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# Development of sensitive cellular assay systems and their application to the identification of "orphan" seven-transmembrane receptors

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Damit das Mögliche entsteht, muss immer wieder
das Unmögliche versucht werden.

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# Contents

# Abbreviations

| | |
|---|---|
| 7TM | Seven-transmembrane |
| ATP | Adenosine 5`-triphosphate |
| $BLT_1R$ | Leukotriene $B_4$ receptor 1 |
| $BLT_2R$ | Leukotriene $B_4$ receptor 2 |
| C5a | Complement factor 5a |
| cAMP | Adenosine 3`,5`-cyclic monophosphate |
| CCR5 | CC chemokine receptor 5 |
| cGMP | Guanosine 3`,5`-cyclic monophosphate |
| CHO | Chinese hamster ovary cells |
| CMV | Cytomegalovirus |
| CRE | cAMP response element |
| CXCR4 | CXC chemokine receptor 4 |
| DNA | Deoxyribonucleic acid |
| $EC_{50}$ | Half-effective concentration |
| EGFP | Enhanced green fluorescence protein |
| EST | Expressed sequence taq |
| FACS | Fluorescence activated cell sorting |
| FFA | Free fatty acid |
| $FFA_1R$ | Free fatty acid 1 receptor |
| FLIPR | Fluorometric imaging plate reader |
| GFP | Green fluorescent protein |
| GIRK | G-protein regulated inwardly rectifying potassium channel |
| GPCR | G-protein coupled receptor |
| GRK | G-protein coupled receptor kinases |
| HeLa | Human cervix carcinoma cell line derived from H.L. |
| HEK | Human embryonic kidney cells |
| HETE | Hydroxy-eicosatetraenoic acid |
| 9-HODE | ±9 Hydroxy octadecadienoic acid |
| IRES | Internal ribosome entry sites |
| $LTB_4$ | 5,12-Dihydroxy-6,8,10,14-eicosatetraenoic acid or leukotriene B4 |
| mRNA | Messenger ribonucleic acid |
| NFAT | Nuclear factor of activated T-cells |
| NF-κB | Nuclear factor κB |
| ORF | Open reading frame |
| PLC | Phospholipase C |
| PMA | Phorbol 12-myristate 13-acetate |
| PKA | Proteinkinase A |
| PKC | Proteinkinase C |
| PPAR | Peroxisome proliferator-activated receptor |
| $R\alpha_{1b}$ | Rat $\alpha_{1b}$ adrenergic receptor |
| RGS | Regulator of G-protein signaling |
| STAT | Signal transducer and activator of transcription |
| TMD | Transmembrane domain |
| TPA | 12-O-tetradecanoyl-phorbol-13-acetate |
| TRE | TPA-responsive element |
| TZD | Thiazolidinedione |

# List of publications

This thesis is based on the following articles which will be referred to in the text by their roman numerals.

**I.** **A chimeric reporter gene allowing for clone selection and high-throughput screening of reporter cell lines expressing G-protein-coupled receptors.** Kotarsky K, Owman C, Olde B. 2001. *Analytical Biochemistry*; 288(2): 209-215.

**II.** **Optimized reporter gene assays based on a synthetic multifunctional promoter and a secreted luciferase.** Kotarsky K, Antonsson L, Owman C, Olde B. 2003. *Analytical Biochemistry*; in press

**III.** **Cloning and characterization of cDNA encoding a novel human leukotriene $B_4$ receptor.** Tryselius Y, Nilsson NE, Kotarsky K, Olde B, Owman C. 2000. *Biochemical and Biophysical Research Communications*; 274(2): 377-382.

**IV.** **A human cell surface receptor activated by free fatty acids and thiazolidinedione drugs.** Kotarsky K , Nilsson NE , Flodgren E, Owman C, Olde B. 2003. *Biochemical and Biophysical Research Communications*; 301: 406-410

These authors contributed equally to the work.

# Introduction

Multicellular organisms, such as human beings, consist of thousands of billions of cells, organized in tissues, organs and organ systems. All of these cells, tissues and organs act in concert. This requires an on-going communication between cells and cell systems throughout the organisms lifetime. Cells communicate by releasing small, inconspicuous chemical molecules, called mediators or neurotransmitters. Even from the outside of the body a steady stream of chemicals bombards the surface of our cells. To understand all the signals arriving at the cell surface, cells have small antennae, called receptors. Cell surface receptors are divided into different classes, based on their primary structure and similarity to other receptors. The largest family by-far is called the seven-transmembrane (7TM), or G-protein coupled receptor, family, with more than a thousand members. All of them wind seven times through the cell membrane. Unfortunately, many receptors are classified as 7TM receptors only by sequence similarity. Lacking knowledge about the endogenous ligand(s) of a receptor makes it impossible to study the receptor and to understand its function.

The identification and characterization of previously unknown, so-called “orphan” 7TM receptors has attracted a lot of interest in the last decade. This focus on orphan 7TM receptors can be explained by two facts. To understand cell communication and, eventually, physiological mechanisms requires both knowledge about mediators and their receptors. And even more importantly, more than fifty percent of all drugs in medical use interact with seven-transmembrane receptors, making this type of receptor a clinically important target for the evaluation of drug candidates and to lead compound development [1].

# Seven-transmembrane receptors

*Common structure and families*

As stated earlier, the cells of multicellular organisms have to communicate with each other. To receive signals, cells have developed a variety of methods. If the signaling molecule can pass through the cell membrane, it may interact with intracellular enzymes or with nuclear receptors. However, if the signaling molecule is hydrophilic and large it can usually not pass the cell membrane and penetrate into the cell. Such signaling molecules often interact with cell surface receptors.

There are three major families of cell surface receptors described: (i) transmitter gated ion channels, (ii) enzyme linked receptors and, (iii) the seven-transmembrane, G-protein-coupled receptor family.

The 7TM, G-protein-coupled receptor family is the largest of these cell surface receptor families. It accounts for approximately 5% of all genes found in *Caenorhabditis elegans* genome, whereas in humans, 3-4% of all genes encode seven-transmembrane receptors [2, 3]. A wide variety of molecules, called ligands, interact with and activate 7TM receptors. The list of these ligands includes signaling molecules used in the body such as hormones, neurotransmitters, chemo-attractants, or signals from outside of the body, such as odorants, pheromones, nutrients, or even light. The ligands display different sizes (from large proteins to small molecules), and belong to different chemical classes (lipids, amino acids, ions, or proteins). Despite these differences, the receptors show a common architecture based on seven transmembrane helices. It is generally assumed that these receptors function in similar ways, even when the ligand binding domains, or pockets, are not totally conserved. So far, the only three-dimensional structure of a 7TM receptor known is that of bovine rhodopsin. This structure was determined by X-ray crystallography of the receptor and shows the receptor molecule in an inactive conformation [4]. Attempts have been made to superimpose other 7TM receptors onto the rhodopsin model *in silico* [5]. However, differences in the primary sequences and in the ligand binding make receptor modeling difficult [6].

The 7TM receptor family is divided into three major sub-families based on sequence similarity. Family 1, also called the rhodopsin type family, includes the vast majority of all 7TM receptors, and is conveniently grouped into three sub-families. Members of this family often display some conserved amino acids, mainly in their transmembrane domains (TMD). Thus, the first TMD often contains a conserved asparagin, TMD II aspartic acid; the boundary between TMD III and the second intracellular loop contains the DRY amino acid motif; extracellular loops 2 and 3 are usually connected by a disulfide bridge; TMD IV has a tryptophane and TMD VI a conserved proline; and TMD VII often contains the so-called NPXXY amino acid motif (for review [7]). The C-terminus often includes residues which are modified by fatty acids. This directs the C-terminus close to the membrane, where an extra, fourth intracellular loop is formed. The members of the three sub-families within family 1 bind their ligands in different ways. Members of sub-family 1a bind their ligands within their TMDs (e.g., retinal, ATP, odorants, opiate). Members of sub-family 1b involve their N-terminus in ligand recognition. The recognized ligands are usually peptides (e.g. chemokines, C3a). Members of sub-family 1c have big N-terminal structures which bind glycoprotein hormones (e.g., luteinizing hormone) [3].

Family 2 includes receptors which bind larger peptides. Here as well, the N-terminus of the receptor is extended and is involved in ligand binding. Examples include the receptors for vasoactive intestinal peptide, secretin, and glucagon. A subtype of family 2 that sometimes is recognized as a group of its

own, includes the so-called EGF-TM7 receptors that all possess large and complicated N-terminal regions. These regions are usually composed of a variety of protein domains, e.g. epidermal-growth-factor domains.

Family 3 includes taste receptors, an ion-receptor (for $Ca^{2+}$), the metabotrobic glutamate receptors, and $GABA_B$ receptors. This family consists of 3 sub-families, which share relative little sequence similarity in their seven TMDs.
There are three additional families of 7TM receptors. They include a family of pheromone receptors, the frizzled/smoothened family, which is involved in embryonic development, and finally a family of cAMP-receptors, found in *Dyctiostelium*. However, evidence that these receptors couple and signal via G-proteins still remains controversial.

*Signaling through 7TM receptors*

As stated earlier, 7TM receptors function as antennas, receiving signals from the outside of the cell. They then convert the arriving signal to a chemical signal within the cell, and might initiate or modulate a variety of cellular responses, such as cell movement (chemotaxis), enzyme activation and metabolic changes, liberation of other signaling molecules from the cell (exocytosis and secretion), or a change in gene transcription. The complicated process of cell signaling involves a large array of proteins and intracellular (second) messengers. The individual reactions of a cell are dependent upon the specific proteins found in that particular cell. Usually, signaling proteins work in sophisticated cascades and networks. Each step of a given signaling cascade might be realized by different members of specialized protein families. Members of a given family may display slightly different, or even opposite signaling preferences.

The classical pathway for signaling by means of 7TM receptors (or G-protein coupled receptors) is mediated, as the name indicates, by so-called heterotrimeric G-proteins, which consist of an α-subunit functionally connected to a βγ-complex. Each of the sub-units is part of a protein family, where each family member has slightly different functions. The α-family has 16 members, the β-family 5, and the γ-family 12 members (for review [8, 9]). G-proteins are designated according to the α-subunit present in the complex. Different α-subunits have different signaling preferences and properties; however even the βγ-complex can perform signaling tasks (Table 1).

The interaction of an extra-cellular signaling molecule with its receptor leads to conformational changes in the receptor. In an active conformation the receptor interacts with G-proteins where the α-subunit exchanges the bound GDP for GTP and causes dissociation of the complex. Both the α-subunit and the βγ-complex are then able to interact with target proteins and thus regulate cellular functions. The α-subunit remains active as long as it binds the GTP. It has,

however, intrinsic GTP-ase activity and when the GTP is broken down into GDP and $P_i$ the α-subunit turns inactive and binds a βγ-complex. The conversion of GTP to GDP in the active α-subunit is enhanced by members of the RGS-protein family.

| **G-protein subunits** | **Protein/Signaling pathway regulated** | **References** |
|---|---|---|
| $G\alpha_s$ $G\alpha_{OLF}$ | stimulates adenylyl-cyclase;<br>RGS-PX1<br>Calcium channels<br>c-Src tyrosine kinase | [10], [11], [12] |
| $G\alpha_q$,$G\alpha_{11}$,$G\alpha_{14,15,16}$ | stimulate Phospholipases C | [13], [14], [15] |
| $G\alpha_i$ 1,2,3 $G\alpha_Z$ $G\alpha_0$ | inhibit Adenylylcyclase;<br>stimulate c-Src tyrosine kinases | [12], [16], [17], [18] |
| $G\alpha_{12, 13}$ | Rho-activation; stress fibre formation; β-catenin release | [13], [14], [19, 20], |
| $G\alpha_T$(Transducin)<br>$G\alpha_{gust}$ (Gustducin) | Stimulates cGMP phosphodiesterase | [21], [22] |
| Gβγ | Stimulates PLCβ 1-3<br>Stimulates adenylylcyclase II, IV<br>G-protein-coupled-receptor kinases (GRK)<br>G-protein regulated inwardly rectifying potassium channel (GIRK)<br>Phosphatidylinositol-3-kinase | [23], [24], [25], [26, 27], [28], [29] |

**Table 1. Signaling targets/pathways affected by G-protein sub-units (adapted from [9]).**

In addition, signaling through 7TM receptors is influenced by different kinases phosphorylating the receptor. Thus, protein kinase C, protein kinase A, and G-protein-coupled receptor kinases (GRKs) can all lead to rapid changes of signaling behavior after phosphorylating the receptor. Phosphorylation by protein kinase A, for example, can change the coupling efficiency of the $\beta_2$-adreneric receptor from $G\alpha_s$ to $G\alpha_i$ [30]. The family of G-protein-coupled receptor kinases has 7 members and quite distinct expression patterns. These kinases can be activated by either protein kinase C, protein kinase A, or βγ-complexes. The phosphorylated receptors can interact with so-called β-arrestin proteins. These proteins are important in receptor internalization, and function also as scaffold proteins, thereby constituting the framework for macromolecular signaling complexes.

In addition to signaling through G-proteins, there is an increasing number of examples of 7TM receptors signaling through mechanisms, independent of G-proteins. The activation of Jak2 by CCR2B has been suggested to be independent from G-proteins (for review [31, 32]). Instead, the involvement of

receptor homodimers and heterodimers has been proposed in this process. On the other hand, recent work suggests that stimulation of proteinase activated receptors, or angiotensin receptors, in smooth muscle cells trigger the activation of the JAK/STAT pathway via the small GTPase Rac [33], which suggests the involvement of trimeric G-proteins. In the suggested model the activation of Jak2 was not performed by the receptor itself. Instead, it was dependent on the activation of NADPH-oxidase and the generation of reactive oxygen species by Rac. Whether the reported differences are due to different cell types, and/or receptors have to be elucidated. A direct interaction of Jak2 and the angiotensin AT1 receptor had been earlier discussed [34].

*Regulation of transcription*

Most 7TM receptors regulate the transcription of genes in the nucleus of the cell. The regulation may be positive, leading to an increase in gene transcription and hence mRNA production, or negative, leading to gene down-regulation.

| **Transcription factor** | **Response element** | **Activated by** | **Reference** |
|---|---|---|---|
| CREB-family | CRE | $G\alpha_s$ | [35] |
| AP-1 (Fos and Jun) | TRE | $G\alpha_q$, $G\alpha_i$ | [36] |
| Serum response factor | SRE | $G\alpha_{12}$, $G\alpha_{13}$ $G\alpha_{q/11}$ | [37, 38] |
| NF-κB | NF-κB | All except of $G\alpha_s$ | [39] |
| STAT | STAT | ? | [33] |
| NFAT | NFAT | $G\alpha_q$ | [40] |

**Table 2 lists some transcription factors and their response motifs, which may be activated by 7TM receptors. ? means: involvement of G-proteins is controversially discussed.**

Receptor stimulation triggers the activation of signaling cascades, which often involve second messengers and usually also the phosphorylation of signaling proteins, which function as kinases themselves. Eventually, transcription factors are activated by the kinases after phosphorylation. Many transcription factors dimerize in response to phosphorylation, either as homodimers or heterodimers, and bind to certain DNA binding motifs. A transcription factor, which has bound via its response motif to the promoter of a gene, may either increase (by recruiting other transcription factors) or diminish transcription. Table 2 lists some transcription factors, their response elements and the G-proteins involved in their activation.

## Identification of uncharacterized “orphan” 7TM receptors

As a considerable number of drugs in clinical use act on 7TM receptors novel, “orphan”, receptors have been considered as potential new drug targets [1]. Hence, the identification of “orphan” 7TM receptors has not been accomplished by pure academic interest, but has been undertaken in a competitive situation between academics and the pharmaceutical industry (for example: [41-45]. Two different strategies have been used for the identification of “orphan” receptors. The pharmaceutical industry and some larger academic laboratories often use an approach known as “reverse pharmacology”. In this strategy huge substance libraries are matched with “orphan” receptor libraries to find positive hits. Smaller laboratories, on the other hand, work mainly on “strange” receptors, often for many years in a laborious procedure in order to identify the ligand for a specific receptor. The second approach usually starts with using a tissue extract as the ligand source, and is often referred to as the “orphan receptor strategy” [46].

While the genetic identification of unknown and new receptors was in the past accomplished using molecular biological techniques (e.g. polymerase chain reaction with degenerated primers), nowadays, after the completion of the human genome sequencing project, sequences with a certain degree of homology to already known 7TM receptors are continuously discovered using *in silico* approaches and are stored in public databases [47-49]. Estimations of the number of remaining 7TM receptors without known endogenous ligand (the orphans) vary to some extent between one hundred and several hundred [50-53]. Considering the huge amount of orphan receptors the question has been raised, as to why so many of them still lack a known ligand. Although the answer probably has to be found for each individual receptor, difficulties in the process of ligand identification may be grouped into three main categories (i) the receptor itself, (ii) the assay systems used for the identification, and (iii) the nature of the ligand.

*The receptors*

First of the problems connected with the receptor itself is the identification of the correct ORF. Often 7TM receptor N-termini are spliced, and different splice variants for receptors might have been described. Some of the splice variants may show different behavior in pharmacology or expression [54-59].

The availability of the complete human genome makes it attractive, at least for family 1 members which often have an unspliced ORF, to clone the receptor gene from the genome. Full-length mRNA sequences, including complete 5´ and 3´ sequences are not always deposited in the databases.

Once the “proper” reading frame of a gene is identified and the gene is cloned, the receptor has to be expressed heterologously to obtain the receptor protein. However, recombinantly expressed receptor proteins are not always correctly targeted to the cell surface. In order to analyze the correct cell surface expression, different approaches have been used, none of them completely satisfying. For example, the receptor can be tagged with either a short peptide (usually on the N-terminus) or EGFP (usually on the receptor C-terminus). However, both of these techniques potentially influence receptor targeting, disturb ligand binding, or alter the signaling behavior of the receptor. Hence, the functional expression of the receptor has to be controlled in functional assays. Another approach to check for a correct cell surface expression of a receptor relies on the signaling behavior of the receptor. Intrinsic signaling of the receptor may either be achieved by over-expression, or by a mutation, which makes the receptor constitutively active [60, 61]. The over-expressed receptor may also attract G-proteins from other endogenously expressed receptors, thus decreasing signals obtained from these receptors [62]. None of the techniques described is straightforward, all requires considerable work and, yet still, do not guarantee success.

Other problems connected to the receptor may arise from its possible interaction with other molecules in the cell membrane. The most prominent examples, until now, have been the complexing of two gene products to form a functional $GABA_B$ receptor [63-65] and the association of the calcitonin receptor like receptor with receptor associated proteins (RAMPs). In the latter case, the interaction of RAMP2 or 3 with the receptor leads to an adrenomedullin receptor, while interaction with RAMP1 results in the formation of a calcitonin-gene-related-peptide receptor [66] (for review [67]). Finally, heterodimerization of taste receptors to form functional entities for sweet or umami tastes has been reported [68, 69].

Another problem associated with receptors is their coupling to effectors inside the cell. Traditional, assay technologies screen for changes in intracellular second messengers, which assume that the receptor couples to G-proteins. However, there is increasing evidence for a functional G-protein independent signaling of 7TM receptors. An example of this is illustrated by the recent identification of the C5L2 receptor, which is a receptor for the anaphylatoxins C5a and $C5a_{desArg}$ [70].

*The assay systems*

In order to identify ligands acting on 7TM receptors, the receptor protein is used as a “fishing tool”, either in binding assays or in functional tests. There is a plethora of different assay systems described in the literature. However, due to the high demands placed on the assay used for the initial characterization, systems actually used for identification purposes are not as numerous. (i) Assays have to display high reliability and quality, a property that is defined by

a high Z-factor. The Z-factor combines the difference between background and signal with the differences in the standard deviation of both values resulting in a number between 0 and 1, where 1 reflects the ideal assay [71]. (ii) The number of orphan receptors and the number of potential ligands collected in substance libraries demand the use of high-throughput approaches. (iii) Assay and instrumentation should be available to a reasonable cost.

Once a ligand to an orphan receptor has been identified, the initial hit has to be verified by secondary assays, usually performed at lower throughputs and with lower assay quality. Ideally, the hit is confirmed by binding studies showing that the labeled ligand indeed interacts with the receptor.

Appendix 1contains a summary of the identification of about 60 7TM receptors, which were de-orphanized in the last 5 years. Taken together, the data presented show that receptors which display a high sequence similarity usually share a common ligand or are activated by related ligands. These receptors are usually discovered at the same time. Interestingly, in many instances the same receptor has been identified nearly simultaneously by several different groups.

Further, appendix 1 demonstrates that more than half of all identifications have been accomplished using techniques to monitor changes in the intracellular $Ca^{2+}$ concentration. Two different techniques, based on either fluorescent indicators or on the $Ca^{2+}$ sensitive luciferase aequorin, are used for that purpose. The dominating method is fluorescence based and requires the loading of the cell with a calcium-sensitive fluorescent dye. The fluorescent indicator, FURA-2, has been used for that purpose for many years. Other dyes, such as FLUO-3 and FLUO-4, have been introduced more recently. The dye is added to the cells as a membrane permeable ester that passes the cell membrane easily. Intracellular esterases then cut the delivered compound and prevent the release of the ready-to-use indicator. After loading, cells are exposed to the test substances. Changes in the intracellular $Ca^{2+}$ concentration are recorded as a shift in the excitation maxima of the dye. The results are usually expressed as the ratio between the two excitation values, which makes this method both sensitive and trouble-free. The assay can be performed in different devices, where the FLIPR instrument (Molecular Device) being the one mainly used by the pharmaceutical industry due to its high throughput capacity. However, its relatively high cost has prevented it from being more widely used in academic laboratories. Other methods of choice are fluorescence plate readers and microscopic fluorescence imaging systems, which, however, have a much lower throughput.

The aequorin method [72] is also used to monitor shifts in the intracellular $Ca^{2+}$ concentration. This method is based on the expression of apoaequorin protein in cells. The cells are subsequently loaded with coelentrazine, a luciferin substrate. When the intracellular $Ca^{2+}$ concentration is increased, aeqourin protein catalyses the oxidation of coelenterazine. During this process light is released, which can then be recorded using luminometers.

The predominant use of these techniques is closely connected to the introduction of chimeric, and/ or promiscuous, G-proteins which are supposed

to redirect signaling of any 7TM receptor into the $Ca^{2+}$ signaling pathway [73-75]. Thus, a mixture of different chimeric and promiscuous G-proteins is often co-expressed with the "orphan" receptor. However, there are several reports questioning the over-all applicability of this "G-protein cocktail" strategy to virtually any GPCR [76].

Besides these two techniques, half a dozen other assay systems have been successfully used. They often have lower throughputs, are hampered by long incubation times, or have other disadvantages.

Reporter gene technology is a widely used screening approach (for review [77]. It requires a DNA-based reporter construct, usually consisting of a promoter and a reporter gene which is stably introduced into the cells. The transcription activity of the reporter gene is eventually regulated by the receptor, which is present on the cell membrane, through the intracellular signal transduction machinery. Certain transcription factors are activated in response to the stimulation of different signaling pathways (Table 2). Thus, $G\alpha_s$ activation has been monitored by reporter constructs based on cAMP-response elements in their promoters [35]. Activation of $G\alpha_q$ and $G\alpha_i$ coupled receptors has been performed using promoters, which contained either TPA response elements (TRE) [36], serum response elements [78], nuclear factor of activated T-cells (NFAT) response elements [79], or nuclear factor κB (NF-κB) response elements (Paper II). Attempts have been undertaken to design reporter gene assays which couple to virtually all [80] or at least several G-proteins [35] (Paper II). Reporter gene assays are trouble-free and are, once established, not labor-intensive. However, they also have disadvantages; (i) they need an incubation period of several hours in order to produce the reporter enzyme, which makes it possible that other targets than just the receptor will be affected (however, this problem may be circumvented by appropriate controls) and (ii) preferentially even the receptor should be stably inserted.

Assay systems that detect changes in cAMP-production are widely used. They are most valuable for receptors coupling to $G\alpha_s$ or $G\alpha_i$ subtypes of G-proteins. Whereas receptors coupling to $G\alpha_s$ increase production and subsequently the concentration of cAMP, $G\alpha_i$ inhibits the production of cAMP. This makes it necessary to stimulate cAMP production, usually with the PKA stimulator, forskolin.

The production of cAMP can be assayed by a variety of methods. Reporter gene assays have, as discussed above, been successfully used [81, 82]. More often changes in the cAMP-concentration have been monitored by radio-immunoassays [83, 84]. More recently, a technique called AlphaScreen (Amplified Luminescence Proximity Homogenous Assay) has been introduced. It can be performed using very small volumes and at higher throughputs than other cAMP-assays and it has already been successfully used [85].

Electrophysiological assays using *Xenopus* oocytes were applied to some ligand identifications on orphan 7TM receptors [86-89]. While such assays are very

sensitive, their throughput is rather low. In the assay, different ion channels are co-expressed with the receptor in the oocytes, to measure the activation of different Gα-pathways. For example, a G-protein-gated, inwardly rectifying potassium channel (GIRK) has been used to measure activation of $G\alpha_{i/o}$ coupled receptors [63, 86, 90]. In another report, a chloride channel (CFTR) sensitive to elevations of cAMP was used to record the activation of $G\alpha_s$ coupled receptors. The activation of $G\alpha_q$ mediated responses is monitored by an increased chloride current and does not depend on the co-expression of other proteins [87].

7TM receptor activation can also influence the pigment distribution in *Xenopus* melanophores [91, 92]. This principle was used as a secondary assay in the identification of the $CysLT_1R$ [89].

Yeast (*S. cerevisiae*) based assays have been used successfully as a primary screening tool at least twice [90, 93]. As it is an eucaryotic organism, the signaling in yeast resembles the main signaling pathways of other eucaryotes in its pheromone response. Normally, activation of the pheromone response pathway results in growth arrest and initiates the yeast mating procedure. The assay based on yeast is primarily a reporter gene assay, using either β-galactosidase or an enzyme required in the biosynthesis of an essential amino acid (e.g. His3) as a reporter. In the latter case, the read-out is simply the growth of the yeast cells. It is, however, necessary to adapt the yeast genetically in several ways. Besides the introduction of the orphan receptor, chimeric G-proteins to ensure coupling of the receptor, as well as the reporter construct have to be introduced. The yeast pheromone receptors may have to be deleted. The response is also greatly enhanced by deleting the SST2 gene, a member of the RGS family. Yeast assays can be performed at high throughputs and are cheap, however, not all mammalian receptors can be functionally expressed in this system [52].

A technique described as a microphysiometer (or a cytosensor) assays the acidification of cell culture medium upon extracellular stimulation. Activation of the receptor is followed by an increase in the energy metabolism of the cell, leading to acid production [94]. It is supposed to work as a generic assay, but due to its low throughput and sensitivity it has not been widely used [41, 43, 95].

Many cells release arachidonic acid in response to receptor stimulation. The secretion of radioactive arachidonic acid can be monitored after incubating cells with radioactively labeled precursers. Besides the involvement of radioisotopes, the technique is also performed with rather low throughput and has, hence, not been widely used as a primary assay.

Finally, binding assays, where a radioactive ligand shows a specific binding to the receptor, have been applied in some de-orphanizations. The receptor often displayed a high sequence homology to previously known receptors and, hence, the ligand could be determined by an educated guess. Receptor binding is,

however, not a true functional assay and it may indeed be possible that a ligand might bind a receptor without activating it (e.g. [96]).

*The ligand(s)*

Different strategies are employed to accomplish the identification of an orphan receptor. Each of the described methods has its particular advantages and drawbacks.

The earlier described “reverse pharmacology” approach has been applied by the pharmaceutical industry. The success of this approach depends much on the substance library, containing known and potential ligands for 7TM receptors. The compound library is tested on a receptor in a functional assay. The sizes of the compound libraries vary greatly, ranging from several dozen to several thousand compounds. Depending on the assay, this strategy may be performed in a high-throughput format. Using this strategy a large number of receptors has already been identified [97-100]. One of this method’s advantages is that it is based on pure substances, which can be applied at relatively high concentrations. The major drawback of the compound library is two-fold: (i) the library is relatively expensive to purchase and (ii) obtained hits depend entirely on the composition of the library. Completely unknown mediators will not be found using this methodology and even peptide ligands may be missed, exemplified by the discovery of the ghrelin peptide (see below).

The opposite approach starts on the receptor and its tissue distribution, and is referred to as the “orphan receptor strategy” [46, 101, 102]. In theory the ligand should be present at locations where the receptor is expressed, and furthermore that extracts of those tissues can be prepared, fractionated, and screened in a functional assay. By means of this strategy, a large number of natural ligands, including strange ligands with unusual features, have been discovered [103-105]. For example, the peptide ghrelin, acting on the GHS receptor was identified using this strategy. This unusual peptide has a covalent bound octanoyl modification, which is required for functional ligand-receptor interaction [106]. Yet another example is the discovery of a bromidated peptide, which is the ligand for the GPR7 receptor [107]. However, in this case the bromidation was not required for an interaction between receptor and ligand.

Because the orphan receptor strategy starts with a tissue extract containing a complex mixture of different molecules it requires a high quality assay, and since other signaling substances present in the extract may activate the cellular assay system used for the receptor identification. This results in a non-specific activation, which is independent of the expression of the orphan receptor studied and quenches the specific signal. Sub-fractionation of tissue extracts, prior to testing, might reduce the background noise caused by other signaling substances.

Yet another problem arises when the tested cell line already express a subtype of the orphan receptor studied. The presence of nucleotide receptors on many cell types has already caused some confusion through the mis-identification of some members [108-112]. Now these receptors are usually studied in 1321N1 cells, which do not endogenously express such receptors. Sphingosylphosphorylcholine (SPC) receptors are, for the same reasons, studied in MCF10A breast carcinoma cells [113]. Taken together, these studies imply that many signaling molecules can not been studied in the cell lines most often used, HEK293, or Chinese Hamster Ovary (CHO) cells.

Other problems may be related to the stability of the ligand, This may complicate the purification of the ligand, particularly if its amount is limited in the starting material. Furthermore, some ligands may consist of several parts which are not covalently bound together and which would be eluted in different fractions during purification. Finally, the identification of a suitable starting material may be difficult, since the natural ligand may be present only during certain physiological conditions.

Receptors, which have been deorphanized in the last five years have for the most part peptides or lipid mediators as ligands. This fact may reflect that the overwhelming majority of 7TM receptors are activated by such substances. However, it may be possible that these ligand classes are the ones most easily isolated and characterized with the techniques presently available.

Taken together, the approaches for the identification of ligands activating 7TM receptors outlined above demonstrate the complexicity of ligand-receptor interaction.

However, identification of receptor-ligand interaction is only the starting point in a demanding mission. To unravel the function of the receptor and eventually to understand its physiological impact is the main goal. And last, but not least, this increasing amount of knowledge has to be used in the development of new and better drugs.

# CURRENT INVESTIGATIONS

## Design of improved reporter gene assays (Papers I and II)

Reporter gene assays have been widely used as a method to study gene regulation. The method is based on a reporter construct that is transiently or stably introduced into a cell, where it is used to report cellular events, such as receptor activation. The construct is composed of a promoter that is functionally connected to a reporter gene (Figure 1). The promoter is regulated by various intracellular events, which in turn might be triggered by the activation of cell-surface receptors. A wide variety of genes have been used as reporter genes depending on the different requirements of the assay performed.

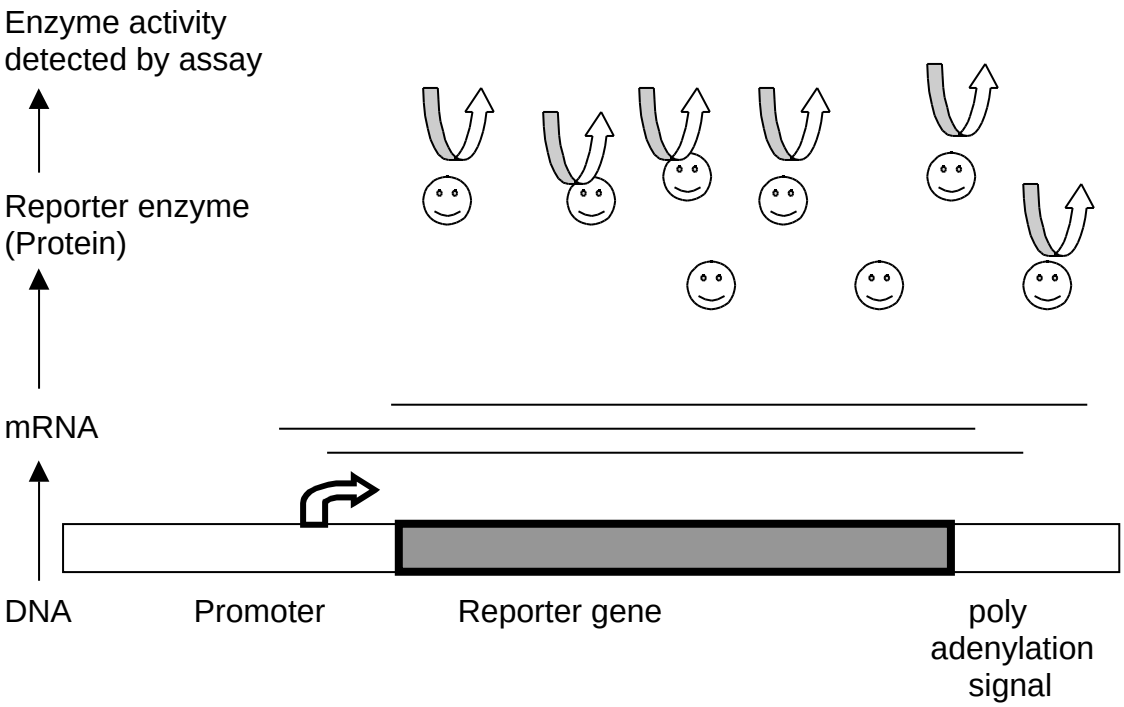


**Figure 1 illustrates the principals of a reporter gene assay. The reporter construct is delivered to the monitored cell. The promoter is the docking place for transcription factors, which start the production of a messenger (mRNA). The messenger is subsequently transcribed into proteins, in this case the reporter enzymes, which are detected based on their enzymatic activity.**

Reporter gene assays have attracted attention in the development of methods to assay functional responses of 7TM receptor activation (for a recent review [77]). Early reporter constructs contained whole promoters of genes, which are regulated upon various stimuli [114-116]. An increasing amount of knowledge, e.g. regarding transcription factor binding sites and signaling pathways, has lead to the use of reporter constructs with synthetic promoters containing one or

several copies of a certain “response motif” [35, 36, 40]. Different response motifs are recognized by different transcription factors that in turn are activated by various intracellular signaling cascades and are hence used to monitor the activation of receptors that are coupled to different G-proteins. For example, receptors which couple to $G\alpha_s$ activation has usually been monitored with reporter constructs containing cAMP-responsive elements (CRE) [35].

Our interest in 7TM receptors displaying a high degree of sequence similarity to receptors for inflammatory mediators in the rhodopsin family led us to use response motifs that were sensitive to $Ca^{2+}$ elevation and MAPK activation (Paper I and II). The first attempts were made with constructs containing TPA-responsive elements (TRE). TPA is a phorbol ester used to stimulate protein kinase C. Constructs containing 1, 5 and 9 response motifs in front of either a minimal FOS or minimal CMV promoter were transiently transfected into different cell lines. HeLa cells turned out to be the cell line that showed the highest increase in luciferase activity when stimulated with phorbol ester. Reporter constructs containing 9 response motifs resulted in the highest increase in luciferase activity. Constructs equipped with the minimal FOS promoter displayed a smaller overall signal than those based on the minimal CMV promoter, and the relative increases upon stimulation were substantially higher. For these reasons stable reporter cell lines were established with constructs containing 9 TRE.

An useful reporter enzyme should display no background in eucaryotic cells, and it should also exhibit a single cell resolution in order to facilitate clone selection. Luciferases, which are often used as reporters, do not have any internal background in mammalian systems and are, hence, very useful in plate reader systems. However, since luciferase activity is analyzed in lysed cells detection at a single cell level is not possible at all.

In order to analyze reporter gene activity at a single cell level, β-galactosidase and β-lactamase have been used [117, 118]. Both require loading cells with substrate molecules, which are then converted to fluorescent products by the enzymatic activity of the reporter enzyme. Beside of the inconvenient loading procedure, cells have an intrinsic fluorescent background, which makes these techniques less sensitive when used in a plate reader.

Green-fluorescent protein (GFP), on the other hand, has an intrinsic fluorescent property due to its three-dimensional structure, which builds up a fluorophore consisting of three amino acids in tight proximity to each other. A variety of GFP variants with different absorbtion and emission spectra have been described [119-123]. GFP is easily detected at a single cell level using fluorescent microscopy or flow cytometry and it has been used in plate reader assays. However, due to the intrinsic fluorescence of the cells the signal window obtained is rather small [124].

In contrast, a fusion gene results in a new fusion protein that combines the advantageous properties of firefly luciferase and GFP. Thus, for the

construction of stable reporter cell lines a fusion gene was made and used as reporter gene. The GFP gene was placed upstream of the firefly luciferase gene. When expressed in mammalian cells both components retained their enzymatic properties. The newly designed reporter gene was placed downstream of the synthetic promoter, containing the 9 x TRE and the minimal CMV promoter. The resulting reporter plasmid was designated pcFUS2 (Figure 3).

The reporter construct was stably transfected into HeLa cells by electroporation. Due to the properties of the chimeric reporter enzyme, reporter gene activity could be measured both in luciferase assays, using a plate reader, and as a fluorescent signal using a fluorescence microscope or flow cytometry. The transfected cell clones showed considerable differences in their ability to respond to stimulation. This combined with the single cell resolution of the assay, allowed for a simple and convenient procedure to select especially well-responding reporter cell lines (Figure 2).

The selected reporter cell line was designated as HF1 (**H**eLa+pc**F**US2, cell clone #**1**) and was shown to increase luciferase activity up to 100 times when stimulated with PMA.

In further experiments, the ability of several receptors to increase reporter enzyme activity was investigated after stimulation by the respective agonist. For this purpose, the first leukotriene B4 receptor ($BLT_1$), the fifth CC chemokine receptor (CCR5) and the rat $\alpha_{1b}$ receptor were transfected into the HF1 reporter cell lines. These gave rise to the HF1pBLT1, HF1pCCR5 and HF1p$R\alpha_{1b}$ reporter cell lines.

The reporter cell lines were subsequently used in ligand stimulation experiments to obtain $EC_{50}$ values from the concentration-response curves. All tested reporter cell lines responded well to full agonist stimulation (CCR5/ RANTES 15 times, rat $\alpha_{1b}$ /epinephrine 50 times, and $BLT_1$/$LTB_4$ 200 times).
In contrast, other described reporter gene assays either pooled the resulting clones or, more often, tested a small number of clones in a rather laborious process [36, 115, 125]. The resulting reporter cell lines increased reporter protein activity at maximum stimulation by a factor of 3 to 20 times, when stimulated with a strong activator of PKC [36, 115].

In an approach to further expand the applicability of the assay described, the reporter construct was optimized (Paper II). Multiple copies of a newly designed response motif, which contained the consensus motifs of NF-κB and STAT binding sites, were introduced into the reporter construct. The reporter vector was further equipped with the minimal FOS promoter and the EGFP-luciferase fusion reporter gene (Figure 3). Stable cell lines were established by the clone election procedure described above. The cloning gave raise to the HFF11 cell line (**H**eLa+pc**F**US3 (**F**OS-promoter), cell clone #**11**). This cell line and the previously described HF1 cell line were used in studies aimed at comparing both cell lines. Experiments were performed using three different 7TM receptors as examples. The receptors were either expressed recombinantly

(complement C5a) or endogenously in both cell lines. It was found that the HFF11-based cell lines increased luciferase expression more strongly in response to stimulation than the HF1-based cell lines.

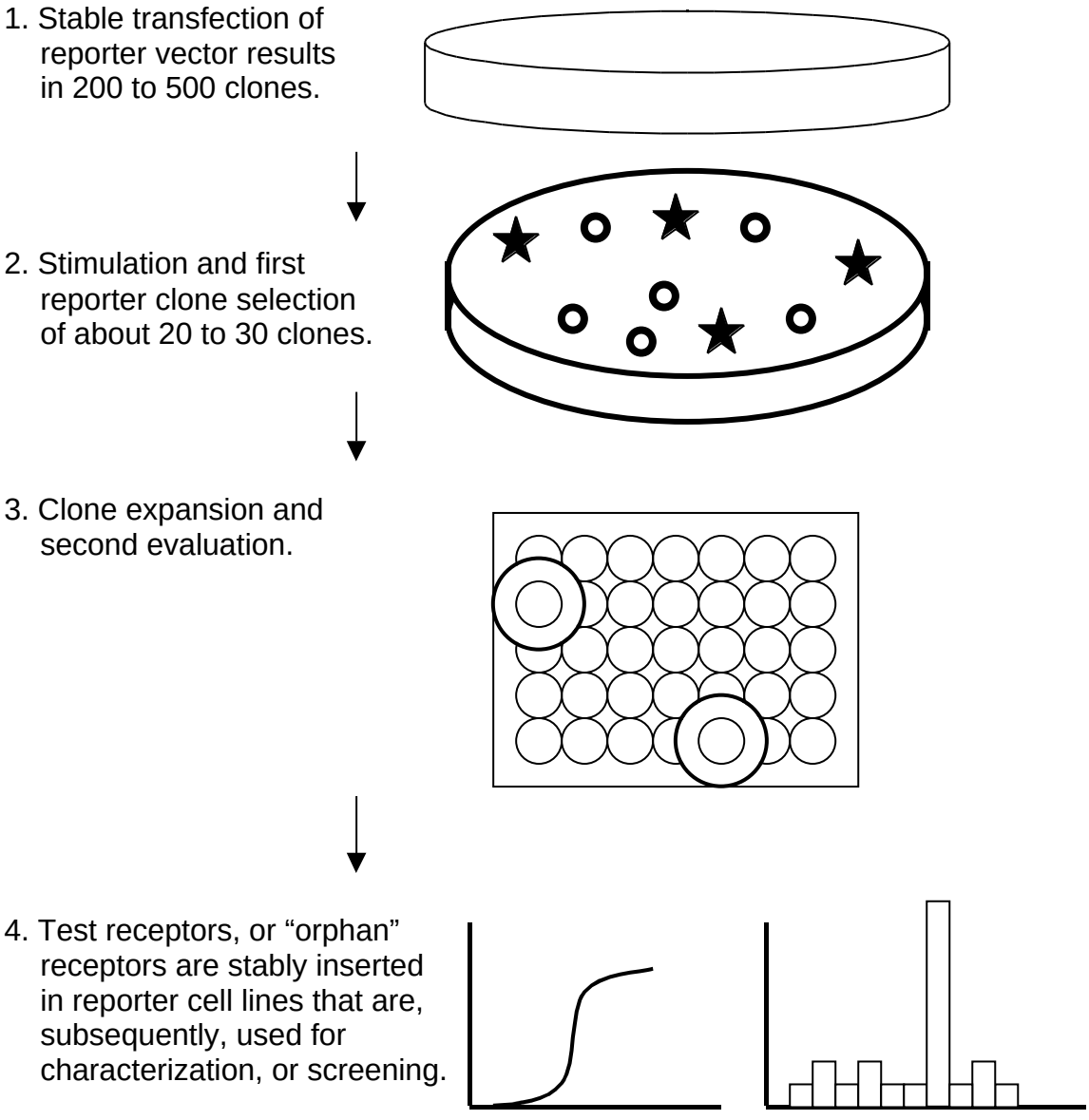


**Figure 2 illustrates the cloning and selection procedure of reporter cell lines for the described reporter gene assays.**

A further reporter construct was assembled to simplify the assay procedure. Due to their intracellular expression, firefly luciferase and many other reporter enzymes used in 96-well plate assays are analyzed in lysed cells. Other reporter enzymes, such as secreted alkaline phosphatase, can be analyzed in the supernatant of the cells [126, 127]. This simplifies the assay procedure in two ways: (i) it is not necessary to lyse the cells for analysis, which eliminates several steps in cell handling, and (ii) normalization can be performed for each

individual culturing well before and after ligand stimulation. This should improve assay quality.

Therefore, a reporter construct was designed containing two reporter enzymes, a secreted, genetically modified *Renilla* luciferase, and a second intracellular reporter, composed of EGFP and firefly luciferase (Figure 3). The expression of both enzymes was connected using an internal ribosomal entry site (IRES) [128, 129].

*Renilla* luciferase enzyme activity was easily detected in the cell supernatant. However, the expression of EGFP could not be detected using fluorescence microscopy or flow cytometery, even in stimulated reporter cells. The cell lysate displayed small, though detectable, amounts of firefly luciferase activity using a plate luminometer. This indicates that the IRES directed the translation machinery to the second, intracellular reporter enzyme in a rather inefficient way. The amount of intracellular reporter protein per cell was too small to allow for an efficient clone selection based on EGFP fluorescence.

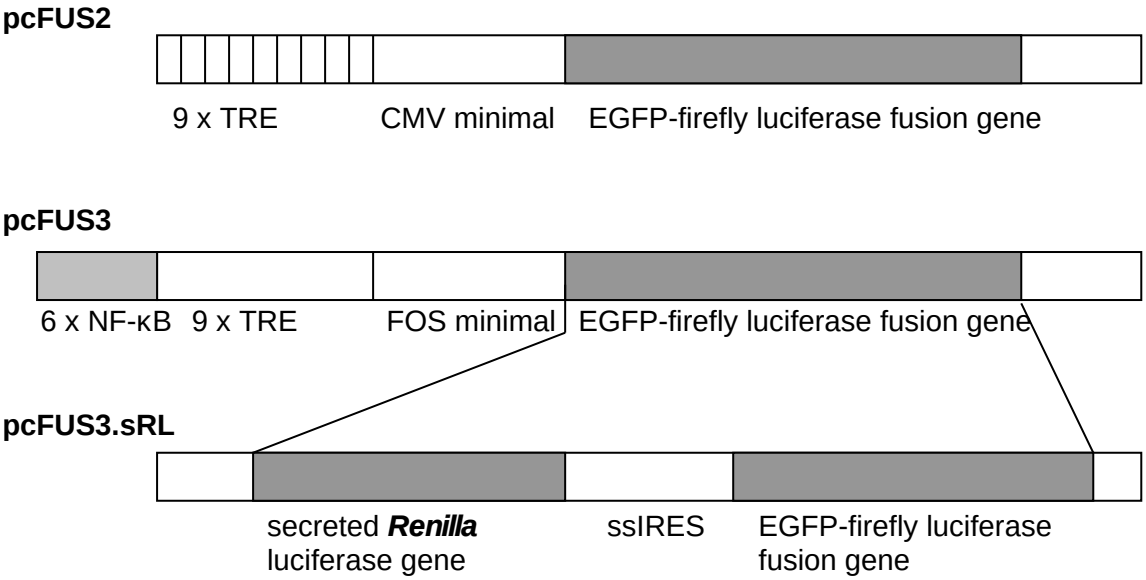


**Figure 3 shows the design of the reporter constructs used to establish the stable cell lines HF1 (pcFUS2), HFF11 (pcFUS3) and HR36 (pcFUS3.sRL).**

Among the 36 clones tested in an old-fashioned clone selection procedure, a clone named HR36 was chosen for comparison of two different assay procedures. In the first approach, cells grown in 96-well plates were exposed to different ATP-concentrations in order to stimulate endogenously expressed ATP-receptors. In a second approach, cells grown in batch culture were suspended using an EDTA solution and added to the ligands present in a 96-well plate. *Renilla* luciferase activity was analyzed in the supernatant. The

second approach simplifies assay performance as it involves fewer steps in cell handling.
Although the use of the secreted reporter enzyme increased the Z-factor [71] slightly to a value of around 0.9 for both approaches, the $EC_{50}$ values obtained were somewhat different for the two assay procedures tested. The reason for this is not fully understood. However, adding the reporter cells, grown in batch culture, to the ligands increased the assay sensitivity.

## The identification of two orphan 7TM receptors (Papers III and IV)

*A second leukotriene B4 receptor, $BLT_2$*

Leukotriene $B_4$ is an arachidonic acid metabolite that acts as a potent chemotactic mediator and is involved in inflammatory processes and diseases [130, 131]. This pharmacologically defined high affinity receptor was cloned [132], subsequently identified [111] and termed BLTR. However, reports suggested a second, low affinity receptor for $LTB_4$ in human granulocytes and murine spleen [133, 134]. It has been suggested that the presently described $BLT_2$ might be identical to this low affinity receptor [135]. However, it is still controversial whether the pharmacologically described low affinity leukotriene B4 receptor is a molecularly defined entity of its own or is rather a form of the already described BLTR (now termed $BLT_1$) [136, 137].

The second receptor for $LTB_4$, BLTR2 (now designated $BLT_2$ receptor [207]) was first identified *in silico* using a homology screening tool (Paper III). An expressed sequence tag (EST) was identified that showed a particularly high sequence homolgy to $BLT_1$. However, the clone from which the EST derived did not contain an ORF coding for a complete 7TM receptor. The C-terminus was lacking, as was revealed after sequence analysis of the complete insert. The fragmented receptor was not functional as it could not elicit $Ca^{2+}$ mobilization when transfected into HeLa cells.

Therefore, the missing part was cloned from a human leukocyte cDNA library and ligated into the earlier cloned receptor fragment. The resulting mRNA contained two possible start codons, resulting in a protein of 389 or 358 amino acids, respectively. The full-length cDNA was inserted into pcDNA3 and the resulting plasmid was used to transform HeLa cells. The transfected cells did, indeed, mediate $Ca^{2+}$ mobilization after stimulation with $LTB_4$, whereas a control vector (pcDNA3 containing the EGFP-gene) failed to do so. Increases in intracellular calcium were recorded using the aequorin method.

High expression of the receptor was detected in the liver, skeletal muscle, heart, spleen, kidney, and weaker levels occurred in the lung, placenta and pancreas. Either a 422 bp fragment or a 673 bp fragment was used as a probe. Both were derived from the 5´ untranslated region and the proximal parts of the gene.

The same receptor was cloned and published simultaneously by three other groups, who presented similar data [84, 135, 138]. Receptor activation was linked to an increase in intracellular $Ca^{2+}$ concentrations in both CHO and HEK cells, and it inhibited adenylate cyclase and mediated chemotaxis in CHO cells. The $EC_{50}$ concentrations were all above those reported for $BLT_1$. Hence, the receptor functions as a low-affinity receptor for $LTB_4$ [84, 135]. Binding studies in these reports further supported this hypothesis.

Later on it was reported that $BLT_2$ is also activated by a variety of ligands similar to $LTB_4$, whereas $BLT_1$ is more restricted and is only efficiently activated by $LTB_4$ [136, 138]. Ligands that activate $BLT_2$, include 12-oxoeicosatetraenoic acid, 12-(S)-hydroxyeicosatetraenoic acid, 12-(S)-hydroperoxyeicosatetraenoic acid, 15-(S)- hydroxyeicosatetraenoic acid and15-(S)-hydroperoxyeicosatetraenoic acid [136].

*The first cell surface receptor activated by free fatty acids, $FFA_1R$*

Fatty acids are carboxylic acids and found in all vertebrate cells. They contribute as building material to the cell membrane and serve as an energy storage medium when esterified (triacylglycerols). Also, they have for a long time been known to act as signaling molecules on the family of nuclear receptors known as peroxisome proliferator-activated receptors (PPARs) [139, 140].

Free fatty acids have an important role in the energy metabolism of many organs. The hormones glucagon and epinephrine stimulate, for example, cell surface receptors on fat cells, which in turn activate hormone-sensitive lipase. In the adipocytes the lipase releases fatty acids from triacylglycerols into the blood stream. The released fatty acids are bound to serum albumin and transported to skeletal muscles, the heart and to the renal cortex where they are degraded by β-oxidation.

Besides their physiological roles, free fatty acids are also involved in pathophysiological mechanisms [141, 142]. High plasma concentrations of FFAs have, for example, been connected to an increase in cancer mortality [143], insulin resistance in obese people [142] [144], and pathological changes in the endothelium [145].

The physiological actions of free fatty acids have usually been explained by either metabolic effects [146] or signaling through the nuclear receptors [139, 140, 147]. However, several reports question this [148] and evidence has accumulated indicating that cell surface receptor(s) may also be involved [149-152].

Using a reverse pharmacology approach, we tested ten reporter cell lines, each of which expressed a different orphan 7TM receptor, in a screen aimed to identify possible fatty acid receptors (Paper IV). In this approach, HFF11 reporter cell lines were used (Paper II). The cell line expressing the orphan receptor, GPR40 [47], showed a significant increase in luciferase activity when stimulated with 50μM linoleic acid (Z-factor 0.8 [71]). This cell line showed no increase in luciferase activity when stimulated with arachidonic acid, the precursor for leukotriene and lipoxin signaling molecules. The signal obtained with linoleic acid showed pertussis toxin sensitivity, indicating a coupling to the $G_i$ class of G-proteins.

Because GPR40 was earlier reported only as an ORF found on human chromosome 19q13.1 displaying sequence similarities to other 7TM receptors, the tissue expression was investigated using a multiple tissue northern blot. Expression of $FFA_1$ was detected in skeletal muscle, the liver and the heart. An apparently strong expression was also detected in pancreatic β-cells (Paper IV).

The initial results obtained from screening experiments were verified using the aequorin system. Thus, HeLa and CHO cells expressing the receptor transiently showed a light flash when stimulated with the agonist in a concentration-dependent manner. This further strengthened the idea that GPR40 was indeed a receptor for free fatty acids.

Numerous fatty acids and fatty acid derivatives were then tested using the GPR40 expressing HFF11 reporter cell line. Fatty acids with varying chain length and different degree of saturation or hydroxylation caused an increase in luciferase activity (Table 3). Short-chain fatty acids (C1-C4) had no effect on cell lines expressing GPR40 (up to 1 mM tested). Because of the activation of this receptor by a broad range of fatty acids along with its tissue distribution and its suggested physiological role, we proposed the name free fatty acid receptor, or $FFA_1R$. It is the first 7TM receptor that seems to serve a function as a "nutrient sensing receptor".

| **Trivial name** | **Systematic name** | **Abbreviation** | **$EC_{50}$ in μM Paper IV** | **$EC_{50}$ in μM [153]** | **$EC_{50}$ in μM [154]** |
|---|---|---|---|---|---|
| Capric acid | Decanoic acid | 10:0 | 12.6 | 14.1 | 43 |
| Lauric acid | Dodecanoic acid | 12:0 | 22.5 | 12.0 | 5.7 |
| Myristic acid | Tetradecanoic acid | 14:0 | 30.3 | 14.5 | 7.7 |
| Palmitic acid | Hexadecanoic acid | 16:0 | 143.2 | 5 | 6.8 |
| Oleic acid | ***cis***-9-Octadecenoic acid | 18:1($\Delta^9$) | 123.1 | 40.7 | 2.0 |
| Elaidic acid | ***trans***-9-Octadecenoic acid | 18:1($\Delta^9$) | 149 | 6.9 | 4.7 |
| Linoleic acid | ***cis***-9,***cis***-12-Octadecadienoic acid | 18:2($\Delta^{9,12}$) | 38.4 | 9.5 | 1.8 |
| Linolenic acid | ***cis***-9, ***cis***-12,***cis-***15-Octadecatrienoic acid | 18:3($\Delta^{9,12,15}$) | 27.1 | 12.5 | 2.0 |
| γ-Linolenic acid | ***cis***-6, ***cis***-9,***cis-***12-Octadecatrienoic acid | 18:3($\Delta^{6,9,12}$) | 28.5 | 8.9 | 4.6 |
| Stearidonic acid | ***cis***-6, ***cis***-9,***cis-***12,***cis***-15-Octadecatetraenoic acid | 18:4($\Delta^{6,9,12,15}$) | 8.9 | n.d. | n.d. |
| Arachidonic acid | ***cis***-5, ***cis***-8,***cis-***11,***cis***-14-Eicosatetraenoic acid | 20:4($\Delta^{5,8,11,14}$) | Not an agonist | 12.0 | 2.4 |

**Table 3. Medium to long chain free fatty acids are ligands to FFAR. Comparison of the $EC_{50}$-values as reported by (IV), [153] and [154]. n.d. not determined.**

The identification of GPR40 as a receptor for medium to long chain free fatty acids was independently described by two other groups [153, 154] and corroborated our main findings. Thus, GPR40 is activated by medium to long chain free fatty acids. There are, however, differences in the reports. The initial screens by Itoh *et al.* and Briscoe *et al.* were performed using the FLIPR system, which records changes in the intracellular $Ca^{2+}$ concentration. The $EC_{50}$ values reported differ noticeably (Table 3). The most remarkable differences included arachidonic acid, which failed to elicit a response mediated by $FFA_1R$ using the transcriptional assay (Paper IV). However, it displayed an $EC_{50}$ value at the μM-range in the other reports [153, 154]. While an experimental explanation of these differences is missing, one could speculate that differences in the assay system may have contributed.

Results of the tissue expression of $FFA_1R$ were almost identical. Although the strongest expression was reported from β-pancreatic cells [153, 154], expression was also detected in the spleen, the liver, the heart and the brain [153].

$FFA_1R$ plays an important physiological role as it regulates insulin secretion from pancreatic β-cells upon stimulation by free fatty acids [154]. In addition, certain fatty acid derivatives emphasize important physiological and patho-physiological functions of this receptor (Paper IV). 9-HODE and a conjugated linoleic acid (CLA) activated the receptor strongly. 9-HODE is a major component of oxo-low density lipoprotein [155] and is involved in the etiology of arteriosclerosis. CLA has attracted a lot of attention in cancer research and research of other life style diseases [156-159].

An even more important finding is the activation of $FFA_1R$ by MEDICA16 and thiazolidinedione-type compounds (TZDs), such as rosiglitazone [160] and MCC-555 [161]. Some of these TZDs were recently introduced as a treatment of type II diabetes. It is generally believed that PPARγ is an important target for these drugs (for review [139, 162]). However, PPARγ-independent effects have been reported [163-165], and the involvement of cell surface receptors has been suggested [166]. Earlier observations even showed a poor correlation between the potency of PPARγ activation and the anti-diabetic effects of these compounds [161].

MEDICA16 is an experimental drug with antidiabetogenic and hypolipidemic actions in the rat model. Its main site of actions is thought to be the liver, although drug actions have also been observed in the heart and in pancreatic β-cells [224, 225].

| **Re-ceptor** | **$FFA_1R$** | **PPARα** | **PPARδ** | **PPARγ** |
|---|---|---|---|---|
| **ligands** | Medium to long chain length; saturated and unsaturated fatty acids $C_{10-20}$; 9-HODE MEDICA 16 thiazolidinediones | Eicosanoids from lipoxygenase pathway: $LTB_4$; 8-HETE; fatty acids $C_{12-20}$ fibrates | fatty acids $C_{12-20}$ Prostaglandins triglycerides | Eicosanoids from lipoxygenase and cycloxygenase pathway: 15-HETE,15-deoxy-Δ12,14-prostaglandine J2; 9-HODE, 13-HODE, fatty acids $C_{12-20}$, Thiazolidinedione, MEDICA16 |

**Table 4. Ligand spectrum of cell surface and intracellular fatty acid receptors. Collected from Paper IV, [153, 167-174].**

Taken together, the activation of $FFA_1R$ by two different types of antidiabetic compounds suggests that $FFA_1R$ might serve as a drug target in the body. Its ligand spectrum is very similar to that found for PPARγ (Table 4), which hitherto has been considered to be the main target for the TZD drugs. Instead, it might be possible that both, the nuclear receptor and the cell surface receptor participate in the transmission of the drug actions. These findings might allow the development of specific agonists and antagonists acting specifically on their targets, thereby contributing to unravel the complicated physiology of the metabolic fatty acids.

# Conclusions

The identification of natural and surrogate ligands which act on 7TM receptors involve mainly cell based assay systems. Hitherto, reporter gene assays have not been used extensively in this process.

The construction of improved reporter gene assays was accomplished by optimizing the promoter region of the construct, the reporter enzyme used, and the assay procedure. Further, a fluorescence based clone selection step was introduced that enables the selection of the most sensitive reporter cell clones. The established test cell lines responded in a very sensitive way upon stimulation of various cell surface receptors. The amplification of reporter enzyme activity was substantially larger than in any earlier described system and the high assay quality makes it suitable as a primary screening tool. The resulting system concept suitable for mass-screenings of 7TM receptors, has been named “High-TRACE” (**high**-**t**hrough-**p**ut **r**eporter **a**ssay with **c**lone **e**lection). The described reporter gene assays turned already out to be useful for the identification of ligands acting on orphan 7TM receptors.

These approaches to identifying ligands for orphan 7TM receptors led to the discovery of the second leukotriene $B_4$ receptor, $BLT_2$ and the first cell surface, free fatty acid receptor $FFA_1$.

While the exact physiological role of $BLT_2$ is still a matter for debate, the identification of $FFA_1$ provides us with an explanation of the many physiological effects attributed to free fatty acids, which had hitherto remained unexplained. The activation of $FFA_1$ by TZD-type anti-diabetic drugs implies an important connection to type II diabetes and will subsequently contribute to the deepening of our understanding of the underlying mechanisms of this disease.

## Sammanfattning på svenska (Swedish Summary)

I alla flercelliga organismer behövs utbyte av information mellan cellerna, som bygger upp kroppen. För detta ändamål är cellerna försedda med små antenner, som kan ta emot signaler och kallas därför receptorer. Receptorer kan vara uppbyggda på olika sätt. De flesta receptorer slingrar sig sju gånger genom membranet och har ett gemensamt sätt att fortleda signaler till insidan av cellen via så kallade G-proteiner. De kallas därför 7TM (transmembran) eller G-protein kopplade receptorer. Dessa receptorer är mycket betydelsefula då de styr många fysiologiska funktioner i kroppen, men även eftersom de utgör måltavlor för många läkemedel. De är faktiskt de mål på cellytan, som flest läkemedel använder sig av.

I detta arbete beskrivs nya förfinade metoder att mäta signalfortledningen genom sådana receptorer (delarbeten I och II). Det kan användas för att identifiera substanser (läkemedel eller naturliga ämnen) som kan binda till, och aktivera receptorer.

Utöver detta beskrivs identifieringen av två nya receptorer (delarbeten III och IV), som ingår i superfamiljen. Den första receptorn blev inledningsvis identifierad från databasen för det humana genomet, som ett potentiell receptor med stor likhet med den redan kända leukotrien-receptorn, $BLT_1$. Vidare utnyttjades receptorn för att hitta den substans, som binder och aktiverar receptorn i kroppen. Substansen visade sig vara leukotriene $B_4$, en signalsubstans som frisätts när till exempel infektioner pågår och som rekryterar immunceller. Fysiologiska funktionen för den andra leukotriene $B_4$ receptorn, $BLT_2$ är ännu inte helt klarlagd.

Den andra receptorn är den första receptorn som aktiveras av fettsyror. Receptorn finns i många organ i kroppen som är förknippade med energiomsättningen och metabolism, såsom levern, muskelvävnad och hjärta. Men receptorn uttrycks också i de insulin-producerande celler, som finns i bukspottkörteln. Receptorn är inblandad i den insulin-frisättning, som äger rum efter en fettrik måltid.

Vad som gör receptorn extra betydelseful är, att även nya läkemedel mot diabetes typ II interagerar med receptorn. Det är möjligt att delar av läkemedelsinverkan överförs genom den nya receptor. Upptäckten kan på så vis påverka utvecklingen av nya och bättre antidiabetes-läkemedel och kanske förkorta utvecklingstiden.

# Deutsche Zusammenfassung (German Summary)

Während der Entwicklung neuer Wirksubstanzen in der Arzneimittelforschung verwendet man oft Massen-Screenings-Technologien, in denen tausende von potentiellen Wirksubstanzen an molekularen Zielstrukturen getestet werden. In unserem Körper liegen diese molekularen Zielstrukturen oft in der Oberfläche von Zellen. Dort nehmen sie ähnlich wie kleine Antennen Botschaften aus dem Körper entgegen. Man nennt solche Antennen auch Rezeptoren. In unserem Körper gibt es tausende, unterschiedliche Rezeptoren. Ein jeder erkennt einige wenige jedoch spezifische Signale und vermittelt Informationen in das Zellinnere. Zellen reagieren dann auf diese Signale, sie können dann selbst Signalsubstanzen freisetzen, sich bewegen, teilen oder wachsen.

Die grösste Klasse von Rezeptoren stellen die sieben-transmembranen (7TM), oder G-protein gekoppelten Rezeptoren dar, mit mehr als tausend Mitgliedern. Diese Rezeptoren winden sich siebenmal durch die Zellmembran und wechselwirken in der Zelle mit sogenannten G-proteinen. Weit mehr als die Hälfte aller heutzutage als Medikamente verwendeten Wirkstoffe beeinflusst solche Rezeptoren. Es ist also nicht verwunderlich, dass dieser Klasse von Rezeptoren und Analysemethoden für deren Aktivierung grosse Aufmerksamkeit geschenkt worden ist.

Die hier vorgelegte Dissertation besteht aus vier Teilarbeiten, von denen die ersten beiden Arbeiten neue und verbesserete Methoden zur funktionellen Analyse dieser Rezeptoren beschreiben. Teilarbeit 3 beschreibt die Identifizierung und Charakterisierung eines neuen Leukotriene $B_4$-Rezeptors $BLT_2$ und Teilarbeit 4 identifiziert GPR40 als den ersten Rezeptor für freie Fettsäuren und zeigt, dass dieser auch von einer Klasse antidiabetischer Arzneimittel (Thiazolidinedione) aktiviert wird.

Die ersten beiden Teilarbeiten beschreiben zellbasierte Werkzeuge, mit deren Hilfe man die Aktivierung von 7TM Rezeptoren analysieren kann. Die hier diskutierten Techniken erreichen eine grössere Empfindlichkeit und Zuverlässigkeit, als bisher beschriebene Methoden. Es handelt sich dabei um ein Reportergen-assay, in dem die Aktivierung von Zelloberflächenrezeptoren zur Steigerung der Transkription an einem Reportergen führt. Um diese Methode verwenden zu können braucht man einen Reportervektor. Dieser enthält als wichtigste Bestandteile einem Promotor und das Reportergen. In der vorliegenden Arbeit wurde der Reportervektor, das Reporterenzym und die Durchfuehrung der Teste optimiert. Es wurde ausserdem eine neue, verbesserte Methode zur Auswahl von Reporterzellklonen entwickelt und angewandt.

Der hier beschriebene Promotor enthält sogenannte TPA-empfindlichen Motife (TRE), kleine (ca. 8 Basenpaare) Stücke DNA, die von speziellen Transkriptionsfaktoren erkannt und gebunden werden können, wenn diese aktiviert wurden. Konstrukte mit einer unterschiedlichen Anzahl von TRE Motifen wurden getestet (1 x TRE, 5 x TRE und 9 x TRE). Konstrukte mit 9 solchen TRE Motifen zeigten die vorteilhaftesten Eigenschaften, wenn sie in Zellkulturen transfektiert wurden.

Als Reportergen wurde ein Fusionsgen, bestehend aus dem grünen fluoreszierendem Protein (EGFP) und der Glühwürmchen-Luziferase, verwendet. Dieses ermöglichte die Analyse von sowohl einzelnen Zellen, wie auch von Zellysaten mit hoher Empfindlichkeit.

HeLa Zellen wurden mittels Elektroporierung stabil mit dem ersten Reporterkonstrukt (pcFUS2) ausgestattet. Zellklone wurden selektiert und dann mit einem Phorbolester stimuliert, um Zellklone zu finden die auf die Stimulierung mit einem besonders grossem Anstieg in der Enzymaktivität reagieren. Die beste so gefundene Zellinie wurde HF1 genannt.

HF1 Zellen wurden mit verschiedenen Rezeptoren stabil transfektiert, um das System zu testen. Nach Stimulierung der Testrezeptoren konnte in jedem Fall ein bedeutender Anstieg der Luziferaseaktivität beobachtet werden, das Niveau variierte: Der Leukotriene $B_4$ Rezeptor $BLT_1$ steigerte die Enzymaktivität 200 mal, der $alpha_{1b}$ Rezeptor der Ratte steigerte sie rund 50 mal und der fünfte CC chemokine Rezeptor CCR5 rund 15 mal.
In einer Weiterentwicklung dieses Systems wurden Motife für andere Transkriptions-faktoren in den Promotor integriert, und zwar sechs NF-κB Motife und sechs STAT Motife.

Auch der neue Reportervektor (pcFUS3) verwendete das oben beschriebene Fusionsreportergen. Der Vektor wurde wiederum in HeLa Zellen stabil transfektiert und die beste Reporterzellinie wurde selektiert (HFF11). Diese und die oben beschriebenen HF1 Zellen wurden nun mit unterschiedlichen Rezeptoren getestet, die entweder schon in den Zellen vorhanden waren (ATP-Rezeptoreren und CXCR4) oder stabil eingesetzt wurden (C5a Rezeptoren). In allen getesteten Fällen waren HFF11-Reporterzellinien den vergleichbaren HF1 Zellinien überlegen.

Teilarbeit drei beschreibt die Konierung und Identifizierung eines bis dahin unbekannte 7TM Rezeptors, der grosse Sequenzähnlichkeit mit dem bereits bekannten Leukotriene $B_4$ Rezeptor (heute $BLT_1$ Rezeptor genannt) aufweisst. Auch dieser Rezeptor wird durch Leuktriene $B_4$ aktiviert. Zellen die diesen Rezeptor exprimierten, zeigten eine erhöhte intrazelluläre Kalzium-freisetzung, wie mit der Kalzium-abhängigen Luziferase Aequorin nachgewiessen werden konnte. Der neue Rezeptor wird heute als $BLT_2$ Rezeptor bezeichnet.

Teilarbeit vier beschreibt die Identifizierung des ersten Oberflächenrezeptors für freie Fettsäuren. Zehn verschiedene Testrezeptoren, ohne bekannte Liganden, wurden in HFF11 Zellen eingesetzt. Die so gewonnenen Zellinien wurden zur Suche nach ihren körper-eigenen Signalmolekülen (Liganden) benutzt. Wurden diese Zellinien mit 50 µM Linolsäure behandelt, so zeigten nur Zellen, die den Rezeptor namens GPR40 enthielten, einen Anstieg der Luziferaseaktivität. Es konnte weiterhin gezeigt werden, dass dieser Rezeptor durch eine grosse Vielfalt von Fettsäuren aktiviert wird, die eine Kettenlänge von 10 bis 18 Kohlenstoffatomen besitzen können. Sowohl gesättigte als auch ungesättigte Fettsäuren und sogar hydroxylierte Fettsäuren aktivierten diesen Rezeptor. Der vorgeschlagene Name lautet deshalb $FFA_1$ Rezeptor (Free Fatty Acid 1 Receptor = erster Rezeptor fuer freie Fettsäuren). Wurde $FFA_1R$ mit der Kalzium-empfindlichen Luziferase

Aequorin in Zellen exprimiert, so konnte ein Anstieg der Kalziumkonzentration nach einer Behandlung mit Linolsäure nachgewiessen werden.

$FFA_1R$ gibt es in mehren Organen, die eng mit der Enegieumsetzung und dem Stoffwechsel verbunden sind, zum Beispiel dem Herzen, der Leber und Skelettmuskelzellen. Auch Beta-Pankreaszellen enthalten diesen Rezeptor. Dieser Rezeptor ist fuer die Erhöhung der Insulinfreisetzung nach einer fettreichen Mahlzeit verantwortlich [154]. Wir schlugen deshalb vor, diesen Rezeptor als den ersten, beschriebenen Nährstoff-Sensor („nutrient sensing receptor“) zu bezeichnen.

Durch das Ligandspektrum und das Expressionsmuster stimuliert, wurden verschiedene Diabetesarzneimittel und experimentelle Substanzen getestet. Im Reportergen Assay stimulierten das Fettsäurederivate MEDICA16 und die Thiazolidinedione Rosiglitazone und MCC-555 den Rezeptor stark. Alle diese Substanzen verbessern den Krankheitsverlauf von Diabetes II im Tierversuch. Eine dieser Substanzen (Rosiglitazone) ist darueber hinaus auch zur Behandlung von Diabetes II und dessen Folgeerkrankungen beim Menschen zugelassen. $FFA_1R$ ist damit ein Ziel für Antidiabetika, neben dem nukleären Rezeptor für freie Fettsäuren PPARγ. Der Rezeptor ist damit eng mit der Behandlung von Typ II Diabetes verbunden. Der Fund dieses molekularen Zieles fuer antidiabetische Wirkstoffe duerfte die Entwicklung neuer, besser Antidiabetesmedikamente vereinfachen und beschleunigen.

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227.

**APPENDIX 1 lists some of human, orphan 7TM receptors identified in the last 5 years, their ligands, and the primary screening technique used. *indicates the identification of the rat receptor only.**

| Ligand type | Receptor name | Synonyms | Accession number | Ligand(s) | Identified using | Reference |
|---|---|---|---|---|---|---|
| peptide | (C5L2) | C5L2, GPR77 | NM 018485 | C5a, $C5a_{desArg}$ | β-hexosaminidase release | [70] |
| | (NPW R) | GPR7 | NM 005285 | NP B | Expression cloning | [107] |
| | | GPR7 andGPR8 | U22491, U22492 | NP W23=L8 NP W30=L8C | Purification of extracts; cAMP-detection by immunoassay | [175] |
| | | GPR7 and GPR8 | U22491, U22492 | L8=NP W23, L8C=NP W30, L7, L7C | $Ca^{2+}$ mobilization (Aequorin) | [176] |
| | (SNS1-6 R) | hMrgX3, hMrgX1,hMrgX4 | AF474987 - AF474992 | BAM22 | Reverse pharmacology; $Ca^{2+}$-mobilization (FLIPR) | [97] |
| | | MrgA1, MrgC11 | NM 153095 NW 000319 | RF-amid-related peptides | Reverse Pharmacology; $Ca^{2+}$ mobilization (FURA-2) | [98] |
| | (LGR7)+ (LGR8) | GPR106 | AF403384 NM 021634 | Relaxin | cAMP-detection by immunoassay | [83] |
| | (LGR8) | GPR106 | | Leydig Insulin like peptide (ISNL3) | cAMP-detection by immunoassay | [177] |
| | PK-R2 | | AF506288 | Prokineticin 2 | | [178] |
| | PK-R1+ PK-R2 | ZAQ+ I5E | AY089976 | EG-VEGF/ prokineticins 1 and 2 | Purification of extracts; $Ca^{2+}$-mobilization (FLIPR) | [179] |
| | (GPR54) | OT7T175, AXOR12, GPR54 | AY029541 | KiSS-1 peptids | Purification of extracts; $Ca^{2+}$-mobilization (FLIPR) | [103] |
| | (GPR54) | OT7T175, AXOR12, GPR54 | AY029541 | KiSS-1 peptide | Reverse pharmacology; $Ca^{2+}$-mobilization (FLIPR) | [99] |
| | (GPR54) | OT7T175, AXOR12, GPR54 | AY029541 | Kisspeptins | Purification of extract; $Ca^{2+}$ mobilization (FURA-2) | [180] |
| | MCH-R2 | | AF399937 | Melanin-concentrating-hormone (MCH) | Radioligand-binding assay | [181] |
| | MCH-R2 | | AY029596 | MCH | $Ca^{2+}$-mobilization (FLIPR) | [58] |
| | MCH-R2 | | AY029596 | MCH | $Ca^{2+}$-mobilization (FLIPR) | [182] |
| | MCH-R2 | | AY029596 | MCH | $Ca^{2+}$ mobilization (Aequorin) | [183] |
| | MCH-R1 | SLC-1, GPR24 | AB063174 | MCH | Purification of extract; $Ca^{2+}$ mobilization (FLIPR) | [104] |
| | MCH-R1 | SLC-1, GPR24 | AB063174 | MCH | Reverse Pharmacology; $Ca^{2+}$ mobilization (FLIPR) | [100] |

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| | MCH-R1 | SLC-1, GPR24 | AB063174 | MCH | Reverse Pharmacology; $Ca^{2+}$ mobilization (FLIPR) | [184] |
| | MCH-R1 | SLC-1, GPR24 | AB063174 | MCH | Purification of extract; Xenopus electro-physiological assay | [86] |
| | NPFF R | HLWAR77 | AF257210 | Neuropeptide sFF and AF | Reverse Pharmacology; $Ca^{2+}$ mobilization (FLIPR) | [185] |
| | NPFF1R and NPFF2R | | | NPFF | Reverse Pharmacology; Xenopus electro-physiological assay | [87] |
| | NPFF R | HLWAR77 | AF257210 | NPFF and related peptides | Reverse Pharmacology; $Ca^{2+}$ mobilization (Aequorin) | [186] |
| | | OT7T022 | AB040104 | RFamide peptides (RFRP) | Cytosensor | [187] |
| | CCR10 | GPR2 | NM 016602 | CCL27 (CTACK) | $Ca^{2+}$ mobilization (FURA-2) | [188] |
| | CCR10 | GPR2 | NM 016602 | CCL27 (ESkine) | $Ca^{2+}$ mobilization (FURA-2) | [189] |
| | CCR10 | GPR2 | NM 016602 | CCL28 | $Ca^{2+}$ mobilization (INDO-1) | [190] |
| | CXCR6 | Bonzo, STRL33, TYMSTR | NM 006564 | CXCL16 | Expression cloning of receptor; binding assay | [191] |
| | CXCR6 | Bonzo, STRL33, TYMSTR | NM 006564 | CXCL16 | Expression cloning of ligand; Chemotaxis assay | [192] |
| | XCR1 | GPR5 | NM 005283 | XCL1 (lymphotactin) | Chemotaxis assay | [193] |
| | $CX_3CR1$ | V28 | | CX3CL1 (fractalkine) | Binding assay | [194] |
| | NMU1R+ NMU2R | GPR66, FM-3+ FM-4 | NM 020167 AF272362 | Neuromedin U | Reverse Pharmacology; $Ca^{2+}$ mobilization (FLIPR) | [42] |
| | NMU1R | GPR66, FM-3 and TGR-1 | AB041228 | Neuromedin U | Microphysiometer | [41, 43] |
| | NMU1R | GPR66, FM-3 | AB041228 | Neuromedin U | Reverse Pharmacology; $Ca^{2+}$ mobilization (FLIPR) | [195] |
| | NMU1R | FM-3, GPR66 | AB041228 | Neuromedin U | Reverse Pharmacology; $Ca^{2+}$ mobilization (FLIPR) | [45] |
| | NMU1R | FM-3, GPR66 | AB041228 | Neuromedin U | Purification of extract; $Ca^{2+}$ mobilization (FLIPR) | [44] |
| | | GPR14, (rat SENR) | NM 019498 | Urotensin II | Reverse Pharmacology; $Ca^{2+}$ mobilization (FLIPR) | [196] |
| | (UII-R1a) | GPR14, (rat SENR) | NM 019498 | Urotensin II | $Ca^{2+}$ mobilization (Aequorin) | [197] |
| | | GPR14, (rat SENR) | NM 019498 | Urtensin II | Purification of extract; $Ca^{2+}$ mobilization (FLIPR) | [198] |
| | OX1R and OX2R | HFGAN72 | NM 001525 NM 001526 | Orexin-a and -b | Purification of extract; $Ca^{2+}$ mobilization (FURA-2) | [199] |

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| | | GHS-R | Q95254 | Ghrelin | Purification of extract; $Ca^{2+}$ mobilization (FLIPR) | [106] |
| | MTL-R1 | GPR38 | NM 001507 | Motilin | Reverse Pharmacology; $Ca^{2+}$ mobilization aequorin | [200] |
| | APJ R | APJ | NM 005161 | Apelin | Purification of extract; Microphysiometer | [95] |
| | | hGR3, | AB015745 | Prolactin releasing peptide (PrRP) | Purification of extract; arachidonic acid release | [201] |
| Lipids and lipid com-pounds | | | | | | |
| | (GPR63) | GPR63 | Q9EQQ3 | Sphingosine 1-phosphate (S1P); dihydrosphingosine 1-phosphate (DHS1P) | Reverse pharmacology; $Ca^{2+}$ mobilization (FLIPR) | [202] |
| | ($FFA_1R$) | GPR40 | NM 005303 | Medium to long chain fatty acids | Reverse pharmacology; reporter gene assay | Paper IV |
| | | GPR40 | NM 005303 | Medium to long chain fatty acids | Reverse pharmacology; $Ca^{2+}$ mobilization (FLIPR) | [153] |
| | ($FFA_2R$) ($FFA_3R$) | GPR43 GPR41 | AR126790 NM 005304 | Short chain fatty acids | Yeast assay | [90] |
| | ($FFA_1R$) | GPR40 | NM 005303 | Medium to long chain fatty acids | Reverse pharmacology; $Ca^{2+}$ mobilization (FLIPR) | [154] |
| | ($FFA_2R$) | GPR43 | NM 005303 | Short chain fatty acids | Reverse pharmacology; reporter gene assay | [226] |
| | | TG1019 | AC013396 .3 | 5(S)-oxo-6E,8Z,11Z,14Z-eicosatetraenoic acid (5-oxo-ETE); arachidonic acid; | GTPγS-binding on receptors expressed in insect cells | [203] |
| | $DP_2R$ | CRTH2, GPR44 | NP 034092 | Prostaglandin $D_2$ | $Ca^{2+}$ mobilization | [223] |
| | ($S1P_6$,$S1P_7$, $S1P_8$) | GPR3,GPR6 ,GPR12 | NM 005281 NM 005284 NM 005288 | Sphingosine 1-phosphate (S1P); dihydrosphingosine 1-phosphate (DHS1P) | Reverse pharmacology; $Ca^{2+}$ mobilization (FLIPR) | [85] |
| | ($S1P_8$) | GPR12 | NM 005288 | Sphingosylphosphorylcholine (SPC) | $Ca^{2+}$ mobilization (aequorin) | [204] |
| | ($SPC_2R$) | GPR4 | NM 005282 | Sphingosylphsphorylcholine (SPC); Lysophosphatidylcholine (LPC) | $Ca^{2+}$ mobilization (FURA-2, spectrofluorometer) | [78] |
| | (LPC R) | G2A | NM 013345 | Lysophosphatidylcholine (LPC), Sphingosylphsphorylcholine (SPC) | $Ca^{2+}$ mobilization (FURA-2, spectrofluorometer) | [205] |
| | (PSY R) | TDAG8 | NM 003608 | Psychosine (D-galactosyl-β- | Reverse pharmacology; $Ca^{2+}$ mobilization | [206] |

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| | | | | 1,1´sphingosine) and related substances | (INDO-1, fluorometer) | |
| | ($SPC_1$ R) | OGR-1 | | Sphingosyl-phosphorylcholine (SPC); | $Ca^{2+}$ mobilization (FURA-2, spectrofluorometer) | [113] |
| | $BLT_2$ R | BLTR2, JULF2, | AB029892 | $LTB_4$ | $^3$H-$LTB_4$ binding | [135] |
| | $BLT_2$ R | BLTR2, JULF2, | AF277230 | $LTB_4$ | $Ca^{2+}$ mobilization (Aequorin) | Paper III |
| | $BLT_2$ R | BLTR2, JULF2, | AB044402 | $LTB_4$ | cAMP-immunoassay | [84] |
| | $BLT_2$ R | BLTR2, JULF2, | AF038571 | $LTB_4$ | Radioligand-binding assay | [138] |
| | $BLT_1$ R | BLTR, CMKRL1, P2y7 | D89078 | $LTB_4$ | Expression cloning of the receptor; radioligand binding | [111] |
| | $BLT_1$ R | BLTR, CMKRL1, P2y7 | | $LTB_4$ | $Ca^{2+}$ mobilization (FURA-2) | [208] |
| | $CysLT_2$ | HG57, PSEC0146 | AF254664 | $LTC_4$=$LTD_4$ | Xenopus oocyte $Cl^-$ fluxes | [88] |
| | $CysLT_2$ | HG57, PSEC0146 | AB038269 | $LTC_4$=$LTD_4$ | $Ca^{2+}$ mobilization (FLIPR) | [209] |
| | $CysLT_1$ | HG55, | AF119711 | $LTD_4$ | Xenopus oocyte $Cl^-$ fluxes | [89] |
| | $CysLT_1$ | HG55 | AF119711 | $LTD_4$ | Reverse pharmacology; $Ca^{2+}$ mobilization (FLIPR) | [210] |
| | $LPA_3$ | Edg-7, HOFNH30 | NP_036284 | Lysophosphatidic acid, Phosphatidic acid | $Ca^{2+}$ mobilization (FLIPR) | [56] |
| | $S1P_4$ | Edg-6, | CAA04118 | Sphingosine 1-phosphate (S1P), Sphingosyl-phosphorylcholine (SPC) | $^3$H-S1P binding and displacement | [211] |
| | $S1P_3$ | Edg-3, | NP_005217 | Sphingosine 1-phosphate (S1P), Sphingosyl-phosphorylcholine (SPC) | $^3$H-S1P binding and displacement | [212] |
| | $LPA_1$ | Edg-2, Vzg-1 | NP_476500 | Lysophosphatidic acid | Yeast reporter gene assay | [213] |
| | $S1P_1$ | Edg-1 | | Sphingosine 1-phosphate (S1P), | $^3$H-cAMP assay | [214] |
| | $S1P_1$ | Edg-1 | NP_001391 | Sphingosine 1-phosphate (S1P), | Purification of extract; Morphological changes in cell growth | [105] |
| Nucleotide type | $P2Y_{13}$ | SP174, GPR86, GPR94 | | Adenosine-diphosphate (ADP) | $Ca^{2+}$ mobilization (FLIPR) | [215] |
| | $P2Y_{13}$ | SP174, GPR86, GPR94 | AF406692 | Adenosine-diphosphate (ADP) | $IP_3$-turnover | [216] |
| | $P2Y_{12}$ | SP1999 | | Adenosine-diphosphate (ADP) | Expression cloning of the receptor; Xenopus oocytes electrophysiological assay | [217] |

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| | $P2Y_{12}$ | SP1999 | AF321815 | Adenosine-diphosphate (ADP) | Purification of extract; $Ca^{2+}$ mobilization (FLIPR) | [218] |
| | $P2Y_{14}$ | KIAA0001 UDP-glc R | D13626 | Uridine 5´-diphosphoglucose (UDP-glc) | Reverse pharmacology; yeast growth | [93, 112] |
| | ($rAA_1$ R) | | AJ311952 | adenine | Purification of extracts; $Ca^{2+}$ mobilization (FLIPR) | [219] |
| Amine | $TA_1$ R | | | Tyramine, β-phenyethylamine (β-PEA) (Trace amines) | Xenopus oocytes; electrophysiological assay | [220] |
| | H4 R | GPRv53, AXOR53 | AB044934 | Histamine | Reporter gene assay | [81] |
| | H4 R | AXOR35,GPRv53 | AF325356 | Histamine | Reporter gene assay | [82] |
| Others | | HM74 and HM74A | AY148884 | Nicotinic acid | GTPγS-binding | [59] |
| | | HM74 | | Nicotinic acid | Expression cloning; $Ca^{2+}$ mobilization (Aequorin) | [221] |
| | | HM74b | AB103062 | Nicotinic acid | Fluorescent cAMP-assay | [227] |
| | | TGR5 | AB089307 | Bile acids | Reverse Pharmacology; Reporter gene assay | [222] |

# PAPERS I-IV

“Äsch! Några enkla idéer bara.”

*Skalman*

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