



## REVIEW ARTICLE

# Orphan G-protein-coupled receptors: the next generation of drug targets?

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The pharmaceutical industry has readily embraced genomics to provide it with new targets for drug discovery. Large scale DNA sequencing has allowed the identification of a plethora of DNA sequences distantly related to known G protein-coupled receptors (GPCRs), a superfamily of receptors that have a proven history of being excellent therapeutic targets. In most cases the extent of sequence homology is insufficient to assign these 'orphan' receptors to a particular receptor subfamily. Consequently, reverse molecular pharmacological and functional genomic strategies are being employed to identify the activating ligands of the cloned receptors. Briefly, the reverse molecular pharmacological methodology includes cloning and expression of orphan GPCRs in mammalian cells and screening these cells for a functional response to cognate or surrogate agonists present in biological extract preparations, peptide libraries, and complex compound collections. The functional genomics approach involves the use of 'humanized yeast cells, where the yeast GPCR transduction system is engineered to permit functional expression and coupling of human GPCRs to the endogenous signalling machinery. Both systems provide an excellent platform for identifying novel receptor ligands. Once activating ligands are identified they can be used as pharmacological tools to explore receptor function and relationship to disease.

**Keywords:** Orphan; novel receptor; 7TM receptor; ligand fishing; novel peptide

## Introduction

Rapid advances in DNA sequencing technologies have led to an exponential increase in the generation of genomic information. Such information holds enormous potential for drug discovery, allowing the identification of a diverse range of novel molecular targets. The challenge for the pharmaceutical industry in dealing with this wealth of new information is to identify the most promising candidates for further biological evaluation and to characterize their potential as drug targets as rapidly and efficiently as possible. This review will focus on one family of novel molecular targets, the family of orphan G-protein coupled receptors (GPCRs) and describe the strategies one can adopt to convert them into therapeutically relevant drug targets.

The superfamily of GPCRs is one of the largest families of genes yet identified. Over 800 members have been cloned to date from a wide range of species. The characteristic motif of this superfamily is the seven distinct hydrophobic regions, each 20–30 amino acids in length, generally regarded as the transmembrane domains of these integral membrane proteins. There is little conservation of amino acid sequence across the entire superfamily of receptors, but key sequence motifs can be found within phylogenetically related sub-families, and these motifs can be used to help classify new members.

Since the first cloning of GPCR cDNAs more than a decade ago, new genes have continued to emerge whose sequences place them firmly within the GPCR superfamily, but whose ligands remain to be identified. These 'orphan' receptors show low levels of homology with known GPCRs (typically less than 40%), too low to classify them with any confidence into a specific receptor subfamily. Many orphan receptors in fact show closer homology to each other than to known GPCRs,

suggesting that they may represent new sub-families of receptors with distinct, possibly novel, ligands. These sub-families are distributed throughout the GPCR superfamily tree, suggesting that they will have a diverse range of functions.

What is the rationale for investing resources in characterizing orphan GPCRs? Simply stated, GPCRs have a proven history of being excellent drug targets. Several hundred drugs launched in the last three decades are directed at known GPCRs. Table 1 shows a representative snapshot of some well-established drugs and their corresponding receptors. It is clear that the therapeutic focus of these drugs spans a wide range of disorders from cardiovascular to gastro-intestinal to CNS and others.

Another example of the significance of GPCRs to drug discovery involves the increasing number of diseases associated with receptor gene mutations (Table 2). Some of the early examples identified involved mutations in receptors which caused gross changes in receptor function, leading to fairly rare but severe inherited disorders (e.g., mutations in vasopressin V<sub>2</sub> receptors associated with X-linked nephrogenic diabetes insipidus); (Birnbaumer, 1995). More recently, mutations have been identified that cause little or no apparent change in receptor function which lead merely to an increased propensity for developing a multifactorial disease (e.g. polymorphisms in the  $\beta_3$ -adrenoceptor associated with increased risk of insulin resistance); (Strosberg, 1997). As genotyping of disease populations becomes more comprehensive it is likely that more GPCR mutations or polymorphisms will be associated with disease states and hence provide additional potential targets for drug intervention. Since knowledge of gene function is not a pre-requisite for carrying out genotyping studies, it is just as likely that such mutations will be found in orphan receptors, which may provide clues to orphan receptor function in physiological and pathophysiological situations.

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**Table 1** Drugs targeting GPCRs

GPCR	Generic	GPCR	Generic
Acetylcholine	Bethanechol	Leukotriene	Pranlukast
	Dicyclomine		Zafirlukast
Adrenoceptor	Ipratropium	Opioid	Buprenorphine
			Butorphanol
			Alfentanil
	Atenolol	Prostaglandin	Morphine
	Clonidine		Epoprostenol
	Propranolol		Misoprostol
Angiotensin II	Terazosin	Somatostatin	Octreotide
	Albuterol		
Dopamine	Carvedilol	Serotonin	Sumatriptan
	Losartan		Ritanserlin
	Eprosartan		Cisapride
	Metoclopramide		Trazodone
Histamine	Ropinirole		Clozapine
	Haloperidol		
	Dimenhydrinate		
	Terfenadine		
	Cimetidine		
	Ranitidine		

**Table 2** Diseases associated with GPCR mutations

Receptor	Disease
Rhodospin	Retinitis pigmentosa
Thyroid stimulating hormone	Hyperfunctioning thyroid adenomas
Lutenizing hormone	Precocious puberty
Vasopressin V <sub>2</sub>	X-linked nephrogenic diabetes
Calcium	Hyperparathyroidism, hypocalciuria, hypercalcemia
Parathyroid hormone	Short limbed dwarfism
$\beta_3$ -Adrenoceptor	Obesity, NIDDM
Growth hormone releasing hormone	Dwarfism
Adrenocorticotropin	Glucocorticoid deficiency
Glucagon	Diabetes, hypertension

Much current research effort within the pharmaceutical industry today continues to focus on GPCRs, as they are justifiably perceived as attractive therapeutic targets. At SmithKline Beecham we have identified over 100 human orphan GPCRs that are distributed throughout the GPCR evolutionary tree. Some of these receptors are selectively expressed in a range of therapeutically relevant tissues, and it thus seems reasonable to speculate that they constitute a source of therapeutic targets with similar potential for drug discovery as seen with known GPCRs.

### Reverse pharmacology approach

The overall strategy for characterizing orphan receptors has often been referred to as a 'reverse pharmacology' approach (Libert *et al.*, 1991) to distinguish it from more conventional drug discovery approaches. The conventional approach was historically initiated by the discovery of a biological activity for which the ligand responsible was identified and then used to characterize tissue pharmacology and physiological role. Subsequently, the ligand was used to clone its corresponding receptor for use as a drug target in high-throughput screening. The reverse approach starts with an orphan receptor of unknown function which is used as a 'hook' to fish out its ligand. The ligand is then used to explore the biological and

patho-physiological role of the receptor. High throughput drug screening is initiated in parallel to develop tool compounds to help determine the therapeutic value of both agonists and antagonists to the receptor.

### Screening strategy

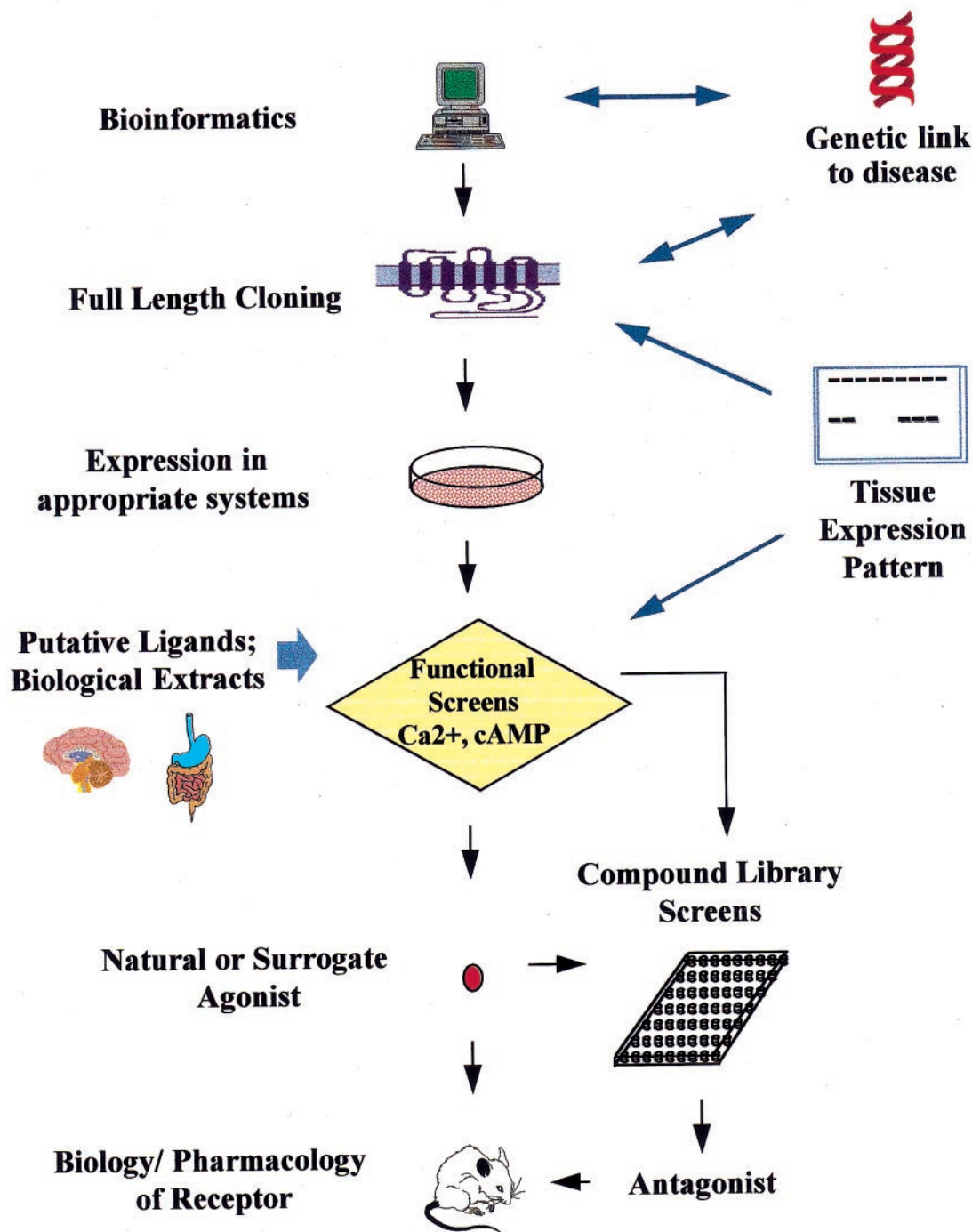
Figure 1 illustrates the reverse pharmacology strategy adopted within SmithKline Beecham. The majority of our orphan receptors have been identified through extensive bioinformatic analysis of expressed sequence tag (EST) databases generated by mass random sequencing of cDNA libraries. The EST approach has previously proven to be a highly productive route for identifying novel genes (Adams *et al.*, 1992).

Since cDNAs identified by EST sequencing are often incomplete, the tissue expression pattern of the EST is analysed *via* Northern blot or RT-PCR to identify the tissue cDNA libraries which should be used to obtain a full length clone. More significantly, the expression pattern can determine whether a receptor is expressed in a normal or diseased tissue of interest as a therapeutic target. A highly selective tissue expression profile can also provide a clue to receptor function. For example, the expression pattern of the orphan receptor FC5 in rat brain as determined by *in situ* hybridization was recognized as resembling that of the Y<sub>1</sub> NPY receptor visualized by ligand autoradiography and hence led to the identification of FC5 as the Y<sub>1</sub> receptor. (Eva *et al.*, 1990). In parallel with obtaining a full length cDNA for the receptor, corresponding genomic clones can be obtained and their sequences from different individuals analysed to look for genetic markers that can be used to investigate possible associations with disease states.

Once a full length cDNA is available it can be expressed in mammalian cell lines for functional analysis. The choice of expression system is crucial to the success of ligand fishing: a system with a good history of GPCR expression which also contains a wide repertoire of G-proteins to allow functional coupling to down-stream effectors is of key importance. CHO (Chinese Hamster Ovary) or HEK (Human Embryonic Kidney) 293 cell lines are often the cells of choice, but as the success of receptor expression and coupling cannot be predicted, a variety of systems may have to be used. Alternative expression systems which can be used to explore different coupling mechanisms include xenopus oocytes, melanophores (Lerner, 1994) and engineered yeast systems (Broach & Thorner, 1996).

In the absence of a ligand to confirm receptor expression it is important to obtain some evidence that receptor transfections have been successful before embarking on the search for a ligand. Northern blotting of cell lines is probably the easiest test, but will only confirm that message for the receptor is present. To be confident that receptor protein is actually expressed one has to generate antibodies to the receptor or else attach an epitope tag to the receptor and assess protein expression *via* FACS analysis or Western blotting. A number of epitope tags have been successfully used to label GPCRs without affecting receptor function. Examples include FLAG and HA tags, for which antibodies are commercially available. (Guan *et al.*, 1992; Koller *et al.*, 1997).

The choice of functional assays used to screen for activating ligands is also critical to the success of ligand fishing. These should be as generic as possible to allow detection of a wide range of coupling mechanisms. Measurement of metabolic activation of cells expressing the orphan *via* the Cytosensor microphysiometer (McConnell *et al.*, 1992) is probably the most generic assay available, but is



**Figure 1** Reverse pharmacology approach to characterizing orphan GPCRs (modified from Stadel *et al.*, 1997).

hampered by its low screening throughput. Alternative assay systems in mammalian cells focus largely on measuring changes in intracellular cyclic AMP or Ca<sup>2+</sup> levels, either directly using standard methods or *via* the use of reporter gene assays. It is becoming increasingly important to use high throughput systems to allow screening of large libraries of ligands, and assay technologies have evolved for each of these second messenger systems to allow high throughput readout in microtitre plate format. More recently, it has become possible to funnel heterologous GPCR signal transduction through a common pathway involving phospholipase C and Ca<sup>2+</sup> mobilization by co-expression of the receptor with the promiscuous G-proteins G<sub>z15/16</sub> or with

chimeric G<sub>q</sub>-proteins such as G<sub>qi5</sub> (Conklin *et al.*, 1996; Offermans & Simon, 1995). Although this approach may not work universally, the diversity of known GPCRs reported to successfully couple *via* these G-proteins suggests that it is a useful method to streamline screening for orphan receptor activation by focusing predominantly on one signal transduction system.

One factor which can complicate the use of heterologous expression systems for ligand fishing involves the presence of endogenous receptors in mammalian cell lines and in particular, clonal variation in the pattern of endogenous receptor expression in cells derived from the same parental cell line. Such variation has probably been responsible for the mis-

identification of a number of orphan receptors in the past. (eg, Cook *et al.*, 1992; Jazin *et al.*, 1993).

The ability to genetically delete endogenous GPCRs from yeast to generate a 'null' background is one of the major advantages in using yeast model systems for orphan receptor screening (Broach & Thorner, 1996). These systems rely on commandeering the endogenous yeast GPCR transduction system, the pheromone mating pathway, to allow coupling of transfected human GPCRs and humanized G-proteins to the endogenous signalling machinery. The manipulations involve conversion of the normal yeast response to pheromone activation (growth arrest) to positive growth on selective media, or to reporter gene expression. Such systems provide rapid, high throughput means of screening orphan receptors.

Once expression of the receptor has been achieved in mammalian or yeast systems and functional assays are in place, the search for activating ligands can begin. The receptors are screened initially against a bank of putative ligands, which includes known GPCR ligands as well as other naturally occurring bioactive molecules of unknown mechanism. The next step is to search for novel ligands by screening biological extracts obtained from tissues, biological fluids and cell supernatants. Another option is to screen peptide or compound libraries for 'surrogate' agonists that can be used as tool compounds. Yeast model systems here again provide unique advantages, in that they can be engineered to express and secrete small peptides from a random peptide library that will permit autocrine activation of heterologously expressed receptors, thus allowing a facile readout for detecting surrogate agonists of the receptor. Once an activating ligand has been found, whether natural or surrogate, it can then be used to explore the biology of the receptor *in vivo* or in tissue preparations, and can also be used to configure a high throughput screen to search for antagonists to support biological studies.

#### Characterization of orphan receptors

Examples are now emerging of the success of the reverse pharmacology approach in identifying orphan GPCRs. A first example is the identification of a calcitonin receptor-like orphan as a CGRP receptor. CGRP (calcitonin gene-related peptide) is a potent vasodilator widely distributed in central and peripheral neurones. It has also been implicated in migraine and non-insulin dependent diabetes. An EST derived from a human synovium library was used to clone a full length cDNA from human lung. Sequence motif analysis placed the receptor firmly within the secretin/VIP subfamily of receptors, with its closest homologue being the calcitonin receptor. However, the message for this novel receptor was expressed predominantly in lung, known to be a source of CGRP receptors. Following expression in HEK293 cells, the receptor was screened against putative ligands using a cyclic AMP stimulation assay (the primary signal transduction pathway for the secretin/VIP receptor family) and was shown to respond to CGRP with high potency ( $EC_{50}$  0.9 nM) (Aiyer *et al.*, 1996).

The pharmacological profile of the receptor determined in the cyclic AMP assay and in radioligand binding assays was subsequently shown to be similar to that observed with endogenous CGRP receptors in human neuroblastoma cells, indicating that the orphan receptor cDNA encoded a CGRP receptor. In contrast, other workers who cloned this receptor failed to detect a CGRP response following expression of the cDNA in COS cells (Fluhmann *et al.*, 1995). The explanation for this discrepancy was initially thought to be due to lack of

appropriate coupling machinery for the receptor in COS cells, as we also failed to detect a functional response with the receptor in this cell line. However, it has been shown more recently that accessory proteins appear to be necessary to allow this receptor to show CGRP responsiveness, so an alternative explanation may lie in differential expression of such accessory proteins in HEK293 vs COS cells (McLatchie *et al.*, 1998). Whatever the explanation, these findings illustrate that the choice of expression system is an important factor in identifying ligands for orphan receptors.

A second example of successful orphan receptor characterization was the identification of the receptor for the anaphylatoxin C3a (Ames *et al.*, 1996). The anaphylatoxins C3a, C4a and C5a are potent inflammatory mediators released during complement activation and they have been implicated in a number of inflammatory diseases. An EST derived from a human neutrophil cDNA library was used to clone an orphan GPCR from the same library. The receptor showed low homology to known GPCRs, with the best match being to the C5a receptor (37% nucleotide identity). However, Northern blot analysis indicated that its expression profile was distinct from that of the C5a receptor. Functional characterization of the C5a receptor was known to be problematic in cell lines such as HEK293 and CHO, but the receptor was known to be functionally active when expressed in RBL-2H3 cells (a rat basophilic cell line).

The orphan receptor was therefore expressed in RBL-2H3 cells and screened against putative ligands in a  $Ca^{2+}$  mobilization assay. It produced a robust  $Ca^{2+}$  mobilization response to C3a but not to C5a or other ligands. In parallel transfections in the same cell line the C5a receptor was shown to elicit a robust  $Ca^{2+}$  response to C5a but not to C3a. Radioligand binding studies using [ $^{125}I$ ]-C3a confirmed that the orphan receptor bound C3a with a  $K_D$  of 0.3 nM. These findings, together with the tissue distribution data, are consistent with this orphan encoding the human C3a receptor (Ames *et al.*, 1996).

CGRP and C3a constitute examples where known ligands have been paired with an orphan receptor. A third example involves the pairing of a novel ligand with an orphan receptor: the identification of a novel neuropeptide as an endogenous ligand for the receptor GPR10. The human orphan receptor GPR10 was first reported by Marchese *et al.* (1995), who cloned the receptor from genomic DNA using a PCR-based approach. Like the majority of orphan receptors, GPR10

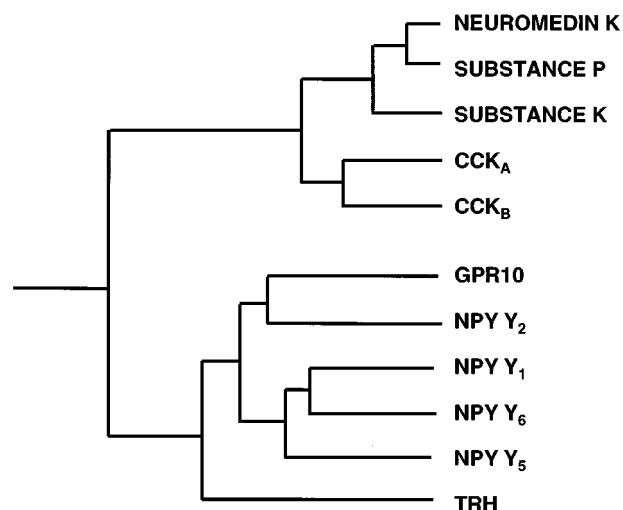
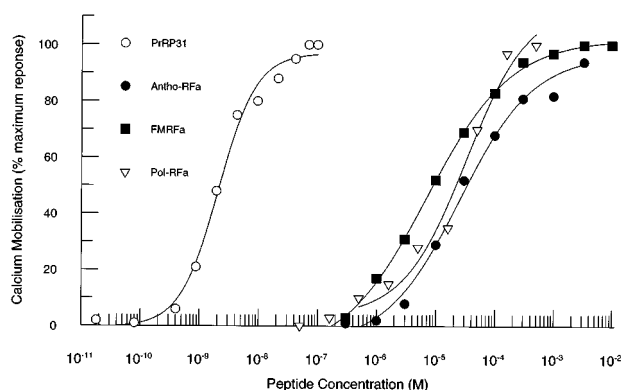


Figure 2 Phylogenetic tree for GPR10 and closely related receptors.

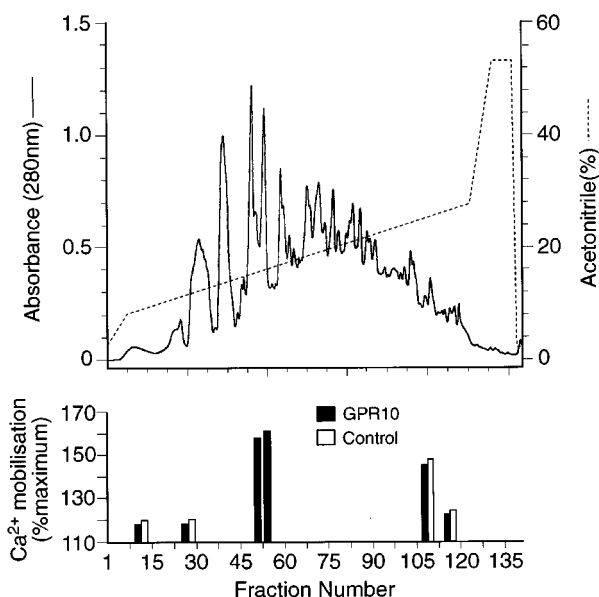


**Figure 3**  $\text{Ca}^{2+}$ -mobilization responses to -RFamide peptides in HEK 293 cells stably expressing GPR10.  $\text{EC}_{50}$  values: PrRP31: 2 nM; Antho-RFa: 24  $\mu\text{M}$ ; FMRFa: 45  $\mu\text{M}$ ; Pol-RFa: 40  $\mu\text{M}$ .

shows a low level of sequence homology with known GPCRs, although its closest match is to members of the NPY family of receptors (Figure 2). When expressed in HEK293 cells and screened against a bank of putative ligands, we found that the receptor failed to respond to NPY, but did produce robust  $\text{Ca}^{2+}$  mobilization responses to a number of short peptides found in invertebrates which are all members of the structurally related -RFamide peptide family (Figure 3). These peptides include FMRFamide, found in a variety of molluscan species, pQGRFamide (antho-RFamide) from *Anthopleura* and pQLLGGRFamide (pol-RFamide) from *Polyorchis* (Greenberg & Price, 1992).

The potency of these peptides at GPR10 was low ( $\text{EC}_{50}$  values in  $\mu\text{M}$  range—see Figure 3); moreover, the two known mammalian members of the -RFamide family, NPAF and NPFF, were inactive at GPR10. We hypothesized that the natural human ligand for GPR10 would be a novel member of the -RFamide family, perhaps a longer peptide which retained the characteristic -RFamide C-terminal motif. We therefore set out to purify the peptide from biological extracts of tissues.

High resolution reverse-phase HPLC fractions of a porcine hypothalamus extract were screened against GPR10 stably expressed in HEK293 cells and specific, robust  $\text{Ca}^{2+}$  mobilization responses were found in adjacent fractions (Figure 4). The activities appeared to be peptidic, as protease treatment of the fractions destroyed activity. In a parallel but unrelated effort, Hinuma *et al.* (1998) used very similar methods to also identify specific, peptidergic activity in hypothalamic extracts screened against GPR10 expressed in CHO cells, using arachidonic acid release as the functional assay. The activity was purified to homogeneity by further rounds of HPLC and ion exchange chromatography, and sequencing revealed a 31 amino acid peptide that, as predicted, contained a C-terminal -RFamide motif: SRAHQHS-MEIRTPDINPAWYAGRGRIPVG-RFamide. Hinuma *et al.* (1998) also found a second, overlapping peptide of 20 amino acids that appeared to be a truncated version of the longer peptide: TPDINPAWYAGRGRIPVG-RFamide. Cloning of the cDNA for the peptide revealed the existence of a pre-peptide containing an N-terminal secretory signal, and confirmed the sequence of the peptide (Hinuma *et al.*, 1998). The authors found that the longer peptide stimulated prolactin release from pituitary cells *in vitro*, and termed the peptides PrRP31 and PrRP20 respectively. PrRP31 shows high potency



**Figure 4** Stimulation of GPR10 by rp-hplc fractions of crude peptide extracts of hypothalamus. Top panel, chromatogram. Acetonitrile gradient and absorbance of eluted materials indicated by dotted and solid lines respective. Bottom panel,  $\text{Ca}^{2+}$  responses in HEK293 cells stably expressing GPR10 or an unrelated orphan receptor (control). Peak  $\text{Ca}^{2+}$  responses were normalized to the maximum response induced by muscarine in the same cells. Note that a number of fractions induced similar responses in both cell lines, presumably acting through an endogenous receptor in HEK293 cells.

at GPR10 ( $\text{EC}_{50}$  2 nM—see Figure 3), contrasting the low potency of the peptides from invertebrates, and suggesting that this peptide is the endogenous mammalian ligand for GPR10. The role of the peptide *in vivo* is under evaluation.

GPR10 is the most recent example of the identification of a novel ligand for an orphan receptor using the reverse pharmacology approach. A second example involved the discovery of the orexins (orexin-A and orexin-B), two related peptides derived from the same precursor by proteolytic processing. Both peptides were purified from brain extracts by screening for  $\text{Ca}^{2+}$  mobilization responses against an orphan receptor expressed in HEK293 cells (Sakurai *et al.*, 1998). Orexin-B showed significantly lower potency than orexin-A at the orphan receptor, termed  $\text{OX}_1$ , which subsequently led to the discovery of a second orexin receptor ( $\text{OX}_2$ ) at which both peptides were equi-potent. The orexin peptides are expressed in the hypothalamus and appear to be involved in the regulation of feeding (Sakurai *et al.*, 1998).

Novel non-peptide ligands have also been identified for orphan receptors, examples being anandamide and 2-arachidonyl glycerol as putative native ligands for cannabinoid receptors (Devane *et al.*, 1992; Stella *et al.*, 1997). As more orphan GPCRs are discovered and subjected to reverse pharmacology approaches, it seems likely that the number of novel ligands identified will increase. Moreover, as the biology of novel ligand/receptor pairs is evaluated and their association with disease states explored, we believe they will yield new drug targets that will allow us to treat a range of as yet unmet medical needs. As a consequence, orphan GPCRs may well provide the pharmaceutical industry with the next generation of drug targets (Stadel *et al.*, 1997).

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