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# *Deorphanization of G-Protein-Coupled Receptors*

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**Abstract.** G-protein-coupled receptors constitute one of the major families of drug targets. Orphan receptors, for which the ligands and function are still unknown, are an attractive set of future targets for presently unmet medical needs. Screening strategies have been developed over the years in order to identify the natural ligands of these receptors. Natural or chimeric G-proteins that can redirect the natural coupling of receptors toward intracellular calcium release are frequently used. Potential problems include poor expression or trafficking to the cell surface, constitutive activity of the receptors, or the presence of endogenous receptors in the cell types used for functional expression, leading to nonspecific responses. Many orphan receptors characterized over the last 10 years have been associated with previously known bioactive molecules. However, new and unpredicted biological mediators have also been purified from complex biological

sources. A few old and recent examples, including nociceptin, chemerin, and the F2L peptide are illustrated. Future challenges for the functional characterization of the remaining orphan receptors include the potential requirement of specific proteins necessary for quality control, trafficking or coupling of specific receptors, the possible formation of obligate heterodimers, and the possibility that some constitutively active receptors may lack ligands or respond only to inverse agonists. Adapted expression and screening strategies will be needed to deal with these issues.

## 1 G-Protein-Coupled Receptors

G-protein-coupled receptors (GPCRs) are the largest family among the membrane receptors. They play a major role in a variety of physiological and pathophysiological processes, such as carbohydrate metabolism, regulation of the cardiovascular system, nociception, feeding behavior, and immune responses. All GPCRs share a common structural organization with seven transmembrane segments, and a common way of modulating cell function by regulating effector systems through a family of heterotrimeric G-proteins (although G-protein-independent signaling has been reported as well). Not considering the olfactory and gustatory receptors, more than 350 G-protein-coupled receptor types and subtypes have been cloned to date in mammalian species. Among these, approximately 250 have been characterized functionally.

## 2 Orphan Receptors as Opportunities for Future Drug Targets

Following the cloning of rhodopsin (Nathans and Hogness 1984), β-adrenergic receptors (Dixon et al. 1986; Yarden et al. 1986), and the M1 muscarinic receptor (Kubo et al. 1986), as a result of protein purification and peptide sequencing approaches, the common transmembrane organization and structural relatedness of GPCRs rapidly became clear. As a consequence, polymerase chain reaction using degenerate primers (Libert et al. 1989) over the early 1990s led to the progressive

accumulation of a large number of orphan receptors, characterized by a typical GPCR structure, but of unknown function. Later on, the systematic sequencing of cDNA libraries (ESTs) and genomes has further expanded the list of orphan receptors. Due to their accessibility from the extracellular space and their key roles in modulating cell functions, G-protein-coupled receptors constitute the targets for about 40% of the active compounds presently used as therapeutic agents. The pharmaceutical industry is keen on the permanent input of new pharmacological targets in their drug development programs. As G-protein-coupled receptors will certainly remain a major avenue for drug design, characterization of orphan receptors are providing original and attractive targets for therapeutic agents and will likely lead to the development of novel drugs in the future (Ribeiro and Horuk 2005).

## 3 Expression and Screening Strategies

The identification of the ligands of orphan receptors, starting from purely genetic data, has been referred to as reverse pharmacology. This process is based on the use of specific and sensitive functional assays. As the signaling cascade activated by orphan receptors cannot be predicted for certain, a generic functional assay, independent of the activation of a specific cascade, is generally used. Several of these assays have been proposed and used in the past. Over the last few years, we have used essentially a high-throughput functional assay based on the luminescence emitted by recombinant aequorin following intracellular calcium release (Stables et al. 1997; Le Poul et al. 2002). In this system, a recombinant cell line is developed that coexpresses an orphan receptor, apoaequorin targeted to mitochondria, and $G_{\alpha 16}$ as a generic coupling protein (Fig. 1). Following preincubation of cells with coelenterazine to reconstitute active aequorin, luminescence is recorded in a luminometer following mixing with potential agonists. This assay has been validated with a number of characterized GPCRs and is now used routinely for orphan receptor screening.

Also widely used in the frame of orphan receptor characterization is the classical calcium mobilization assay using fluorescent dyes in a microplate format, following coexpression of the receptor, and $G_{\alpha 15}$, $G_{\alpha 16}$

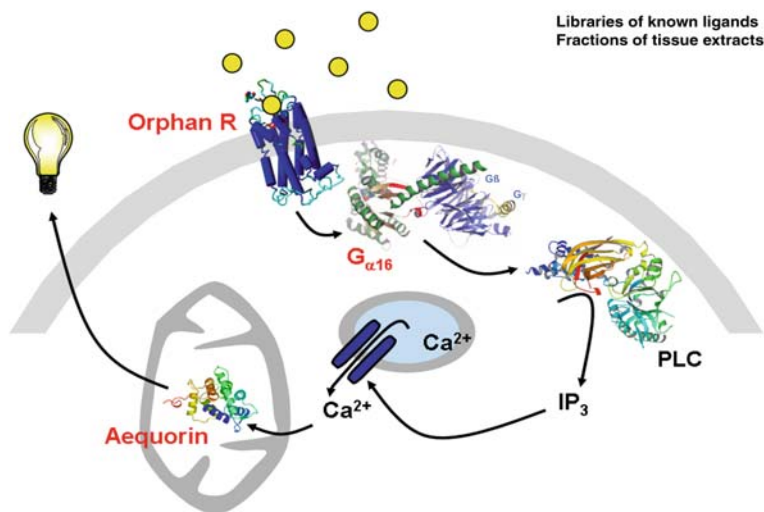


**Fig. 1.** Aequorin-based assay. The main components of the aequorin-based assay are represented schematically. Three proteins (in *red*) are coexpressed in a CHO-K1 cell line: the orphan receptor, $G_{\alpha 16}$ and apoaequorin. $G_{\alpha 16}$ allows the coupling of most GPCRs to the phospholipase C (PLC)-$IP_3$ pathway, irrespective of the natural pathways activated by the receptors. Aequorin is formed by the association of apoaequorin (targeted to mitochondria) and its cofactor coelenterazine. Following receptor activation, the release of $Ca^{2+}$ from intracellular stores results in the activation of aequorin, and the emission of photons recorded by a luminometer. This assay is adapted to 96- and 384-well microplate formats

or hybrid G-proteins (i.e., $G_{\alpha qi5}$) (Offermanns and Simon 1995; Conklin et al. 1993). However, in our hands this technique is less sensitive and less robust than the aequorin-based approach. Other generic techniques include the use of frog melanocytes (Lerner 1994), the internalization of receptor-GFP fusion proteins, or the translocation of a β-arrestin–GFP fusion. Alternatively, cascade-specific assays have been used as well for the deorphanization of specific receptors, including cAMP, GTPγS, and arachidonic acid measurements. These various approaches have been detailed elsewhere (Wise et al. 2004).

All these assays have their specific advantages and limitations, and not all receptors will provide a robust signal in each of them. Using the aequorin-based assay in CHO-K1 cells, we have identified a number of orphan receptors that express poorly in this system, as indicated by particularly low frequencies of clones displaying high transcript levels, the frequent rearrangement of the coding sequence generating the synthesis of nonfunctional receptors, or the low FACS signal obtained on cell lines when monoclonal antibodies are available. Such expression problems are frequently correlated with the demonstration that the receptor displays an apparent constitutive activity. Constitutive activity is usually detected following transient expression of the receptor, and the measurement of cAMP ($G_s$-coupled receptors), inositol phosphates ($G_q$-coupled receptors), or GTP$\gamma$S binding ($G_i$-coupled), which are recorded as significantly different from the basal levels of untransfected cells. The constitutive activity of the receptor therefore appears as a factor that counter-selects the cell lines expressing it at high levels. We have also determined that some receptors naturally coupled to $G_s$ do not couple efficiently to $G_{\alpha 16}$.

Potentially troublesome receptors may be expressed as fusions with a tag, which allow analyzing cell surface expression. As the tag itself may modify the expression or the binding of the receptor ligand, we find it useful to express an untagged receptor in parallel.

We also use an inducible expression vector, based on the tet-on technique. CHO-K1 cell lines coexpressing apoaequorin, $G_{\alpha 16}$, and the tet repressor have been established and validated with a number of model receptors. The selected cell line is being used for the expression of the orphan receptors for which constitutive activity and/or other expression problems have been encountered. For each receptor, the doxycycline concentration is adapted by measuring the constitutive activation of intracellular cascades, before screening.

## 4 Known Molecules and New Biological Mediators

Once established, the cell line expressing an orphan receptor is tested for its functional response to a set of potential ligands. These potential ligands can be well-known biological mediators, for which the pre-

cise binding site has not been characterized yet, collections of natural peptides, lipids, or metabolic intermediates with a poorly established role in signaling, or complex biological mixtures. Structural similarities with characterized receptors can of course focus the selection of potential ligands onto specific mediators or chemical classes of potential ligands. Over the years, a large number of orphan receptors have been matched with a well-characterized pharmacology. Other orphan receptors were identified as responding to known ligands, but were characterized either by a novel pharmacology or an original tissue distribution, leading to the multiplication of subtypes in some families, such as the serotonin and chemokine receptors. Finally, a set of orphan receptors were found to respond to previously unknown molecules, as the result of the isolation of these molecules from complex biological sources, on the basis of their biological activity on the recombinant receptor. The first example was the identification of a novel neuropeptide, nociceptin, as the natural agonist of an orphan receptor related to the opioid receptor (see below). Subsequently, orexins, prolactin-releasing peptide, apelin, melanin-concentrating hormone, ghrelin, motilin, urotensin II, prokineticins, kisspeptin/metastin, relaxin-3, and an RFamide peptide have been identified as the natural ligands of previously orphan receptors (Table 1). This is in our view the most attractive side of the orphan receptor field, since naturally processed forms of peptides and proteins, containing necessary tertiary structures and post-translational modifications can be discovered. It is likely that other novel molecules will be similarly discovered in the future, following the analysis of the remaining orphan receptors. With this aim in mind, we are presently expanding our extract preparation and purification schemes, primarily selected to retain peptides and small proteins, in order to focus on other classes of potential ligands, such as bioamines and other small molecules, medium- to large-sized proteins, and lipids. In addition, we also use human clinical samples that have allowed the identification of ligands for some orphan receptors (see below).

**Table 1** Natural ligands of human receptors identified through their purification from complex biological sources (tissue extracts of biological fluids)

| Receptor | Ligand | Year | Assay | References |
|---|---|---|---|---|
| ORL1 | Nociceptin | 1995 | cAMP | Meunier et al. 1995<br>Reinscheid et al. 1995 |
| HFGAN72 | Orexins | 1998 | $Ca^{2+}$ | Sakurai et al. 1998 |
| APJ | Apelin | 1998 | Micr. | Tatemoto et al. 1998 |
| GPR10 | PrRP | 1998 | AA | Hinuma et al. 1998 |
| GHSH | Ghrelin | 1999 | $Ca^{2+}$ | Kojima et al. 1999 |
| GPR14 | Urotensin II | 1999 | $Ca^{2+}$ | Mori et al. 1999 |
| GPR24 | MCH | 1999 | $Ca^{2+}$ | Saito et al. 1999 |
| GPR66 | Neuromedin U | 2000 | $Ca^{2+}$ | Kojima et al. 2000 |
| CCR5 | CCL14[9–74] | 2000 | $Ca^{2+}$ | Detheux et al. 1999 |
| GPR54 | Metastin/kisspeptins | 2001 | $Ca^{2+}$ | Ohtaki et al. 2001<br>Kotani et al. 2001 |
| GPR8 | NPW | 2002 | cAMP | Shimonura et al. 2002<br>Tanaka et al. 2003 |
| GPR7 | NPB | 2002 | cAMP | Fujii et al. 2002<br>Tanaka et al. 2003 |
| GPR73 | Prokineticin | 2003 | cAMP | Lin et al. 2002 |
| ChemR23 | Chemerin | 2003 | $Ca^{2+}$ | Wittamer et al. 2003 |
| GPCR135 | Relaxin-3/INSL7 | 2003 | GTP$\gamma$S | Liu et al. 2003a |
| GPCR142 | Relaxin-3/INSL7 | 2003 | GTP$\gamma$S | Liu et al. 2003b |
| GPR91 | Succinate | 2004 | $Ca^{2+}$ | He et al. 2004 |
| GPR154 | Neuropeptide S | 2004 | $Ca^{2+}$ | Xu et al. 2004 |
| FPRL2 | F2L | 2005 | $Ca^{2+}$ | Migeotte et al. 2005 |
| GPR103 | QRFP | 2006 | Luc. | Takayasu et al. 2006 |

The assay used for the follow-up of their purification is given, together with the year of the reporting in the literature. AA, arachidonic acid assay; Luc., luciferase reporter assay; Micr, microphysiometer

## 5 ORL1 and Nociceptin

Endogenous opioid peptides are widely distributed in the central and peripheral nervous systems and play important roles in modulating endocrine, cardiovascular, gastrointestinal, and immune functions. Pharmacological studies have defined three classes of opioid receptors

termed δ, κ, and μ, which differ in their affinity for various opioid ligands and their distribution in the nervous system (Reisine and Bell 1993). Following the cloning of the δ receptor, reported simultaneously by two groups (Kieffer et al. 1992; Evans et al. 1992), an orphan receptor was cloned by low stringency PCR, and named ORL1 (Mollereau et al. 1994). ORL1 was significantly related to the three classical opioid receptors and to a lesser extent to somatostatin receptors. In situ hybridization demonstrated a large distribution in the central nervous system, distinct from that of opiate receptors. The human recombinant receptor was expressed in CHO-K1 cells, and a large number of natural and synthetic ligands were tested for their potential interaction with ORL1 in binding and functional assays. None of the natural opiate peptides was active, but a functional response (inhibition of forskolin-induced cAMP accumulation) was obtained with high doses of the potent opiate agonist etorphin. The concentrations of etorphin required for the activation of ORL1 ($EC_{50}$ around 1 μM) were two to three orders of magnitude higher than what is necessary to achieve a similar effect on opiate receptors (Mollereau et al. 1994). These results demonstrated, however, that ORL1 was coupled, like opiate receptors, to the inhibition of adenylyl cyclase, and that the cell line expressing the orphan receptor could be used as a functional assay to detect the activity of agonists. The cell line expressing human ORL1 was therefore used as a bioassay to detect biological activities in extracts from rat brain. Following a gel filtration step, a fraction was found to be active, and the biological activity was purified to homogeneity by FPLC and HPLC. The active compound was characterized by mass spectrometry as a novel heptadecapeptide, FGGFTGARKSARKLANQ, sharing similarity with the endogenous opioid peptide dynorphin A (Meunier et al. 1995). The synthetic peptide exhibits nanomolar potency in inhibiting forskolin-induced accumulation of cAMP. When administered intra-cerebro-ventricularly in mice, the peptide was shown to induce hyperalgia in a hot plate assay, and was therefore termed nociceptin (Meunier et al. 1995). The same peptide was isolated independently by Reinscheid et al. (1995) and named orphanin FQ. The prepronociceptin (pPNOC) gene displays organizational and structural features that are very similar to those of the genes encoding the precursors to endogenous opioid peptides, enkephalins (pPENK), dynorphins/neo-endorphins (pP-

DYN), and β-endorphin (pPOMC), demonstrating its evolution from a common ancestor (Mollereau et al. 1996). Pronociceptin contains cleavage sites suggesting the generation of other potentially bioactive peptides. A C-terminal peptide of 28 amino acids, whose sequence is strictly conserved across murine and human species, was later described as nocistatin, displaying analgesic properties in vivo (Okuda-Ashitaka et al. 1998).

Nociceptin has since been described to display a range of activities, as a consequence of the broad distribution of ORL1 in the central nervous system. Nociceptin can exhibit both antiopiate as well as analgesic properties, depending on the experimental setting and its site of action (Heinricher 2005). The nociceptin-ORL1 system is now considered as a target for the development of drugs in the fields of pain, anxiety, drug dependence, and obesity (Zaveri et al. 2005; Reinscheid 2006).

## 6 Characterization of Chemerin as the Natural Ligand of ChemR23

A large number of G-protein-coupled receptors contribute to the mounting of immune responses by regulating the trafficking of leukocyte populations. Chemokines constitute one of the major classes of signaling proteins in this frame (Rossi and Zlotnik 2000; Sallusto et al. 2000), with over 40 chemokines and 19 chemokine receptors described so far (Murphy et al. 2000). Other chemoattractant molecules include the formyl peptides, complement fragments (C3a, C5a), and leukotrienes, among others. A number of orphan human receptors are structurally related to chemoattractant receptors.

ChemR23 is a receptor that was initially described to be expressed in immature dendritic cells (DCs) and macrophages (Samson et al. 1998). Given this distribution pattern and the rapid down-modulation following maturation of DCs, we speculated that the ligand was generated in inflammatory conditions. We therefore used the receptor in a bioassay, and tested fractions derived from human inflammatory samples. A biological activity, specific for ChemR23, was identified in a human ascitic fluid secondary to an ovarian carcinoma (Wittamer et al. 2003). The purification of this activity led to the characterization of the

bioactive molecule as the product of *TIG-2* (tazarotene-induced gene-2), a gene previously shown to be induced in keratinocytes by analogs of vitamin A, and overexpressed in patients with psoriasis (Nagpal et al. 1997). This natural ligand of ChemR23 was named chemerin. Chemerin is structurally related to the cathelicidin precursors (antibacterial peptides), cystatins (cysteine protease inhibitors), and kininogens (Fig. 2). Like other chemoattractant receptors, chemerin was shown to act through the $G_i$ class of G-proteins.

Chemerin is synthesized as a secreted precursor, prochemerin, which is poorly active, but converted into a full agonist of ChemR23 by the proteolytic removal of the last six or seven amino acids. We have determined that a synthetic nonapeptide corresponding to the C-terminal end of mature chemerin is able to activate the receptor with limited loss of potency as compared to the full-size protein (Wittamer et al. 2004). Neutrophil cathepsin G and elastase were identified as two proteases able to activate prochemerin, generating two chemerin forms differing by a single amino acid at their C-terminus (Wittamer et al. 2005). Enzymes of the coagulation cascades have been described as processing

**Fig. 2a,b.** Structure of chemerin. **a** The amino acid sequence of human preprochemerin (*Chem*) is aligned with other proteins containing a cystatin fold. This includes the precursors of the human cathelicidin FALL39 (*FA39*), the mouse Cramp (*CRAM*) and porcine protegrin (*PTG*), the first domain of bovine kininogen (*KNNG*), and the chicken egg-white cystatin (*CYST*). The signal peptides are represented in *lowercase italics*. The cysteines involved in disulfide bonding (of which four are conserved across the family) are in *green*. The *red arrowheads* indicate (when known) the position of the introns in the structure of the respective genes. In all cases, the introns interrupt the coding sequences between codons. C-terminal peptides that are cleaved by proteolysis are represented in *blue*. This results in the generation of active chemerin (N-terminal domain), while for cathelicidin precursors, the released C-terminal peptides display bactericidal properties. The nonapeptide represented in *red* and underlined is chemerin-9, the synthetic peptide that binds and activates the ChemR23 receptor with low nanomolar potency. **b** Tridimensional structure of the cystatin-like domain of porcine protegrin, a cathelicidin precursor (PDB 1PFP). Chemerin is expected to adopt a similar conformation

prochemerin as well (Zabel et al. 2005). The receptor was also shown to recruit both myeloid and plasmacytoid dendritic cells (Vermi et al. 2005). Resolvin E1, an omega-3 lipid mediator, was also proposed recently as a ligand for ChemR23 (Arita et al. 2005), although it is not yet clear whether the anti-inflammatory activities of resolvin E1 are indeed mediated through ChemR23.

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hChem            mrrlliplalwlgavgvGVAELTEAQRRGLQVALEEFHKHPPVQWAFQETS
hFA39 mktqrdghslgrwslvllllglvmplaiiaQVLSYKEAVLRAIDGINQRSSDANLYRLLD
mCRAM    mqfqrdvpslwlwrslslllllglgfsQTPSYRDAVLRAVDDFNQQSLDTNLYRLLD
pPTG   metqraslclgrwslwllllalvvpsasaQALSYREAVLRAVDRLNEQSSEANLYRLLE
bKNNG       mklitilflcsrllpsltQESSQEIDCNDQDVFKAVDAALTKYNSENKSGNQFVLY
cCYST magarg...vgavlgSEDRSRLLGAPVPVDENDEGLQRALQFAMAEYNRASNDKYSSRVV

Chem VESAVDTPFPAGIFVRLEFKLQQTSCRKRDWKKPE-CKVRP---NGRKRKCLACIKLG-SED
FA39 LDPRPTMDGDPDTPKPVSFTVKETVCPRTTQQSPEDCDFKK---DGLVKRCMGTVTLN---Q
CRAM LDPEPQGDEDPDTPKSVRFRVKETVCGKAERQLPEQCAFKE---QGVVKQCMGAVTLN---P
PTG  LDQPPKADEDPGTPKPVSFTVKETVCPRPTRQPPELCDFKE---NGRVKQCVGTVTLD---Q
KNNG RITEVARMDNPDTFYSLKYQIKEGDCPFQSNKTWQDCDYKD-SAQAATGQCTATVAKR-GNM
CYST RVISAKRQLVSGIKYILQVEIGRTTCPKSS-GDLQSCEFHDEPEMAKYTTCTFVVYSIPWLN

Chem KVLGRLVHCPIETQVLREAEEHQETQCLRVQRAGEDPSFYFPGQFAFSKALPRS
FA39 ARGSFDISCDKDNKRFALLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES
CRAM AADSFDISCNEPGAQPFRFKKISRLAGLLRKGGEKIGEKLKKIGQKIKNFFQKLVPQPEQ
PTG  IKDPLDITCNEVQGVRGGGLCYCRRRFCVCVGRG
KNNG KFSVAIQTCLITPAEGPVVTAQY....
CYST QIKLLESKCQ
```

a

N-term

C-term

b

Clear orthologs can be found in rodents for both the ChemR23 receptor and prochemerin. The binding and functional parameters of the mouse system were very similar to those observed in human ($K_D$ and $EC_{50}$ around 1 nM for chemerin), with full cross-reactivity between the human and mouse components.

Therefore, chemerin appears as a potent chemoattractant of a novel class. Expression of ChemR23 is essentially restricted to macrophages and dendritic cells and its agonist chemerin can be found at a high level in human inflammatory fluids. We therefore postulate that chemerin is involved in the recruitment of APCs and regulates the inflammatory process and the development of adaptive immune responses. The characterization of a knock-out model for ChemR23 is ongoing and will make it possible to determine the phenotype associated with ChemR23 deficiency and its involvement in disease.

## 7 Characterization of F2L and Humanin as High-Affinity Endogenous Agonists of FPRL2

FPRL2 belongs to the family of receptors similar to FPR, the receptor for formylated peptides of bacterial origin. Formyl peptide receptors play an essential role in host defense mechanisms against bacterial infection and in the regulation of inflammatory reactions. In human, this family includes three receptors: FPR, FPRL1, and FPRL2. FPR is expressed in neutrophils, monocytes, and DCs, FPRL1 in neutrophils and monocytes, and FPRL2 in monocytes and DCs. FPRL1 is a promiscuous receptor, responding to a large variety of ligands of endogenous and exogenous origins and high structural diversity, including lipoxin A4, serum amyloid 4, and bacterial peptides (Le et al. 2001, 2002). Many of these ligands are low-affinity ligands, and their functional relevance is therefore questionable. A few of these FPRL1 agonists were found to activate FPRL2 at high (μM) concentrations as well, but no high-affinity ligands have been described so far for this receptor. Using a CHO-K1 cell line expressing human FPRL2, $G_{\alpha16}$ and apoaequorin, we tested fractions of tissue extracts for biological activities, focusing on lymphoid organs. Two specific activities were found in fractions of human and porcine spleen, and the bioactive porcine compounds

were purified to homogeneity. These peptidic ligands were characterized as N-terminal peptides of the intracellular heme-binding protein (HBP). The most active peptide was an acetylated 21 amino acid peptide (Ac-MLGMIKNSLFGSVETWPWQVL), perfectly conserved between human and pig, and was named F2L (Fig. 3). It displayed a 5- to 10-nM $EC_{50}$ for human FPRL2 according to the assay. Acetylation of the peptide is not essential for its activity. The second activity corresponded to a longer peptide, incompletely characterized, but presenting a much lower potency. F2L was demonstrated to trigger intracellular calcium release, inhibition of cAMP accumulation, and phosphorylation of ERK1/2 MAP kinases through the $G_i$ class of G-proteins in FPRL2-expressing cells. It activates and chemoattracts monocytes and immature dendritic cells. F2L is inactive on FPR, and poorly active on FPRL1 (Migeotte et al. 2005). HBP, described as binding various molecules containing a tetrapyrrole structure, is poorly characterized functionally (Taketani et al. 1998; Jacob Blackmon et al. 2002). F2L therefore appeared as a new natural chemoattractant peptide for DCs and monocytes in human, and the first potent and specific agonist of FPRL2. In parallel, another human endogenous peptide, humanin, was described as a high-affinity ligand of FPRL2, but this ligand is shared with FPRL1 (Harada et al. 2004).

As HBP is an intracellular protein, it is unclear at this stage what mechanism is involved in the release of the F2L peptide. Our hypothesis is that cell death by apoptosis or necrosis would be responsible for the proteolytic generation of F2L from HBP and its release in the extracellular space. This hypothesis is presently being tested.

## 8 Future Challenges

Besides olfactory receptors, approximately 100 GPCRs presently remain orphan. A number of associations between ligands and orphan receptors proposed over the recent years are still a matter of debate, and it is likely that some receptors presently considered deorphanized will find more convincing agonists in the future. It is also likely that the easy ligands have been identified, and that the remaining set of orphan receptors concentrates a number of obstacles that will make their characteri-

```
Human                   MLGMIKNSLFGSVETWPWQVLSKGDKEEVAYEERACEGGKFATVEVTDKPVDEALREAMPKVA
Pig                     MLGMIKNSLFGSVETWPWQVLSKGDKQDISYEERACEGGKFATVEVTDKPVDEALREAMPKVM
Mouse                   MLGMIRNSLFGSVETWPWQVLSTGGKEDVSYEERACEGGKFATVEVTDKPVDEALREAMPKIM
Rat                     MLGMIRNSLFGSVETWPWQVLSTGGKEDVSYEERACEGGKFATVEVTDKPVDEALREAMPKIM
Chicken                 MLGMIKNSLLSTVETWPYRVLSKGEKEQLSYEERECEGGQFAVVEVTGKPFDEASKEAALKLL
Xenopus                 MFGMIKNSLLGGVENNEGKLVSKGEKDGVAFEEREYEGGKFISTEVSGKPFDEASKEGVLRLL
Tetraodon               MFGMIKNSLFGNTEKTEYKLLSSETKDGVSFEVRRYDAAKYATVSSEGRTFDQISGELVRKLL
Soul (Hum) MAEPLQPDPGAAEDAAAQAVETPGWKAPEDAGPQPGSYEIRHYGPAKWVSTSVESMDWDSAIQTGFTKLN

Human      KYAGGTNDKGIGMGMTVPISFAVFPNEDGSLQKKLKVWFRIPNQFQSDPPAPSDKSVKIEEREGITVYSM
Pig        KYVGGSNDKGIGMGMTVPISFAVFPSDGGSLQKKLKVWFRIPNEFQSNPPVPSDDSIKIEERESITVYSL
Mouse      KYVGGTNDKGVGMGMTVPVSFAVFPNEDGSLQKKLKVWFRIPNQFQGSPPAPSDESVKIEEREGITVYST
Rat        KYVGGTNDKGVGMGMTVPISFAVFPNEDGSLQKKLKVWFRIPNQFQGSPPTPSDQSVKIEEREGITVYST
Chicken    KYVGGSNDKGTGMGMTAPVSITAFPAADGSLQQKVKVYLRIPNQFQASPPCPSDESIKIEERQGMTIYST
Xenopus    KYVGGSNNKSAGMGMTSPVIINSYPSENDTLQPNVKVLLRIPSQYQADPPVPTDNTIQIEDRESVTLYST
Tetraodon  MYIGGSNEQGEAMGTATPI-------------------------YQQSPPTPSDTAVKIEERPGMTVYAL
Soul (Hum) SYIQGKNEKEMKIKMTAPVTSYVEPGSGPFSESTITISLYIPSEQQFDPPRPLESDVFIEDRAEMTVFVR

Human      QFGGYAKEADYVAQATRLRAALEGT-ATYRGDIYFCTGYDPPMKPYGRRNEIWLLKT
Pig        QFGGYAKEADYVARAAQLRTALEGI-ATCRSDVYFCTGYDPPMKPYGRRNEVWLVKA
Mouse      QFGGYAKEADYVAHATQLRTTLEGTPATYQGDVYYCAGYDPPMKPYGRRNEVWLVKA
Rat        QFGGYAKEADYVAHATQLRTTLEGTPATYQGDVYYCAGYDPPMKPYGRRNEVWLVKV
Chicken    QFGGYAKEVDYVNYAAKLKTAL-GSEAAYRKDFYFCNGYDPPMKPYGRRNEVWFVKE
Xenopus    QFGGYAKEADYVSHAAKLRSCL-GPDISYHSDYYMCCGYDPPMKPYGRRNEVWFIKNN
Tetraodon  QFGGFAGESEYRAEALRLTRTL-GETAPYQRKQYFCCSYDPPLKPYGRCNEVWFLQDEP
Soul (Hum) SFDGFSSAQKNQEQLLTLASILREDGKVFDEKVYYTAGYNSPVKLLNRNNEVWLIQKNEPTKENE
```

**Fig. 3.** The F2L peptide and its HBP precursor. Alignment of the heme-binding protein (HBP) from various species, together with the most closely related human protein, SOUL. Identities with the human HBP sequence are represented in *red*. The underlined peptide in *blue* is F2L, the N-terminally acetylated peptide isolated from porcine spleen as a natural agonist of human FPRL2. The sequence of F2L is identical in human, and well conserved (as the remaining part of HBP) in more distant species

zation difficult. Indeed, all orphan receptors have been tested by numerous groups for their response to large collections of bioactive molecules; they have also been tested for their response to tissue extracts, particularly peptidic extracts. There is still a set of ligands that have not found their receptor, including CART, nocistatin, motilin-associated peptide, neuropeptide GE, neuropeptide EI, NocII, BRAK, and EMAPII among others. However, it is likely that the ligands of most of the remaining orphan receptors are unknown biological mediators that are either unstable, expressed at low levels or in restricted regions, tightly regulated in specific situations, or only present at precise developmental stages. Our experience and the experience of others have shown that many new ligands could not be predicted from standard genomic analysis, as the active compounds required post-translational modifications such as pro-

teolytic processing or grafting of lipid moieties. This suggests that many of the ligands expected for orphan receptors will require their purification from complex biological samples.

Another potential issue that may hinder the characterization of the remaining orphan receptors is the potential requirement for protein partners that may affect their folding, trafficking, pharmacology, or the efficiency of their coupling to signal transduction cascades. The first example of this type was RAMPs, single-pass membrane proteins that determine which agonists are able to activate the complex, but are also required for its traffic from the endoplasmic reticulum to the plasma membrane (McLatchie et al. 1998). The association of RAMP1 with the calcitonin-receptor-like receptor (CRLR) results in a CGRP receptor, while the association of RAMP2 with the same GPCR results in an adrenomedullin receptor. Although the RAMP family is limited and influences the function of a restricted number of receptors (Parameswaran and Spielman 2006), various proteins belonging to diverse structural and functional families have been shown over the years to influence GPCR properties. The MC2 (ACTH) receptor was reported to require another single-pass transmembrane protein, MRAP, for its trafficking to the cell surface (Metherell et al. 2005). Olfactory receptors, which for years have proven to be extremely tedious to express functionally in classical heterologous systems, have been shown to require chaperones such as RTP1, RTP2, and REEP1 in order to traffic properly to the plasma membrane (Saito et al. 2004).

G-protein-coupled receptors have been shown over recent years to form homo- and heterodimers (Bulenger et al. 2005). Some receptors require the heterodimerization of two different polypeptides in order to form functional receptors. The first well-established example is the $GABA_B$ receptor, for which the $GABA_{B1}$ polypeptide, which is able to bind GABA, contains a ER-retention signal that prevents its trafficking to the plasma membrane. The association with the $GABA_{B2}$ polypeptide, which does not bind GABA, masks the retention signal and allows the trafficking of the heterodimer and its functional response (Pin et al. 2003). A similar situation is found in taste receptors. A common $T1R_1$ polypeptide forms a sweet receptor when associated with $T1R_2$ and a L-amino acid sensor when associated with $T1R_3$ (Nelson et al. 2001, 2002). Obligate heterodimers have also been described for insect

olfactory receptors (Benton et al. 2006), although this property does not seem to be shared by mammalian olfactory receptors. Besides obligate heterodimers, there is growing evidence that heterodimerization of GPCRs that are fully functional when expressed alone can modify the pharmacology of the partners involved. This was first demonstrated for opiate receptors, for which heterodimers can form original binding sites (Jordan and Devi 1999). We have demonstrated that heterodimerization of chemokine receptors could also modify the pharmacology of both receptors through an allosteric interaction between the binding sites of each protomer (Springael et al. 2005, 2006). It is therefore possible that some orphan receptors might be functional only when coexpressed as heterodimers, or alternatively that the only function of an orphan GPCR might be to modify the pharmacology of a presently characterized receptor. Characterization of these potential situations will require detailed analyses of expression patterns, in order to determine which receptors are coexpressed in each cell type, and raise hypotheses regarding possible heterodimers.

A last set of conditions that may render the characterization of orphan receptors troublesome is that some of them may exhibit signaling properties different from the classical pathways activated by GPCRs. It is now well established that GPCRs may activate intracellular cascades independently from G proteins (Luttrell 2006). There is so far no well-established example of a GPCR acting solely through G-protein-independent pathways, but this possibility must be considered. Also, many receptors display constitutive activities when overexpressed, and some, including a number of orphans, keep such constitutivity when expressed at physiological levels. This suggests that some of these receptors might regulate the cells as a result of their expression, without the need for agonists. Alternatively, some constitutively active receptors may be regulated by natural inverse agonists, rather than by agonists. This might be considered as an example for GPR3, displaying strong constitutivity toward the adenylyl cyclase pathway (Eggerickx et al. 1995), and involved in the blockade of the cell cycle during oogenesis (Mehlmann et al. 2004; Ledent et al. 2005). Finally, some receptors might bind ligands without promoting signaling in the cell. Such behavior has been convincingly proposed for several receptors belonging to the chemokine receptor family, such as DARC and D6. These

receptors are considered as decoy receptors, able to bind a diverse set of chemokines, internalize them, and drive them to degradation, thereby contributing to the dampening of inflammatory responses (Middelton et al. 2002; Locati et al. 2005). These receptors have also been proposed as promoting transcytosis of chemokines across endothelial cells. Each of these situations will require the design of specific assays in order to test the various hypotheses specifically.

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