1 INTRODUCTION

1.1 G Protein-Coupled Receptors – An Overview

Probably the most important proteins connecting the cell with environmental influences are the G protein-coupled receptors (GPCRs). They accept a vast array of stimuli including light, neurotransmitters, odorants, pheromones, nucleotides, amino acids, biogenic amines, lipids, hormones or chemokines (Bockaert and Pin, 1999). GPCRs are involved in a diversity of biological processes:

- Visual sense: photons lead to photoisomerization reactions of chromophor in opsin
- Sense of smell: receptors in the olfactory endothelium bind odorants and pheromones
- Behavior and mood regulation: binding of neurotransmitters, *e.g.* serotonin and dopamine
- Immune system: chemokine receptors enable intercellular communication of immune cells or histamine receptors, which interact with inflammatory mediators, summon target cells for inflammatory response.

GPCRs have high scientific importance because of their widespread localization and functionality in almost every organism. In most vertebrates ≥ 1% of the genomes code for GPCRs (Bockaert and Pin, 1999). They also have been found in invertebrates (fly, nematodes), plants (*Arabidopsis thaliana*), and protozoa (amoeba, yeast).

The Human Genome Project identified so far 375 non-olfactory and 347 olfactory GPCRs. 160 non-olfactory receptors were annotated as functionally uncharacterized: the orphan GPCRs (Foord, 2002). These orphan receptors are of high pharmacological interest as new drug targets.

GPCRs represent also therapeutic drug targets of choice in cancer, cardiac dysfunction, diabetes, inflammation, pain, etc. For this reason, they are currently the target of 40 to 50% of modern pharmaceuticals (Filmore, 2004), although only 10% of GPCRs are known drug targets so far (Vassilatis *et al.*, 2003).

1.1.1 Classification of G Protein-Coupled Receptors

One of the major goals in GPCR research is the generation of more structural data at atomic level. So far, the structure of only one GPCR is known: the X-ray crystallographic structure of bovine rhodopsin (Palczewski *et al.*, 2000; Stenkamp *et al.*, 2002; Teller *et al.*, 2001). It consists of an extracellular N-terminus, 7 membrane-spanning helices (serpentine domain) and an intracellular C-terminus (see Fig. 1-1). Structural information on other GPCRs is based on their homology to rhodopsin.

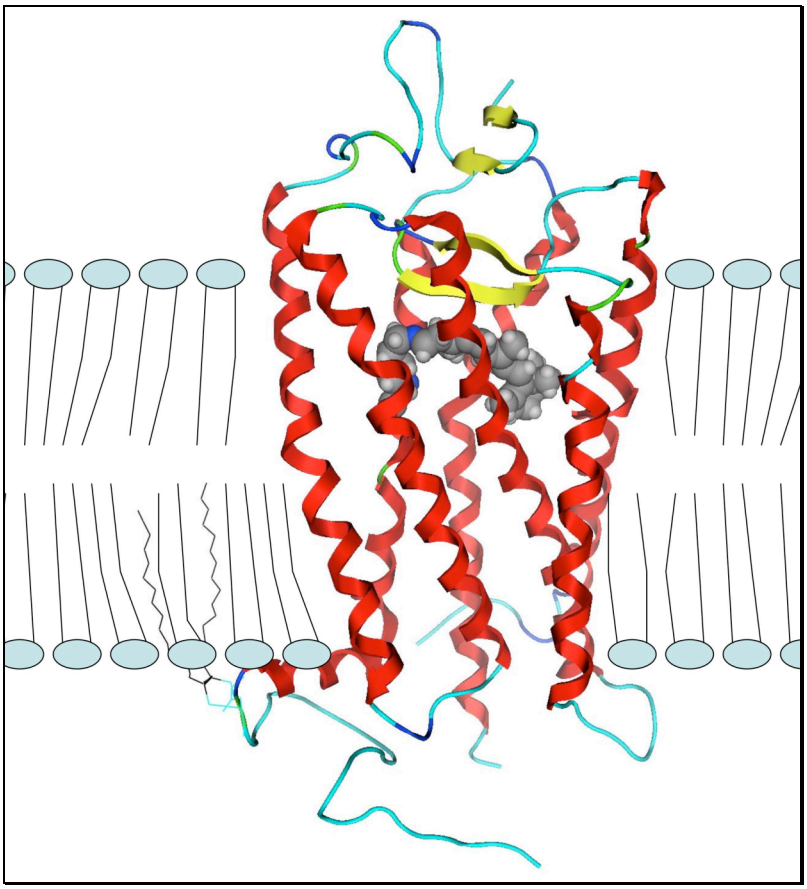

Figure 1-1:
The backbone structure of bovine rhodopsin (PDB entry: 1HZX) and its probable orientation in the cellular membrane: A bundle of 7 membrane-spanning helices (red) is connected by 3 extracellular loops ECL1, ECL2, ECL3 and 3 intracellular loops ICL1, ICL2, ICL3. The highly structured extracellular N-terminus interacts with the extracellular loops. The intracellular C-terminus forms an 8th helix, which is tethered to the membrane by palmitoylated cysteine residues.

Based on sequence similarity all known GPCRs were classified into 5 clans (Attwood and Findlay, 1994; Gether, 2000; Kolakowski, 1994; Kristiansen, 2004):

- Clan A: the rhodopsin-like receptors
- Clan B: the secretin-like receptors
- Clan C: the metabotropic glutamate receptors
- Clan D: the cAMP receptors
- Clan E: the fungal mating pheromone receptors

In a second classification system 342 unique, non-olfactory, human GPCRs were classified into 5 families (Fredriksson *et al.*, 2003):

- Family G: the glutamate receptor family
- Family R: the rhodopsin receptor family
- Family A: the adhesion receptor family
- Family F: the frizzled/taste2 receptor family
- Family S: the secretin receptor family

Common to both classification systems is a group of rhodopsin-like receptors. Model structures of GPCRs, which are not closely related to rhodopsin, are keenly discussed and quite often not well accepted because of the low sequence similarity. The small amount of structural information on the superfamily of GPCRs leads to a large involvement of bioinformatics and molecular modeling approaches in order to understand the action of GPCRs.

The GPCRs of this study – the endothelin receptor subtypes ETA and ETB as well as the nicotinic acid receptors GPR109A and GPR109B – are members of the rhodopsin-like GPCRs in both classification systems and thus suitable targets for homology modeling.

1.1.2 The Mechanism of Action of G Protein-Coupled Receptors

Initiation of Activation

Some receptors were pre-assembled with prosthetic groups. Rhodopsin (a member of the opsin-receptors) has the chromophor 11-cis-retinal covalently bound by a protonated Schiff base linkage to a lysine residue within the transmembrane binding cleft (Gether, 2000; Sakmar, 1998). The extracellular portions of the receptor protect the transmembrane binding cleft against influences from the environment by forming a lid: ECL2 covers the binding cleft, whereas the N-terminus is on top of this loop (Fig. 1-1). The initial step in activation of the opsin-receptors is a photoisomerization of the chromophor. The isomerization product, all-trans-retinal, behaves as an agonist and activates the GPCR. The pre-assembled 11-cis-retinal behaves as an antagonist, with negative intrinsic activity. It restrains the receptor in an inactive state.

Most other GPCRs do not have a pre-assembled ligand (*e.g.* the GPCRs of this study). The inactive state of the receptor has to be restrained by interactions of amino acid side chains, which usually appear in the transmembrane region. In the process of ligand interaction at the receptor, the initiation of activation takes place either by inducing a conformational change to the active state of the receptor or by stabilization of the receptor's active conformation.

GPCRs that are selective for small ligands (*e.g.* the biogenic amine receptors) may have similar extracellular portions like rhodopsin. The ligands of these receptors are small and flexible enough to pass these portions. Similar to retinal in rhodopsin, these ligands use a binding crevice buried into the transmembrane region, where no interactions to the extracellular loops or the N-terminus are established. For example, the binding cavity in the β2-adrenergic receptor is located between transmembrane helices TMH3 (Strader *et al.*, 1991), TMH5 (Strader *et al.*, 1989), TMH6 (Tota *et al.*, 1990; Wieland *et al.*, 1996) and TMH7 (Suryanarayana *et al.*, 1991).

GPCRs using larger ligands (*e.g.* peptides) need to have different extracellular portions than rhodopsin. Depending on the receptor, several transmembrane helices were experimentally shown to be relevant for ligand interaction (*e.g.* in endothelin receptor subtype A where TMH2 (Lee *et al.*, 1994b; Webb *et al.*, 1996), TMH3 (Breu *et al.*, 1995; Lee *et al.*, 1994a) and TMH6 (Breu *et al.*, 1995) are involved into ligand binding). The structures of the extracellular portions of those GPCRs are discussed as being different from the rhodopsin template, offering additional interaction patterns for peptidic ligands (Gether, 2000). Nevertheless, the structural information of rhodopsin highly suggests interactions between the extracellular portions and the existence of a disulfide bridge network. At least one disulfide bridge is conserved in the rhodopsin-family, tethering ECL2 at transmembrane helix TMH3. In several receptors, site-directed mutation experiments of the cysteine residues involved in this disulfide bridge resulted in decreased ligand binding and/or misfolded receptors (Dohlman *et al.*, 1990; Fraser, 1989; Perlman *et al.*, 1995; Savarese *et al.*, 1992). The restricted position of ECL2 seems to be more relevant for the GPCR than those of the other extracellular loops or the N-terminus. Additionally, the N-terminus of certain GPCRs may be cleaved by proteases during ligand binding as experimentally demonstrated for the endothelin receptor subtype B (Grantcharova *et al.*, 2002).

Depending on the GPCR, its patterns of ligand recognition may be localized only in the transmembrane region (for those interacting with small ligands) or may be distributed at both the transmembrane region and the extracellular portions.

Transmission of the Activation Signal Into the Cell

Following the initiation of activation, intramolecular changes including side chain movements, rearrangements of hydrogen bonds and even rotations of one (TMH6) or two transmembrane helices (TMH3 and TMH6) lead to the transmission of the extracellular signal to the cytoplasmic side (Ballesteros *et al.*, 2001a; Singh *et al.*, 2002). In addition, conformational changes of the transmembrane region are propagated to the intracellular loops

structures of these receptors. Thereby, these loops move and expose a binding domain with a hydrophobic core motif in the neighborhood of TMH6 for the Gα C-terminus of trimeric G proteins (Janz and Farrens, 2004). These trimeric G proteins are complexes of a Gα-subunit (where guanosine diphosphate is bound) and a Gβγ-complex.

Activation of G Proteins Inside the Cell

Binding to the active state of a GPCR activates the trimeric G protein and initiates the exchange of guanosine diphosphate (GDP) to guanosine triphosphate (GTP) within the Gα subunit. This process is followed by conformational changes at the interface of Gα and Gβ and result in dissociation of Gα, Gβγ and the GPCR. Gα and Gβγ now regulate downstream signaling. The intrinsic hydrolytic function of the Gα subunit hydrolyzes GTP to GDP and phosphate (P_i), leading to the formation of an inactive Gα-protein and the re-assembly of Gα and Gβγ into the trimeric G protein.

Many steps of this simple-looking process are not understood. One of the most interesting is the selectivity between GPCRs and G proteins, which is hard to investigate because of the high amount of different G protein-subunits. In humans, 18 different Gα-, 5 Gβ- and 11 Gγ-subunits were found (Hermans, 2003). Another reason is the unresolved mechanism of GPCRs to select between the different combinations of Gαβγ-complexes. For characterization of the receptor's function it is necessary to know that every receptor (and even receptor subtype) has its own favorite G protein-interaction (Wess, 1998).

1.1.3 G Protein-Signaling

Based on the differences in signaling, G proteins were separated into different families. Due to the strong signaling effects mediated by Gα-proteins, investigation of trimeric G proteins is mainly based on studies of these subunits. Gα subunits are separated into four main families: Gαs, Gα12/13, Gαq and Gαi. In general, the folds of these proteins are conserved as pointed out in their structures (PDB ID codes: 1GIA (Coleman *et al.*, 1994), 1GG2 (Wall *et al.*, 1995), 1GP2 (Wall *et al.*, 1995) for Gαi and 1AZS (Tesmer *et al.*, 1997), 1AZT (Sunahara *et al.*, 1997), 1CJK (Tesmer *et al.*, 1999) for Gαs). However, their mechanisms of selective interaction with GPCRs and in regulation of other target proteins are still not completely solved. Trimeric G proteins are known to affect several intracellular target proteins. Many of them generate second messengers – molecules activating, modulating or inhibiting various other proteins. The use of these signaling cascades results in a very fast acceleration of the

whole signaling process and to a faster cellular response. Depending on the family of G proteins involved different second messenger-producing molecules are targeted.

Proteins of the Gαs family are stimulators of cAMP production by binding and activation of adenylyl cyclase (AC). AC, also target of Gαi and Gβγ, catalyzes the formation of cAMP from cytoplasmic ATP. The primary function of cAMP is the activation of protein kinase A (PKA). PKA phosphorylates several other proteins, such as Gαq-selective GPCRs and phospholipase C (PLC). Relaxation of blood vessels, gene regulation and modulation of ion channels are results of the Gαs-induced signaling cascade.

In contrast, Gαi acts as negative modulator of Gαs-induced signaling. Gαi decreases the level of cAMP by inhibition of AC. Additionally, Gαi regulates ion channels, activates phospholipases (PLCβ) and protein kinase C (PKC). Stimulation of growth and contraction of blood vessels are the results of Gαi-induced signaling.

The proteins of the Gαq family increase the concentration of diacyl glycerol (DAG) and inositol-3-phosphate (IP3). IP3 as well as DAG (the latter using activation of PKC) synergistically lead to an increase of intracellular calcium (Ca^{2+}) concentration. Similar to Gαi, contraction of blood vessels and stimulation of growth are results from Gαq-induced signaling.

Gα12/13 is a family of proteins that activates Rho (a regulation factor in the transcription of DNA to RNA) and is thereby directly involved in intranuclear processes (not shown).

Recent experiments demonstrated the functions of Gβγ complexes involved in regulation of ion channels, phospholipases, adenylyl cyclases and receptor kinases. The interaction of GPCRs with G proteins, often discussed as an interaction of GPCRs with Gα, seems to be very sensitive to the presence of the right Gβγ-complex (Hamm, 2001).

1.2 Structure-Function Studies on G Protein-Coupled Receptors

The characterization of ligand binding, receptor activation and G protein-interaction of known GPCRs as well as the description of functions of orphan GPCRs are focus of current pharmacological research. Currently, the main problem in this field of research is the small amount of structural data on these proteins. This is a common problem in investigations of membrane proteins. The experimental techniques (X-ray crystallography and Nuclear Magnetic Resonance (NMR)) need relatively high amounts of proteins, which for membrane-located proteins are usually scarcely available. Additionally, other experimental conditions are required (*e.g.* solubilization), which often lead to structural changes or distortions resulting in missing or corrupt structural data (Muller, 2000).

Thus, in the field of GPCR research the amount of theoretical techniques is immense. A first basis of such approaches was structural data of bacteriorhodopsin (Grigorieff *et al.*, 1996). Thereby, approaches using bioinformatics and homology modeling, as well as tailor-made protein simulation methodologies, complement the experimentally derived data (Muller, 2000). Since the year 2000, the X-ray crystallographic structures of bovine rhodopsin, primarily published by Palczewski and colleagues (Palczewski *et al.*, 2000), are the origin of such investigations. The use of bovine rhodopsin instead of bacteriorhodopsin allows the use of a real GPCR for investigations, which shares both the typical 7 membrane-spanning helices and the ability to activate G proteins (the latter is not a part of bacteriorhodopsin). Due to the high-resolution 3D data of bovine rhodopsin as well as the information on several GPCRs, experimental data of one receptor allows predictions to the functional behavior of another (Archer *et al.*, 2003). It also allows the generation of GPCR structures from sequence as demonstrated with the PREDICT technology (Shacham *et al.*, 2001).

Another basis for this kind of research are multiple sequence alignment investigations that primarily demonstrated a separation of GPCRs into several groups (Attwood and Findlay, 1994; Gether, 2000; Kolakowski, 1994; Kristiansen, 2004; Shi and Javitch, 2006). Rhodopsin-like receptors are the most interesting group, due to the available structural data of rhodopsin, and the fact that this family also includes the most members of GPCRs. The 7 membrane-spanning helices of GPCRs of this family are very conserved. A common numbering system allows conclusions on all receptors of this group based on investigations on only one or a few receptors (Ballesteros *et al.*, 2001b). The most conserved residues get numbered X.50 (X marks the number of the transmembrane helix). N-terminally and C-terminally located residues of X.50 get the preceding and succeeding numbers to 50, respectively. Besides the very conserved transmembrane helices in GPCR structures, the

portions of N-terminus, extracellular loops, intracellular loops and C-terminus often vary largely even between receptors of the rhodopsin-like family (Gether, 2000; Kristiansen, 2004). As a conclusion, additional information has to be used in the generation of GPCR structure models. State-of-the-art is the use of structural information of other proteins from the Protein Data Bank (Berman *et al.*, 2000). Molecular models based on such constructions provide a more detailed view of the structural groundwork of GPCRs and their functional behavior, allowing suggestions for experimental validation as well as implications in drug design (Patny *et al.*, 2006). This way, mechanisms of sequence dependent functional differences are able to detect, finally leading to the experimentally observed differences in ligand binding, receptor activation and G protein-interaction.

This study focuses on the mechanisms of ligand binding, initial receptor activation and G protein-interaction of homologous GPCRs. The endothelin receptor subtypes ETA and ETB are the main topic of these investigations. They were chosen because of their very homologous sequences, suggesting a general similarity in structure and function (*e.g.* in the process of signal transmission through the membrane). Anyhow, the remaining sequence differences lead to observable dissimilarities in function of ligand interaction and G protein-selectivity. The second topic of this work is the ligand binding of nicotinic acid receptor subtypes GPR109A and GPR109B. The extremely small sequence differences and their very different ligand selectivity make them suitable targets for such structural investigations.

1.2.1 The Endothelin Receptor Subtypes ETA and ETB

Background

From mammalian tissues two endothelin receptor subtypes have been isolated and cloned so far: the ETA and ETB (according to the receptor database nomenclature of IUPHAR, the International Union of Basic and Clinical Pharmacology). Both are widely expressed in almost every tissue including vascular and even non-vascular structures (epithelial cells, neurons, glia cells). This is consistent with the physiological role of endothelin-1 (ET-1) – the most active vasoconstrictor known – contributing to the maintenance of normal vascular tone and its localization as ubiquitous element in the endothelium (Davenport, 2002). Besides ET-1, which is the most abundant form, endothelin-2 (ET-2) and -3 (ET-3) are also members of this isopeptide family targeting the endothelin receptors. While ETA favors binding of ET-1, ETB binds all endothelins with high affinity. Thus, the high affinity for all three endothelin isotypes as well as the ETB's localization in kidney give further evidence that receptor subtype ETB may function as a "clearing receptor" to remove endothelin from the blood

(Davenport *et al.*, 1997). The physiological roles of endothelin receptors vary depending on tissue localization and species. In human, ETA regulates vasoconstriction (Maguire and Davenport, 1995), whereas ETB is involved in processes of vasodilatation, probably by release of endothelium-derived relaxing factors such as prostanoids or nitric oxide (Warner *et al.*, 1989). But this scheme is not fixed. In other mammals the influences may be vice versa, as in rabbit, where ETB regulates vasoconstriction (Davenport, 2002). Furthermore it is known that endothelin is necessary in cellular development processes such as proliferation of smooth muscle cells by use of ETA or proliferation of astrocytes by ETB subtype (Davenport, 2002).

Ligand Binding

The endothelin receptors ETA and ETB are activated following binding of the endothelin isopeptides ET-1, ET-2 and ET-3. ETA binds ET-1 and ET-2 with similar affinity but ET-3 with a 100-fold lower affinity (Davenport and Battistini, 2002; Nakajima *et al.*, 1989b). In contrast, the ETB binds all endothelin isoforms with similar affinity (de Nucci *et al.*, 1988). A unique feature of ETB, not shared by ETA, is the quasi-irreversible interaction of ET-1 – with the exception of ETB of rat (Takasuka *et al.*, 1994). The quasi-irreversibility of this complex is based on the formation of a super-stable complex that is intact even in the presence of 2% SDS and in an acidic environment (Takasuka *et al.*, 1994). As a consequence, the ETB/ET-1 complex remains tightly bound even in the late endosomes and lysosomes (Oksche *et al.*, 2000). In addition to the endothelins, the sarafotoxins represent further naturally occurring agonists. Sarafotoxins, which comprise four isoforms (Sfx6a, Sfx6b, Sfx6c, Sfx6d), were isolated from the snake venom of *Atractaspis engaddensis*.

Structural information on two native peptide ligands are available at the Protein Data Bank PDB (Berman *et al.*, 2000): an NMR structure of sarafotoxin Sfx6b (entry 1SRB) as well as NMR and X-ray data of ET-1 (entries 1EDN, 1EDP, 1V6R) (Andersen *et al.*, 1992; Atkins *et al.*, 1995; Janes *et al.*, 1994; Takashima *et al.*, 2004). All of the native endothelin-receptor interacting peptides consist of 21 residues and share a common fold with a conserved disulfide-linkage (Nakajima *et al.*, 1989a). Two disulfide-bridges (Cys1-Cys15 and Cys3-Cys11) link the peptide's N-terminus in an anti-parallel orientation to a middle helix, formed by amino acid residues Lys9 through Cys15 (Atkins *et al.*, 1995).

Our own preparatory work revealed that this helix is N-terminally stabilized by a helix capping also common to native peptides. Thereby, Sfx6b adopts a slightly changed loop conformation between the N-terminal tail and the helix. This is caused by a helix capping of Thr7 to the backbone hydrogen of Glu10, instead of Asp8 to the backbone hydrogen of Cys11

in case of ET-1 (Fig. 1-2).

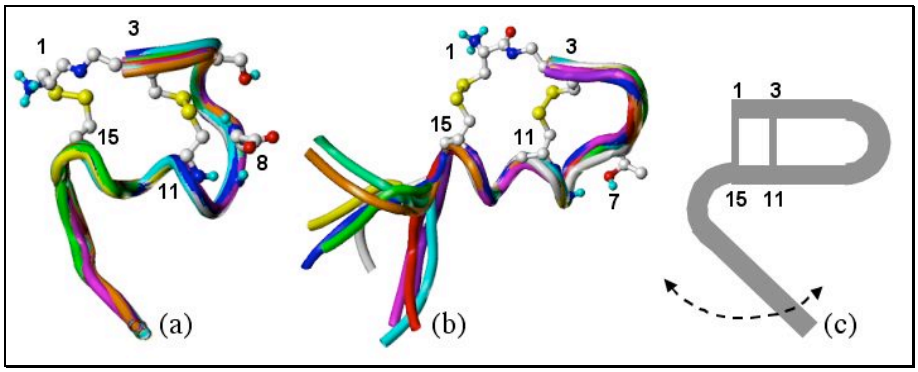

Figure 1-2:
Our own preparatory work analyzing structural features of endothelin-receptor peptide ligands: (a) an ensemble of NMR structures of ET-1 from PDB entry 1V6R. Determinants of its fold are the two disulfide-bridges between Cys1-Cys15 and Cys3-Cys11 as well as the helix capping by Asp8. (b) Superposition of NMR structures of Sfx6b from PDB entry 1SRB. As in ET-1 the structure is restrained by the two disulfide-bridges, but the different helix capping of Thr7 results in a slightly shifted orientation of the N-terminal 7 residues. Furthermore, the C-terminus shows large flexibility. We suggest that the C-terminus of peptide ligands is rather mobile (c) and we suppose that the difference between ET-1 structure and Sfx6b structure may be based on experimental differences.

The reported NMR structure of the ETB-selective agonist IRL1620, which lacks the seven N-terminal residues of ET-1 (and also the disulfide network), shows mainly α-helical orientation (Atkins *et al.*, 1995). Marked structural differences were obtained for the orientation of the C-terminus in ET-1 and Sfx6b, most likely origin in different experimental conditions.

Studies with ETA/ETB chimeras revealed that the N-terminal regions of peptide ligands interact with transmembrane helices TMH1, TMH2, TMH3 and TMH7, whereas the C-termini interact with TMH4 to TMH6 (Sakamoto *et al.*, 1993). In photo-labeling studies with TTA-386 and IRL1620 an orientation placing the C-terminus of peptide ligands towards TMH5 was suggested (Boivin *et al.*, 2004). Comparisons of the amino acid sequences of both receptor subtypes in their respective transmembrane regions reveal a high degree of similarity. Only few differences are found, which could contribute to selective ligand binding. For instance, the replacement of Tyr129 in position 2.53 of ETA with ETB's histidine residue resulted in a slight increase of the affinity for ET-3 and Sfx6c (Krystek *et al.*, 1994; Lee *et al.*, 1994b; Webb *et al.*, 1996). This and other mutations of position 2.53 in ETA and ETB did not alter the affinities for ET-1 and ET-2 (Lee *et al.*, 1994b; Webb *et al.*, 1996). For four additional residues in the transmembrane regions, a role in ligand binding and/or signaling was demonstrated: Asp2.50, Asp2.57, Lys2.64, Asp7.35 (Adachi *et al.*, 1994a; Adachi *et al.*, 1994b; Breu *et al.*, 1995; Rose *et al.*, 1995; Vichi *et al.*, 1999). However, these variant residues cannot explain the high selectivity of ligand binding. Thus, it is assumed that the extracellular regions, such as the N-terminus and the three extracellular loops contribute to ligand selectivity (Menziani *et al.*, 1995). This may further explain the remarkably high apparent binding affinities for peptide ligands (*e.g.* ET-1: 0.01-0.03 nM), while the affinities

for small molecules such as Bosentan (4.7 nM) are an order of magnitude lower (Clozel *et al.*, 1994; Gregan *et al.*, 2004). However, data on single residues for ligand/receptor interaction and the mechanisms underlying the selective binding of agonists and antagonists remain unclear.

G Protein Interaction

Like other GPCR subtypes, ETA and ETB show different preferences for trimeric G protein-complexes (Wong, 2003). ETA binds to Gαq, Gαs and Gα12/13, whereas ETB prefers Gαq and Gαi but not Gαs. The exact mechanisms of the interactions between the endothelin receptors' intracellular loops and the G proteins are still not completely understood.

Chimera studies revealed an involvement of ICL2 and ICL3 as a major determinants in selective coupling of hETA with Gαs and hETB with Gαi (Takagi *et al.*, 1995). Additionally, mutation studies described an involvement of ICL1 of ETB in the interaction with Gα13 (Liu and Wu, 2003). An interaction of at least the C-terminus of ETB with G proteins is assumed because of disrupted G protein-interactions that follow the removal of the cysteine-attached C-terminal palmitoylation (Aquilla *et al.*, 1996). Recent studies on activation of bovine rhodopsin revealed a hydrophobic area at the inner side of TMH6 involved into G protein-activation (Janz and Farrens, 2004). These information as well as structural models on ETA and ETB was used for the identification of the receptors' patterns for selective G protein-interactions.

In the case of G proteins, chimera studies showed that the last five C-terminal residues of the Gα subunit can recognize selective signaling of GPCRs towards Gαi, Gαq and Gαz (Blahos *et al.*, 1998). In addition, areas in the vicinity of the C-terminus and in the N-terminal helix of Gα, as well as the C-terminus of Gγ and regions of Gβ, were identified as interaction epitopes for GPCRs at the G protein (Azpiazu and Gautam, 2001; Gilchrist *et al.*, 2001; Hamm *et al.*, 1988; Kostenis *et al.*, 1998; Muradov and Artemyev, 2000; Onrust *et al.*, 1997).

However, the high sequence divergences between the intracellular loops of hundreds of GPCRs with different G protein-selectivity complicate the identification of general selectivity patterns in the interactions of GPCRs with G proteins.

Interestingly, also peptides and small molecules were described to directly modulate G proteins (*e.g.* secretagogues like mastoparans, substance P or bradykinin (Bueb *et al.*, 1990; Higashijima *et al.*, 1988; Mousli *et al.*, 1990; Sukumar and Higashijima, 1992). In the case of mastoparan-X (MPX) it is assumed that it affects the C-terminus of Gαi in the same way as Gαi-selective GPCRs do. The sensitivity for pertussis-toxin (PTX) in MPX induced activation of Gαi indicates a very similar interaction region for MPX and GPCRs, closely

located to the cysteine at the C-terminal tail of Gαi (*e.g.* Cys351 of SwissProt entry: gbi2_human). The PTX-dependent ADP-ribosylation of the cysteine at the very C-terminus of Gα, resulting in disturbed activation, is an established test for Gαi-activating GPCRs.

Modification of mastoparan-based peptides resulted in Gαs-selective mastoparan-S (MPS). The differences compared to MPX are the following substitutions: Ala8→Ser, Ala10→Aib (Aib, U, α-amino isobutyric acid), Lys11→Arg, Lys12→Gln and Leu13→Val. A disturbed helical structure at residue Met9 differs the NMR structures of the G protein-interacting MPS from MPX (Sukumar and Higashijima, 1992; Sukumar *et al.*, 1997) (Fig. 1-3).

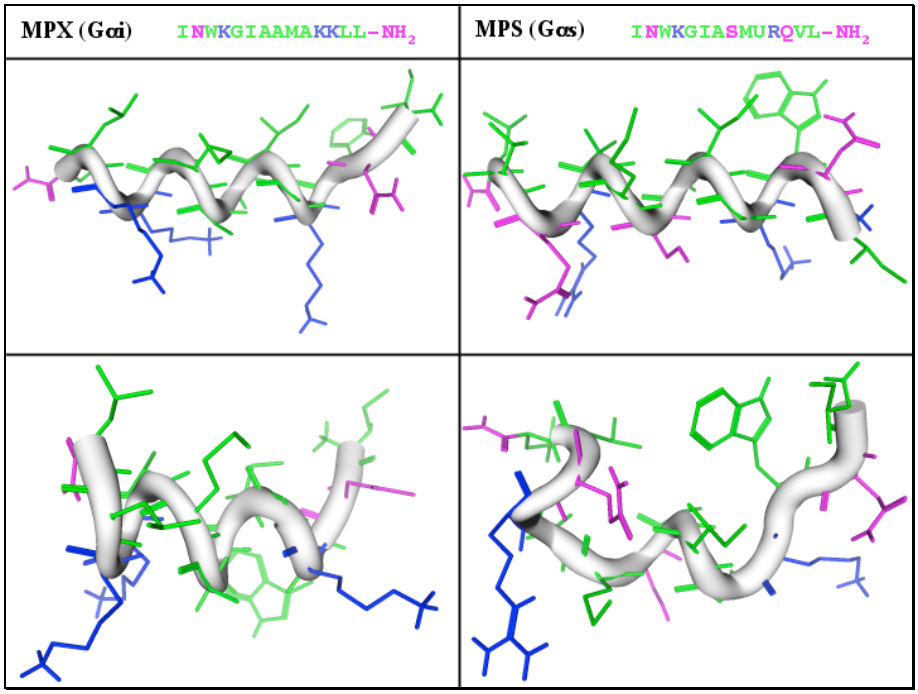

Figure 1-3:
Our own preparatory work comparing NMR-structures of mastoparan-X (MPX) und mastoparan-S (MPS): Top row: G protein-selectivity and sequences of both receptor mimetic peptides. Middle row: Structures from studies using DPPC micelles as environment for MPX and MPS. Bottom row: Structures of both peptides as investigated at adequate G proteins. (General residue coloring: green - hydrophobic, magenta/red - hydrophilic, blue – basic)

Even small synthetic compounds like alkyl-substituted amino acid derivatives can stimulate G proteins (Breitweg-Lehmann *et al.*, 2002; Leschke *et al.*, 1997; Nurnberg *et al.*, 1999). The interaction site of Gαi-selective forms of these molecules is similar to those of Gαi-selective receptor mimetic peptides and GPCRs (Breitweg-Lehmann *et al.*, 2002; Bueb *et al.*, 1990; Leschke *et al.*, 1997; Nurnberg *et al.*, 1999). Their few degrees of freedom and demonstrated selectivity regarding different Gα subtypes makes them highly interesting as potential tools for characterizing the interaction patterns of G proteins.

However, the mechanism of G protein-activation by alkyl-substituted amino acid derivatives is different to GPCRs, because of a somehow detergent-like way of Gα-activation independent from a Gβ and Gγ.

1.2.2 Nicotinic Acid Receptors GPR109A and GPR109B

Background

The water soluble B-complex vitamin nicotinic acid (niacin) has been used to treat different dyslipidemic disorders (Knopp, 1999; Olsson, 1994) for decades in clinical practice. But the exact mechanism of nicotinic acid-induced effects on lipid metabolism is still not completely understood. However, the inhibition of fat cell lipolysis via the activation of a Gαi-coupled receptor and subsequent inhibition of cAMP formation (Aktories *et al.*, 1980; Aktories *et al.*, 1982) has been postulated to be significant (Tornvall *et al.*, 1990). Recently, a GPCR has been identified, which binds nicotinic acid with the expected affinity (Soga *et al.*, 2003; Tunaru *et al.*, 2003; Wise *et al.*, 2003). The receptor termed GPR109A (HM74A in human and PUMA-G in mice) is expressed in adipocytes and immune cells and couples to G proteins of the Gαi family. This receptor's activation through nicotinic acid decreases the activity of hormone-sensitive lipase by lowering the cAMP levels, resulting in a reduced hydrolysis of triglycerides to free fatty acids. In mice lacking the murine form of the nicotinic acid receptor, the anti-lipolytic effects of nicotinic acid, the decrease in free fatty acid and triglyceride plasma levels, are abrogated (Tunaru *et al.*, 2003). Thus, GPR109A (HM74A/PUMA-G) is the receptor mediating the anti-lipolytic effects of nicotinic acid.

Sequence investigations indicate that GPR109A is a member of a subfamily of GPCRs, which comprise GPR109A (HM74A/PUMA-G) and GPR81, both existing in human and in rodent species. In addition, a third member of this receptor family, GPR109B (HM74) has been found exclusively in human. GPR109A, GPR81, and GPR109B are co-localized on human chromosome 12q24.31 and are most likely the result of gene duplications.

Nicotinic acid receptor GPR109A and its homologous derivative GPR109B are functional GPCRs, which can be activated by furan-carboxylic acid derivate Acifran (Carlson and Oro, 1962). In contrast, pyridine-3-carboxylic acid (nicotinic acid, niacin) as well as pyrazine-carboxylic acid derivatives, like Acipimox (5-methylpyrazine-carboxylic acid-4-oxide), are high-affinity agonists exclusively for GPR109A. The physiological and pharmacological importance of GPR109B remains unclear.

Besides its shorter C-terminus, GPR109A differs from GPR109B only by 17 amino acids, 14 of those are conserved in human, mouse and rat. Due to this and the above-mentioned ligand selectivity, it is likely that these residues are involved in ligand binding. Structurally, they cluster around extracellular loops ECL1 and ECL2. Before starting this project, each of these 14 amino acid residues in GPR109A was systematically mutated into the corresponding residue of GPR109B (done by Sorin Tunaru and Stefan Offermanns, University of

Heidelberg). Nicotinic acid-induced activation of mutant receptors was tested in cells co-expressing receptor mutants and the promiscuous G-protein α-subunit $G\alpha_{15}$ in a Ca^{2+} reporter assay (Tunaru *et al.*, 2003). All receptor mutants were N-terminally tagged with the FLAG epitope; confocal microscopy and ELISA verified their expression levels as well as membrane localization. Only receptor mutants Asn86→Tyr, Trp91→Ser, and Ser178→Ile lost their ability to respond to nicotinic acid (Fig. 1-4, Tab. 1-1) but were well expressed and showed membranous localization.

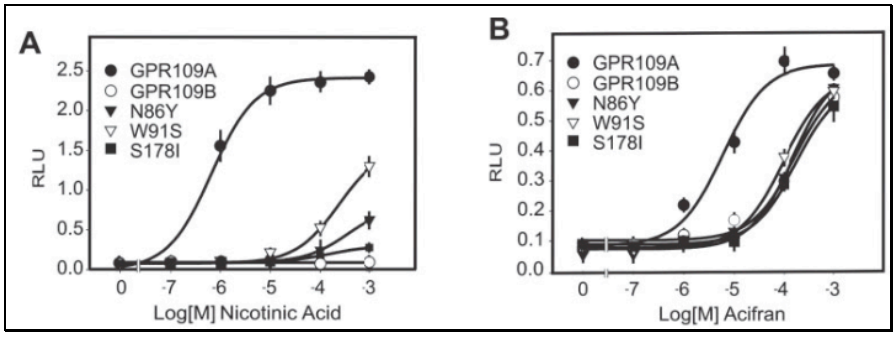

Figure 1-4:
Binding of nicotinic acid (A) and acifran (B) at nicotinic acid receptors GPR109A and GPR109B and their mutants. (A) GPR109A receptor binds nicotinic acid very well, while GPR109B does not. Mutants of GPR109A bearing residues Asn86, Trp91, Ser178, which were exchanged to complementary residues of GPR109B Tyr86, Ser91, Ile178, show affinity comparable to GPR109B receptor. (B) In contrast, both receptors GPR109A and GPR109B bind acifran. Nevertheless, the given mutants also demonstrate a shift from a GPR109A pattern to GPR109B. (Tunaru *et al.*, 2003)

Interestingly, the response of these mutants to Acifran was unchanged, indicating that the mutants were functionally active. Radioligand-binding assays using ^{3}H-labelled nicotinic acid (Tab. 1-1) demonstrated all three mutants were unable to bind nicotinic acid.

Table 1-1: Binding Studies of nicotinic acid and acifran at GPR109A receptor mutants including the corresponding residues from GPR109B receptor (Tunaru *et al.*, 2003).

	EC_{50}		K_D (Binding of [^{3}H]-Nicotinic Acid)
	Nicotinic Acid	Acifran	
Wild-type Receptors			
GPR109A	0.7 ± 0.2 µM	1.9 ± 0.4 µM	60 ± 8 nM
GPR109B	Inactive	90 ± 12 µM	> 500 nM
Receptor Mutants			
Leu83→Val	3.0 ± 0.5 µM	2.0 ± 0.3 µM	
Asn86→Tyr	> 100 µM	88 ± 20 µM	> 500 nM
Trp91→Ser	> 100 µM	96.8 ± 10 µM	> 500 nM
Lys94→Asn	1.4 ± 0.8 µM	4 ± 0.7 µM	
Met103→Val	5.3 ± 1.1 µM	22 ± 0.5 µM	
Leu107→Phe	3.1 ± 0.5 µM	2.9 ± 0.3 µM	
Arg142→Trp	1.0 ± 0.3 µM	4.3 ± 0.5 µM	
Ile156→Val	0.5 ± 0.1 µM	2.8 ± 0.4 µM	
Met167→Leu	2.3 ± 0.3 µM	5.6 ± 0.2 µM	
Pro168→Leu	2.3 ± 0.4 µM	4.7 ± 0.6 µM	
Gly173→Pro	1.1 ± 0.2 µM	2.4 ± 0.3 µM	
Leu176→Val	3.0 ± 0.4 µM	18.7 ± 2 µM	
Ser178→Ile	> 100 µM	80 ± 14 µM	> 500 nM
Phe198→Leu	0.7 ± 0.1 µM	1.9 ± 0.4 µM	

Thus, asparagine Asn86, tryptophane Trp91, and serine Ser178 of GPR109A are required for nicotinic acid binding but are not necessary for Acifran-induced receptor activation (Tunaru

et al., 2003). Additional chimera studies of GPR109A and GPR109B receptors, as well as the re-introduction of residues missing in the adequate part of the chimeras, support these findings on Asn86, Trp91 and Ser178 (Tunaru *et al.*, 2003).

Furthermore, the conserved disulfide-bridge of rhodopsin-like GPCRs between transmembrane helix TMH3 and extracellular loop ECL2 was characterized to be formed by residues Cys100 in TMH3 and Cys177 in ECL2 (Tunaru *et al.*, 2003).

1.3 Objectives of this Study

In almost every organism GPCRs are widespread in localization and function. Today, already 40-50% of modern drugs rely on GPCRs, although only 10% of known GPCRs are targeted. The increasing need for highly effective and sensitive drugs requires detailed information of the receptors' ligand binding and G protein coupling. While ligand binding of small molecular ligands at GPCRs is quite well characterized, the interaction of larger ligands, such as peptides or proteins, is not. Additionally, experimental results proving patterns for selective recognition of G protein-subtypes were rarely known prior to this study.

Since 3D X-ray crystallographic data is available for a single GPCR, namely rhodopsin, homology modeling combined with mutation/substitution studies of receptors and ligands are a promising approach to delineate structure-function relationships for understanding the molecular mechanisms of GPCRs. In this case, we are relying on the close sequence homology between GPCR subtypes (ETA/ETB and GPR109A/GPR109B). Additionally, their different biological response to ligand binding and G protein coupling was used to reveal structural determinants for selective and high-affinity ligand binding as well as selective interactions with G proteins.

To achieve this goal, the following specific aims were outlined:

- Binding site studies of peptide agonists and antagonists to endothelin receptor subtypes ETA and ETB in order to characterize highly selective ligand binding at ETA as well as rather unselective ligand recognition at ETB.
- Characterization of the binding sites of GPR109A and GPR109B to explain high-affinity binding of nicotinic acid to the former and to suggest possible ligands for the latter. Supporting experimentalists to deorphanize the function of GPR109B.
- Delineation of structural determinants and patterns of G protein-recognition utilizing small G protein-interacting peptides, alkyl-substituted amino acid derivatives and homologous GPCR subtypes ETA and ETB.