, Matsumoto M, Saito T, Ohishi T, et al. 2000. Molecular cloning and characterization of another leukotriene B4 receptor. *J. Biol. Chem.* 275:27000–4
33. Hinuma S, Habata Y, Fujii R, Kawamata Y, Hosoya N, et al. 1998. A prolactin-releasing peptide in the brain. *Nature* 393:272–76
34. Mori M, Sugo T, Abe M, Shimomura Y, Kurihara M, et al. 1999. Urotensin II is the endogenous ligand of a G-protein-coupled orphan receptor, SENR (GPR14). *Biochem. Biophys. Res. Commun.* 265:123–29
35. Lembo PMC, Grazzini E, Cao J, Hubatsch DA, Pelletier M, et al. 1999. The receptor for the orexigenic peptide melanin-concentrating hormone is a G-protein-coupled receptor. *Nat. Cell Biol.* 1:267–71
36. Im DS, Heise CE, Nguyen T, O'Dowd BF, Lynch KR. 2001. Identification of a molecular target of psychosine and its role in globoid cell formation. *J. Cell Biol.* 153:429–34
37. Zhu K, Baudhuin LM, Hong G, Williams FS, Cristina KL, et al. 2001. Sphingosylphosphorylcholine and lysophosphatidylcholine are ligands for the G protein-coupled receptor GPR4. *J. Biol. Chem.* 276:41325–35
38. Pausch MH. 1997. G-protein-coupled receptors in *Saccharomyces cerevisiae*: high-throughput screening assays for drug discovery. *Trends Biotechnol.* 15:487–94

39. Zhu Y, Michalovich D, Wu HL, Tan KB, Dytko GM, et al. 2001. Cloning, expression, and pharmacological characterization of a novel human histamine receptor. *Mol. Pharmacol.* 59:434–41
40. Xu Y, Zhu K, Hong GY, Wu WH, Baudhuin LM, et al. 2000. Sphingosylphosphorylcholine is a ligand for ovarian cancer G protein-coupled receptor 1. *Nat. Cell Biol.* 2:261–67
41. Kabarowski JHS, Zhu K, Le LQ, Witte ON, Xu Y. 2001. Lysophosphatidylcholine as a ligand for the immunoregulatory receptor G2A. *Science* 293:702–5
42. Kotarsky K, Nilsson NE, Flodgren E, Owman C, Olde B. 2003. A human cell surface receptor activated by free fatty acids and thiazolidinedione drugs. *Biochem. Biophys. Res. Commun.* 301:406–10
43. Itoh Y, Kawamata Y, Harada M, Kobayashi M, Fujii R, et al. 2003. Free fatty acids regulate insulin secretion from pancreatic β cells through GPR40. *Nature* 422:173–76
44. Briscoe CP, Tadayyon M, Andrews JL, Benson WG, Chambers JK, et al. 2003. The orphan G protein-coupled receptor GPR40 is activated by medium and long-chain fatty acids. *J. Biol. Chem.* 278: 11303–11
45. Brown AJ, Goldsworthy SM, Barnes AA, Eilert M, Tcheang L, et al. 2003. The orphan G protein-coupled receptors GPR41 and 43 are activated by propionate and other short-chain carboxylic acids. *J. Biol. Chem.* 278:11312–19
46. Sawzdargo M, George SR, Nguyen T, Xu SJ, Kolakowski LF, Odowd BF. 1997. A cluster of four novel human G protein-coupled receptor genes occurring in close proximity to CD22 gene on chromosome 19Q13.1. *Biochem. Biophys. Res. Commun.* 239:543–47
47. Chambers JK, Macdonald LE, Sarau HM, Ames RS, Freeman K, et al. 2000. A G protein-coupled receptor for UDP-glucose. *J. Biol. Chem.* 275:10767–71
48. Lee MJ, Van Brocklyn JR, Thangada S, Liu CH, Hand AR, et al. 1998. Sphingosine-1-phosphate as a ligand for the G protein-coupled receptor EDG-1. *Science* 279:1552–55
49. An S, Bleu T, Huang W, Hallmark OG, Coughlin SR, Goetzl EJ. 1997. Identification of cDNAs encoding two G protein-coupled receptors for lysosphingolipids. *FEBS Lett.* 417:279–82
50. Van Brocklyn JR, Tu ZX, Edsall LC, Schmidt RR, Spiegel S. 1999. Sphingosine 1-phosphate-induced cell rounding and neurite retraction are mediated by the G protein-coupled receptor H218. *J. Biol. Chem.* 274:4626–32
51. Yamazaki Y, Kon J, Sato K, Tomura H, Sato M, et al. 2000. Edg-6 as a putative sphingosine 1-phosphate receptor coupling to Ca(2+) signaling pathway. *Biochem. Biophys. Res. Commun.* 268: 583–89
52. Im DS, Heise CE, Ancellin N, O'Dowd BF, Shei GJ, et al. 2000. Identification of a novel sphingosine 1-phosphate receptor, Edg-8. *J. Biol. Chem.* 275:14281–86
53. Erickson JP, Wu JJ, Goddard JG, Tigyi G, Kawanishi K, et al. 1998. Edg-2/Vzg-1 couples to the yeast pheromone response pathway selectively in response to lysophosphatidic acid. *J. Biol. Chem.* 273:1506–10
54. An S, Bleu T, Hallmark OG, Goetzl EJ. 1998. Characterization of a novel subtype of human G protein-coupled receptor for lysophosphatidic acid. *J. Biol. Chem.* 273:7906–10
55. Im DS, Heise CE, Harding MA, George SR, O'Dowd BF, et al. 2000. Molecular cloning and characterization of a lysophosphatidic acid receptor, Edg-7, expressed in prostate. *Mol. Pharmacol.* 57:753–59
56. Joost P, Methner A. 2002. Phylogenetic analysis of 277 human G-protein-coupled receptors as a tool for the prediction of orphan receptor ligands. *Genome Biol.* 3:1–16

57. Chou KC, Elrod DW. 2002. Bioinformatical analysis of G-protein-coupled receptors. *J. Proteome Res.* 1:429–33
58. Bock JR, Gough DA. 2002. A new method to estimate ligand-receptor energetics. *Mol. Cell Proteomics* 1:904–10
59. Eva C, Keinänen K, Monyer H, Seeburg P, Sprengel R. 1990. Molecular cloning of a novel G protein-coupled receptor that may belong to the neuropeptide receptor family. *FEBS Lett.* 271:81–84
60. Larhammar D, Blomqvist AG, Yee F, Jazin E, Yoo H, Wahlested C. 1992. Cloning and functional expression of a human neuropeptide Y/peptide YY receptor of the Y1 type. *J. Biol. Chem.* 267:10935–38
61. Green A, Milligan G, Dobias SB. 1992. Gi down-regulation as a mechanism for heterologous desensitization in adipocytes. *J. Biol. Chem.* 267:3223–29
62. Lorenzen A, Stannek C, Lang H, Andrianov V, Kalvinsh I, Schwabe U. 2001. Characterization of a G protein-coupled receptor for nicotinic acid. *Mol. Pharmacol.* 59:349–57
63. Hiraiwa M, Campana WM, Martin BM, O'Brien JS. 1997. Prosaposin receptor: evidence for a G-protein-associated receptor. *Biochem. Biophys. Res. Commun.* 240:415–18

63a. Devi LA. 2001. Heterodimerization of G-protein coupled receptors: pharmacology, signalling and trafficking. *Trends Pharmacol. Sci.* 22:532–37

64. Jones KA, Borowsky B, Tamm JA, Craig DA, Durkin MM, et al. 1998. GABAB receptors function as a heteromeric assembly of the subunits GABABR1 and GABABR2. *Nature* 396:674–79
65. Kaupmann K, Malitschek B, Schuler V, Heid J, Froest W, et al. 1998. GABAB-receptor subtypes assemble into functional heteromeric complexes. *Nature* 396:683–87

65a. McVey M, Ramsey D, Kellett E, Rees S, Wilson S, et al. 2001, Monitoring receptor oligomerization using time-resolved fluorescence resonance energy transfer and bioluminescence resonance energy transfer. *J. Biol. Chem.* 276:14092–99

66. Cain SA, Monk PN. 2002. The orphan receptor C5L2 has high affinity binding sites for complement fragments C5a and C5a desArg^{74}. *J. Biol. Chem.* 277:7165–69
67. Kalant D, Cain SA, Maslowska M, Sniderman AD, Cianflone K, Monk PN. 2003. The chemoattractant receptor-like protein, C5L2, binds C3a des Arg^{77}/Acylation stimulating protein. *J. Biol. Chem.* 278:11123–29

67a. Hall RA, Ostedgaard LS, Premont RT, Blitzer JT, Rahman N, et al. 1998. A C-terminal motif found in the β2-adrenoceptor, P2Y1 receptor and cystic fibrosis transmembrane conductance regulator determines binding to the Na+/H+ exchanger regulatory family of PDZ proteins. *Proc. Natl. Acad. Sci. USA* 95:8496–501

67b. Hur E-M, Kim K-T. 2001. G-protein coupled receptor signalling and cross-talk: achieving rapidity and specificity. *Cell Signal.* 14:397–405

67c. Yamauchi T, Kamon J, Ito Y, Tsuchida A, Yokomizo T, et al. 2003. Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. *Nature* 423:762–69

68. Smit MJ, Verzijl D, Casarosa P, Navis M, Timmerman H, Leurs R. 2002. Kaposi's sarcoma-associated herpesvirus-encoded G protein-coupled receptor ORF74 constitutively activates p44/p42 MAPK and Akt via G(i) and phospholipase C-dependent signaling pathways. *J. Virol.* 76:1744–52
69. Sakurai T, Amemiya A, Ishii M, Matsuzaki I, Chemelli R, et al. 1998. Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behaviour. *Cell* 92:573–85
70. de Lecea L, Kilduff TS, Peyron C, Gao XB, Foye PE, et al. 1998. The hypocretins: hypothalamic-specific peptides with

neuroexcitatory activity. *Proc. Natl. Acad. Sci. USA* 95:322–27
71. Tatemoto K, Hosoya M, Habata Y, Fujii R, Kakegawa T, et al. 1998. Isolation and characterization of a novel endogenous peptide ligand for the human APJ receptor. *Biochem. Biophys. Res. Commun.* 251:471–76
72. Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawaet K. 1999. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* 402:656–60
73. Ames RS, Sarau HM, Chambers JK, Willette RN, Alyar NV, et al. 1999. Human urotensin-II is a potent vasoconstrictor and agonist for the orphan receptor GPR14. *Nature* 401:282–86
74. Ohtaki T, Shintani Y, Honda S, Matsumoto H, Horiet A, et al. 2001. Metastasis suppressor gene KiSS-1 encodes peptide ligand of a G-protein-coupled receptor. *Nature* 411:613–17
75. Shimomura Y, Harada M, Goto M, Sugo T, Matsumoto Y, et al. 2002. Identification of neuropeptide W as the endogenous ligand for orphan G-protein-coupled receptors GPR7 and GPR8. *J. Biol. Chem.* 277:35826–32
76. Fujii R, Yoshida H, Fukusumi S, Habata Y, Hosoya M, et al. 2002. Identification of a neuropeptide modified with bromine as an endogenous ligand for GPR7. *J. Biol. Chem.* 277:34010–16
77. Brezillon S, Lannoy V, Franssen J-D, Le Poul E, Dupriez V, et al. 2003. Identification of natural ligands for the orphan G protein-coupled receptors GPR7 and GPR8. *J. Biol. Chem.* 278:776–83
78. Krasnoperov VG, Bittner MA, Beavis R, Kuang Y, Salnikow KV, et al. 1997. Alpha-latrotoxin stimulates exocytosis by the interaction with a neuronal G-protein-coupled receptor. *Neuron* 18:925–37
79. Fathi Z, Corjay MH, Shapira H, Wada E, Benya R, et al. 1993. BRS-3: a novel bombesin receptor subtype selectively expressed in testis and lung carcinoma cells. *J. Biol. Chem.* 268:5979–84
80. Ames RS, Li Y, Sarau HM, Nuthulaganti P, Foley JJ, et al. 1996. Molecular cloning and characterization of the human anaphylatoxin C3a receptor. *J. Biol. Chem.* 271:20231–34
81. Lynch KR, O'Neill GP, Liu Q, Im DS, Sawyer N, et al. 1999. Characterization of the human cysteinyl leukotriene CysLT1 receptor. *Nature* 399:789–93
82. Feighner SD, Tan CP, McKee KK, Palyha OC, Hreniuk DL, et al. 1999. Receptor for motilin identified in the human gastrointestinal system. *Science* 284:2184–88
83. Chambers J, Ames RS, Bergsma D, Muir AI, Fitzgerald LR, et al. 1999. Melanin-concentrating hormone is the cognate ligand for the orphan G protein-coupled receptor SLC-1. *Nature* 400:261–65
84. Heise CE, O'Dowd BF, Figueroa DJ, Sawyer N, Nguyen T, et al. 2000. Characterization of the human leukotriene 2 (CysLT2) receptor. *J. Biol. Chem.* 275: 30531–36
85. Nothacker HP, Wang Z, Zhu Y, Reinscheid RK, Lin SHS, Civelli O. 2000. Molecular cloning and characterization of a second cysteinyl leukotriene receptor: discovery of a subtype selective agonist. *Mol. Pharmacol.* 58:1601–8
86. Nothacker HP, Wang Z, McNeill AM, Saito Y, Merten S, et al. 1999. Identification of the natural ligand of an orphan G protein-coupled receptor involved in the regulation of vasoconstriction. *Nat. Cell Biol.* 1:383–85
87. Kojima M, Haruno R, Nakazato M, Date Y, Murakami N, et al. 2000. Purification and identification of neuromedin U as an endogenous ligand for an orphan receptor GPR66. *Biochem. Biophys. Res. Commun.* 276:435–38
88. Liu CL, Ma XJ, Jiang X, Wilson SJ, Hofstra CL, et al. 2001. Cloning and pharmacological characterization of a fourth histamine receptor (H(4)) expressed in

bone marrow. *Mol. Pharmacol.* 59:420–26
89. Morse KL, Behan J, Laz TM, West RE, Greenfeder SA, et al. 2001. Cloning and characterization of a novel human histamine receptor. *J. Pharmacol. Exp. Ther.* 296:1058–66
90. Nakamura T, Itadani H, Hidaka Y, Ohta M, Tanaka K. 2000. Molecular cloning and characterization of a new human histamine receptor, HH4R. *Biochem. Biophys. Res. Commun.* 279:615–20
91. Nguyen T, Shapiro DA, George SR, Setola V, Lee DK, et al. 2001. Discovery of a novel member of the histamine receptor family. *Mol. Pharmacol.* 59:427–33
92. Elshourbagy NA, Ames RS, Fitzgerald LR, Foley JJ, Chambers JK, et al. 2000. Receptor for the pain modulatory peptides NPFF and NPAF is an orphan G protein-coupled receptor. *J. Biol. Chem.* 275:25965–71
93. Hollopeter G, Jantzen HM, Vincent D, Li G, England L, et al. 2001. Identification of the platelet ADP receptor targeted by antithrombotic drugs. *Nature* 409: 202–7
94. Hirai H, Tanaka K, Yoshie O, Ogawa K, Kenmotsu K, et al. 2001. Prostaglandin D2 selectively induces chemotaxis in T Helper Type 2 cells, eosinophils and basophils via seven-transmembrane receptor CRTH2. *J. Exp. Med.* 193:255–61
95. Hill J, Duckworth M, Murdock P, Rennie G, Sabido-David C, et al. 2001. Molecular cloning and functional characterization of MCH2, a novel human MCH receptor. *J. Biol. Chem.* 276:20125–29
96. Hosoi T, Koguchi Y, Sugikawa E, Chikada A, Ogawa K, et al. 2002. Identification of a novel human eicosanoid receptor coupled to $G_{i/o}$. *J. Biol. Chem.* 277:31459–65
97. Jones CE, Holden S, Tenaillon L, Bhatia U, Seuwen K, et al. 2003. Expression and characterization of a 5-oxo-6E,8Z, 11Z,14Z-eicosatetraenoic acid receptor highly expressed on human eosinophils and neutrophils. *Mol. Pharmacol.* 63: 471–77
98. Hsu SY, Nakabayashi K, Nishi S, Kumagai J, Kudo M, et al. 2002. Activation of orphan receptors by the hormone relaxin. *Science* 295:671–74
99. Maruyama T, Miyamoto Y, Nakamura T, Tamai Y, Okada H, et al. 2002. Identification of membrane-type receptor for bile acids (M-BAR). *Biochem. Biophys. Res. Commun.* 298:714–19
100. Kawamata Y, Fujii R, Hosoya M, Harada M, Yoshida H, et al. 2003. A G protein-coupled receptor responsive to bile acids. *J. Biol. Chem.* 278:9435–40
101. Soga T, Kamohara M, Takasaki J, Matsumoto S, Saito T, et al. 2003. Molecular identification of nicotinic acid receptor. *Biochem. Biophys. Res. Commun.* 303:364–69
102. Ueda H, Inoue M. 2000. In vivo signal transduction of nociceptive response by kyotorphin (tyrosine-arginine) through G alpha (I)- and inositol trisphosphate-mediated Ca2+ influx. *Mol. Pharmacol.* 57:108–15
103. Zeilhofer HU, Muth-Selbach U, Guhring H, Erb K, Ahmadi S. 2000. Selective suppression of inhibitory synaptic transmission by nocistatin in the rat spinal cord dorsal horn. *J. Neurosci.* 20:4922–29
104. Galindo E, Mendez M, Calvo S, Gonzalez-Garcia C, Cena V, et al. 1992. Chromostatin receptors control calcium channel activity in adrenal chromaffin cells. *J. Biol. Chem.* 267:407–12
105. Gonzalez-Yanes C, Santos-Alvarez J, Sanchez-Margalet V. 2001. Pancreastatin, a chromogranin A-derived peptide, activates G alpha (16) and phospholipase C-beta(2) by interacting with specific receptors in rat heart membranes. *Cell Signal.* 13:43–49
106. Gonzalez-Yanes C, Santos-Alvarez J, Sanchez-Margalet V. 1999. Characterization of pancreastatin receptors and signaling in adipocyte membranes. *Biochim. Biophys. Acta* 1451:153–62