G protein-coupled receptor regulation: The role of protein interactions and receptor trafficking

Sandén, Caroline

2009

Link to publication

Citation for published version (APA):
G protein-coupled receptor regulation:
The role of protein interactions and receptor trafficking

by

Caroline Sandén

Group of Drug Target Discovery
Department of Experimental Medical Science
Faculty of Medicine
Lund University
Caroline Sandén
Group of Drug Target Discovery
Department of Experimental Medical Science
Faculty of Medicine
Lund University
Biomedical Center, A12
Tornavägen 10
S-221 84 Lund
Sweden

Phone: +46 46 222 05 21
Fax: +46 46 222 05 68
E-mail: caroline.sanden@med.lu.se

Cover image: Bradykinin B2 receptors expressed in HEK293 cells as visualized by confocal immunofluorescence microscopy.

Printed by Media-Tryck, Lund University, Sweden
© Caroline Sandén
Paper I and II: Reprinted with permission of the American Society for Pharmacology and Experimental Therapeutics. All rights reserved.

ISSN 1652-8220
Lund University, Faculty of Medicine
Doctoral Dissertation Series 2009:74
To my parents, for endless support.
If we knew what we were doing, it wouldn’t be called research.

(A.Einstein)
# Table of contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table of contents</td>
<td>5</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>7</td>
</tr>
<tr>
<td>List of included publications</td>
<td>11</td>
</tr>
<tr>
<td><strong>Introduction</strong></td>
<td>13</td>
</tr>
<tr>
<td>The family of G protein-coupled receptors</td>
<td>13</td>
</tr>
<tr>
<td>Receptor activation and signaling</td>
<td>14</td>
</tr>
<tr>
<td>Receptor maturation and transport to the cell surface</td>
<td>17</td>
</tr>
<tr>
<td>Receptor oligomerization</td>
<td>19</td>
</tr>
<tr>
<td>Receptor oligomerization – effect on maturation</td>
<td>20</td>
</tr>
<tr>
<td>Receptor oligomerization – effect on trafficking and signaling</td>
<td>20</td>
</tr>
<tr>
<td><strong>Bradykinin receptors</strong></td>
<td>23</td>
</tr>
<tr>
<td>The components of the kallikrein-kinin system</td>
<td>23</td>
</tr>
<tr>
<td>Kinin receptor expression and signaling</td>
<td>24</td>
</tr>
<tr>
<td>Post-translational kinin receptor modifications</td>
<td>25</td>
</tr>
<tr>
<td>Kinin receptor trafficking</td>
<td>26</td>
</tr>
<tr>
<td>Protein-protein interactions in kinin receptor signaling</td>
<td>27</td>
</tr>
<tr>
<td>Physiological relevance of kinin receptors</td>
<td>28</td>
</tr>
<tr>
<td><strong>GPER1</strong></td>
<td>31</td>
</tr>
<tr>
<td>GPER1 signaling</td>
<td>32</td>
</tr>
<tr>
<td>Subcellular localization of GPER1</td>
<td>33</td>
</tr>
<tr>
<td>GPER1 and breast cancer association</td>
<td>34</td>
</tr>
<tr>
<td><strong>Present investigation</strong></td>
<td>37</td>
</tr>
<tr>
<td>Aims</td>
<td>37</td>
</tr>
<tr>
<td>Summary</td>
<td>37</td>
</tr>
<tr>
<td>Paper I</td>
<td>37</td>
</tr>
<tr>
<td>Paper II</td>
<td>39</td>
</tr>
<tr>
<td>Paper III</td>
<td>40</td>
</tr>
<tr>
<td>Paper IV</td>
<td>41</td>
</tr>
<tr>
<td>Conclusions</td>
<td>42</td>
</tr>
<tr>
<td>Discussion</td>
<td>42</td>
</tr>
<tr>
<td>Future directions</td>
<td>44</td>
</tr>
<tr>
<td>Is oligomerization important for B1R maturation in breast cancer cells?</td>
<td>44</td>
</tr>
<tr>
<td>Is E2 the true agonist for GPER1?</td>
<td>45</td>
</tr>
<tr>
<td><strong>Svensk sammanfattning (Swedish summary)</strong></td>
<td>47</td>
</tr>
</tbody>
</table>
# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE</td>
<td>Angiotensin converting enzyme</td>
</tr>
<tr>
<td>AT&lt;sub&gt;1&lt;/sub&gt;R</td>
<td>Angiotensin II type 1 receptor</td>
</tr>
<tr>
<td>AT&lt;sub&gt;2&lt;/sub&gt;R</td>
<td>Angiotensin II type 2 receptor</td>
</tr>
<tr>
<td>B1R</td>
<td>Bradykinin B&lt;sub&gt;1&lt;/sub&gt; receptor</td>
</tr>
<tr>
<td>B2R</td>
<td>Bradykinin B&lt;sub&gt;2&lt;/sub&gt; receptor</td>
</tr>
<tr>
<td>β-arr</td>
<td>β-arrestin</td>
</tr>
<tr>
<td>BK</td>
<td>Bradykinin</td>
</tr>
<tr>
<td>BRET</td>
<td>Bioluminescence resonance energy transfer</td>
</tr>
<tr>
<td>C&lt;sub&gt;2&lt;/sub&gt;C&lt;sub&gt;12&lt;/sub&gt;</td>
<td>Mouse myotube cells</td>
</tr>
<tr>
<td>CHO-KI</td>
<td>Chinese hamster ovary cells</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>COS-7</td>
<td>Monkey kidney cells</td>
</tr>
<tr>
<td>CTGF</td>
<td>Connective tissue growth factor</td>
</tr>
<tr>
<td>CXCR1</td>
<td>Chemokine receptor 1</td>
</tr>
<tr>
<td>CXCR2</td>
<td>Chemokine receptor 2</td>
</tr>
<tr>
<td>Cys</td>
<td>Cysteine</td>
</tr>
<tr>
<td>D1R</td>
<td>Dopamine receptor 1</td>
</tr>
<tr>
<td>D2R</td>
<td>Dopamine receptor 2</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DABK</td>
<td>Des-Arg&lt;sup&gt;9&lt;/sup&gt;-bradykinin</td>
</tr>
<tr>
<td>DAKD</td>
<td>Des-Arg&lt;sup&gt;10&lt;/sup&gt;-kallidin</td>
</tr>
<tr>
<td>DDT&lt;sub&gt;1&lt;/sub&gt; MF-2</td>
<td>Hamster smooth muscle cells</td>
</tr>
<tr>
<td>DOPR</td>
<td>δ-opioid receptor</td>
</tr>
<tr>
<td>E2</td>
<td>17-β-estradiol</td>
</tr>
<tr>
<td>EL</td>
<td>Extracellular loops</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial-specific nitric oxide synthase</td>
</tr>
<tr>
<td>EP24.15</td>
<td>Thimet oligopeptidase</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmatic reticulum</td>
</tr>
<tr>
<td>ERAD</td>
<td>ER-associated degradation</td>
</tr>
<tr>
<td>ERα</td>
<td>Estrogen receptor α</td>
</tr>
<tr>
<td>ERβ</td>
<td>Estrogen receptor β</td>
</tr>
<tr>
<td>ERGIC</td>
<td>ER-Golgi intermediate complex</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal regulated protein kinase</td>
</tr>
<tr>
<td>FB1</td>
<td>FLAG-tagged B1R</td>
</tr>
<tr>
<td>FB1stop135</td>
<td>FLAG-tagged B1R fragment</td>
</tr>
<tr>
<td>FEV</td>
<td>Forced expiratory volume</td>
</tr>
</tbody>
</table>
FRET  Fluorescence resonance energy transfer
FSH  Follicle-stimulating homoron
GABABR  \(\gamma\)-aminobutyric acid type B receptor
GFP  Green fluorescence protein
GPCR  G protein-coupled receptor
GPER1  G protein-coupled estrogen receptor
GPR30  G protein-coupled estrogen receptor
GRK  G protein-coupled receptor kinase
HB1  HA-tagged B1R
HB1stop135  HA-tagged B1R fragment
HEK293  Human embryonic kidney cells
HEK293T  HEK293 cells expressing the large T-antigen of SV40
HER 2  Human epidermal growth factor receptor 2
HMWK  High-molecular-weight kininogen
HRP  Horseradish peroxidase
IL  Intracellular loops
IL-1\(\beta\)  Interleukin-1\(\beta\)
IP\(_3\)  Inositol 1,4,5-trisphosphate
JAK  Janus-activated kinase
JNK  c-Jun N-terminal kinase
KD  Kallidin, or Lys-bradykinin
LMWK  Low-molecular-weight kininogen
LH  Luteinizing hormone
LHRH  LH-releasing hormone
LiCl  Lithium chloride
MAPK  Mitogen activated protein kinase
MOPR  \(\mu\)-opioid receptor
NO  Nitric oxide
OHT  Hydroxytamoxifen
P  Phosphate
PC-12  Rat pheochromocytoma cells
PC-12W  Modified PC-12 cells expressing more
PI  Phosphoinositide
PI3K  Phosphatidylinositol 3-kinase
PIP\(_2\)  Phosphatidyl-4,5-bisphosphate
PKA  Protein kinase A
PKC  Protein kinase C
PLA\(_2\)  Phospholipase A\(_2\)
PLC  Phospho lipase C
PLD  Phospholipase D
PM  Plasma membrane
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNGaseF</td>
<td>Peptide-N-glycosidase F</td>
</tr>
<tr>
<td>RAMP</td>
<td>Receptor activity modifying protein</td>
</tr>
<tr>
<td>RGS</td>
<td>Regulators of G protein signaling</td>
</tr>
<tr>
<td>SKBR3</td>
<td>Human breast cancer cells</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal-transducers and activation of transcription</td>
</tr>
<tr>
<td>TRHR1</td>
<td>Thyrotropin-releasing hormone receptor 1</td>
</tr>
<tr>
<td>TRHR2</td>
<td>Thyrotropin-releasing hormone receptor 2</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
</tr>
</tbody>
</table>
List of included publications

Paper I  
**B₁ bradykinin receptor homo-oligomers in receptor cell surface expression and signaling: effects of receptor fragments.**
Dong Soo Kang, Caroline Gustafsson, Mattias Mörgelin, and L.M. Fredrik Leeb-Lundberg.

Paper II  
**Kinin B₂ receptor-mediated bradykinin internalization and metalloendopeptidase EP24.15-dependent intracellular bradykinin degradation.**
Caroline Sandén, Johan Enquist, Sara H Bengtson, Heiko Herwald, L.M. Fredrik Leeb-Lundberg.

Paper III  
**A B₁ kinin receptor fragment exerts dominant-negative behavior by disrupting intact B₁ receptor homo-oligomers in the endoplasmatic reticulum and increasing receptor degradation.**
Caroline Sandén and L.M. Fredrik Leeb-Lundberg.
Manuscript

Paper IV  
**GPER1 in the plasma membrane is subject to rapid agonist-independent receptor endocytosis and recycling.**
Manuscript
Introduction

Communication is very important for all living beings. Among humans, phone calls, meetings, conferences, or late night conversations – curled up in the sofa with a cup of tea – are required for a functional society and individual satisfaction. Communication also occurs inside our bodies, which is crucial for our survival!

The family of G protein-coupled receptors

To be able to communicate, cells project molecular antennas (receptors) on their cell surface. These receptors are able to pick up signals from the extracellular environment, transfer the signals into the cells, and trigger signal-specific cellular responses. The super-family of G protein-coupled receptors (GPCRs) is one of the largest protein groups in vertebrates and the largest single gene family in the human genome. They are activated by numerous ligands, including hormones, light, pheromones, odorants, lipids, neurotransmitters, etc (Schiöth and Fredriksson, 2005; Luttrell, 2008). GPCR-mediated signaling operates in every human cell, and it is involved in processes like metabolism, blood pressure regulation, inflammation and reproduction. Not surprisingly, GPCRs are important drug targets for the pharmaceutical industry, and about 50% of existing clinically useful drugs act through GPCRs (Insel et al., 2007; Luttrell, 2008).

GPCRs have been divided into five main families according to the GRAFS classification system: Glutamate, Rhodopsin, Adhesion, Frizzled/Taste2, and Secretin receptors (Shiöth and Fredriksson, 2005). The structural diversity within the family of GPCRs is substantial, but they share some common structural elements. This includes seven hydrophobic transmembrane-spanning regions that are interconnected by three intracellular and three extracellular loops with an extracellular N-terminal and an intracellular C-terminal domain (Fig. 1).
Receptor activation and signaling

GPCRs transfer extracellular signals across the membrane lipid bilayer through the activation of intracellular heterotrimeric G proteins. G proteins consist of three subunits, α, β and γ, where the β and γ subunits form an undissociable complex and represent a functional unit. Agonist binding to the receptor results in a receptor conformational change, which leads to receptor activation. This promotes coupling between the receptor and Gαβγ, which catalyzes the exchange of GDP for GTP on the Gα subunit. The Gα and the Gβγ subunits then dissociate from the receptor and each regulates the activity of enzyme and ion channel effectors, generating small second messenger molecules that regulate key enzymes involved in intermediary metabolism (Offermanns, 2003; Luttrell, 2008). The signaling continues until the G proteins are inactivated by a mechanism dependent on the intrinsic GTPase activity of the Gα subunit, which is facilitated by the direct binding of regulators of G protein signaling (RGS) to activated Gα-GTP (Fig. 2) (Hepler, 2003). One activated GPCR may activate multiple G-proteins, and each G-protein may activate numerous effector proteins, resulting in a considerable amplification of the signal (Offermanns, 2003; Luttrell, 2008).

Figure 1. Representation of a GPCR. The GPCR winds seven times through the membrane lipid bilayer. The hydrophobic transmembrane domains are interconnected by three intracellular and three extracellular loops with an extracellular N-terminus and an intracellular C-terminal domain.
Figure 2. The G protein cycle. Agonist binding to the receptor leads to receptor activation and coupling between the receptor and Gαβγ, which catalyzes the exchange of GDP for GTP on the Gα subunit. This leads to G protein dissociation, resulting in the activation of effector proteins and intracellular signal transduction. The G protein-subunits are then inactivated and reassOCIated, thereby terminating the signaling.

Within seconds after agonist binding many receptors become inactivated (desensitized). This occurs through phosphorylation of the receptors by G protein-coupled receptor kinases (GRKs). Phosphorylation then enables the recruitment of β-arrestin to the receptor, which physically blocks the interaction between G-protein and receptor leading to uncoupling of the G-protein. β-arrestin also directs the receptor to clathrin-coated pits, which leads to internalization of the receptor in a dynamin- and AP2-dependent manner. Receptors can also be phosphorylated and modulated by second messenger-dependent protein kinases (PKA/C), which may also favor β-arrestin recruitment. Internalized receptors enter early endosomes, and then either return to the plasma membrane through recycling endosomes, through which they become dephosphorylated, or are transported to late
endosomes, followed by degradation in the lysosomes (Fig. 3) (van Knoppen and Jakobs, 2004; Luttrell, 2008).

**Figure 3.** Postendocytic sorting-routes. $\beta$-arr = $\beta$-arrestin, GRK= G protein coupled receptor kinase, p = phosphate. Phosphorylation of the receptor may favor $\beta$-arrestin recruitment and internalization. Internalized receptors reach early endosomes and then either enter a recycling pathway, retargeting the plasma membrane, or a degradation pathway, that involves late endosomes and results in lysosomal receptor degradation.
There are 16 $\alpha$, 5 $\beta$, and 12 $\gamma$ subunit genes in the human genome. The $\alpha$ subunits are divided into four main groups, including $\alpha_s$, $\alpha_{i/o}$, $\alpha_q$, and $\alpha_{12}$. $\alpha_s$ stimulates, and $\alpha_{i/o}$ inhibits, the activity of the integral plasma membrane protein adenylate cyclase, which catalyzes the production of cAMP from ATP, raising the cytosolic level of this second messenger. $\alpha_q$ activates phospholipase C (PLC) that catalyzes the hydrolysis of membrane phosphatidylyl-4,5-bisphosphate (PIP2) to yield two intracellular second messengers, inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). Both contribute to the activation of protein kinase C (PKC), DAG by directly activating the enzyme, and IP3 by raising the cytosolic level of Ca$^{2+}$. The increase in cytosolic calcium also activates other Ca$^{2+}$-dependent enzymes.

The $\alpha_{12}$ family activates the c-Jun N-terminal kinase (JNK), the Na$^+$/H$^+$ exchanger, and phospholipase D. They have also been shown to induce the formation of actin stress fibers in a Rho-dependent manner (Offermanns, 2003; Luttrell, 2008). The role of the $\beta\gamma$ subunit is less well known, but it has been shown that some $\beta\gamma$ subunits are able to activate PLC and the IKACH potassium channel, and to regulate the activity of G protein-coupled receptor kinases (GRK) 2 and 3, which are important for GPCR desenztisation (Luttrell, 2008). GPCRs also carry out G protein-independent signaling. This signaling may involve phosphorylation of the receptor by GRKs, which enables $\beta$-arrestin recruitment and receptor internalization, followed by the activation of the mitogen activated protein kinase (MAPK) ERK (extracellular signal regulated protein kinase) (Luttrell and Lefkowitz, 2002; Lefkowitz and Shenoy, 2005). Another G protein-independent signaling mechanism is the activation of tyrosine kinases through direct interaction with the receptor C-terminus (McGarrigle and Huang, 2007). One example of this mechanism is the angiotensin II type I receptor (AT1R), which has been reported to activate the tyrosine kinase in a G protein-independent manner (Seta et al., 2002).

Receptor maturation and transport to the cell surface

GPCRs are synthesized, folded, and assembled in the endoplasmatic reticulum (ER). After proper folding, the receptors exit the ER through COPII-coated vesicles and are transported through the ER-Golgi intermediate complex (ERGIC), the Golgi apparatus, and the trans-Golgi network, before they reach the plasma membrane. During this tightly regulated process, the receptors undergo several modifications such as glycosylation and palmitoylation. These post-translational modifications are important to attain mature and functional receptors. N-glycosylation of asparagine residues begins in the ER, and makes the extracellular
parts of the receptor more hydrophilic, but may also affect ligand affinity, receptor oligomerization, G protein-coupling, receptor degradation, maturation and/or intracellular trafficking. Other modifications that occur in the ER are cleavage of existing signal sequences and formation of disulphide bonds between cysteine (Cys) residues, which is involved in stabilization of the receptor structure. The stabilization of the receptor structure and proper folding of the receptor is a process that is controlled by one or more chaperones. During the remaining transport through the Golgi to the plasma membrane, final processing occurs by the means of further N-glycosylation, O-glycosylation, palmitoylation, phosphorylation, and sulfation (Dong et al., 2007; Petäjä-Repo and Bouvier, 2005). O-glycosylation adds N-acetyl-galactosamines to serine and threonine residues and occurs in the lumen of the Golgi apparatus. Palmitoylation acylates cysteines in the C-terminal domains of GPCRs, which may modulate G protein-coupling, internalization, resensitization, and/or intracellular trafficking of the receptors (Leeb-Lundberg et al., 2005).

Conserved sequences, so called ER export motifs, in GPCR C-termini or intracellular loops are essential for GPCR export from the ER. The mechanisms by which different ER export motifs aid in receptor maturation and export are not clear, but it may involve processes like proper receptor folding in the ER, interaction with chaperones, or receptor dimerization. There are also other conserved sequences, called ER retention motifs, which prevent GPCRs from leaving the ER. RSRR is the ER retention motif of γ-aminobutyric acid type B receptor (GABABR) 1. When expressed alone, this receptor is retained intracellularly, but when co-expressed with GABABR2, both receptors are expressed on the cell surface. Co-expression of GABABR1 and GABABR2 is thus obligatory for receptor cell surface expression. The mechanism for this is proposed to be hetero-oligomerization between the two receptor subtypes, leading to a conformational change that masks the ER retention motif, thus allowing the receptors to leave the ER (Dong et al., 2007; Petäjä-Repo and Bouvier, 2005).

The ER quality control machinery monitors folding and maturation of GPCRs. Misfolded or incompletely folded receptors are retained in the ER, and only receptors with a native conformation are allowed to exit. Chaperones assist in protein folding and are important participants in this process. The best-characterized chaperones are calnexin, calreticulin and BiP (also called Grp78). These proteins recognize immature GPCRs by their exposed hydrophobic regions, unpaired cysteins, or immature glycans. Chaperone-bound misfolded proteins are covalently ubiquitinylated and targeted for degradation by the proteasome in a process known as ER-associated degradation (ERAD) (Dong et al., 2007; Petäjä-Repo and Bouvier, 2005). Accessory proteins are a novel group of proteins that
assist in receptor maturation, although the exact mechanisms for their actions are still unclear. One example of accessory proteins is receptor activity modifying proteins (RAMPS) that are necessary for the cell-surface expression of the calcitonin-like receptor (Cooray et al., 2009). Misfolded mutant GPCRs that are retained in the ER are the cause of several diseases, such as the autosomal dominant forms of retinitis pigmentosa, X-linked nephrogenic diabetes insipidus, and hypogonadotropic hypogonadism (Conn et al., 2007). It has been reported that certain ligands can serve as pharmacological chaperones to rescue misfolded mutant GPCRs and indeed limit symptoms of disease. One example is the intracellular L57R V2 vasopressin receptor (V2R) mutant, which causes X-linked nephrogenic diabetes insipidus and is partially rescued to the cell surface by administration of the non-peptide V2R ligands SR121463 in transfected HEK293 cells (Ranadive et al., 2008).

Receptor oligomerization

For many years, GPCRs were considered to exist and function as monomers. However, during the last ten years this dogma has gradually been reversed, and a large number of reports now favor the concept that GPCRs exist, and potentially functions, as receptor homo- or hetero-dimers or higher order oligomers (Milligan, 2008). Oligomerization of GPCRs has also been used as a possible explanation for the curvilinear scatchard plots of radioligand binding that suggests negative or positive cooperativity (Albizu et al., 2006). However, it is still not clear whether this event is fundamental to all GPCRs, as there are some recent reports still indicating or arguing for the concept of monomeric GPCRs (Gurevich and Gurevich, 2008; Milligan, 2008; Szidonya et al., 2008). Furthermore, full support that receptor oligomers exist in vivo is still lacking (Milligan, 2008).

Many studies supporting oligomerization have been performed using techniques such as bioluminescence resonance energy transfer (BRET), fluorescence resonance energy transfer (FRET), crosslinking-studies, and co-immunoprecipitation (Rios et al., 2001; Angers et al., 2002; Szidonya et al., 2008). While each method alone generally provides only circumstantial evidence, the combined results from theses methods support the existence of receptor oligomers. As mentioned above, the GABAB receptor system represents one important example of GPCR oligomerization, where both the type 1 and 2 receptor forms are required for cell surface expression of a functional receptor hetero-oligomer (Marshall et al., 1999; Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998). Another example is rhodopsin, which has been shown by atomic force microscopy to be organized as dimers and higher order structures in native
retinal membranes (Fotiadis et al., 2003, 2004; Liang et al., 2003). Since GPCR are important drug targets, an increased understanding of GPCR homo- and hetero-oligomerization is likely to aid in the search for novel therapeutic avenues based on GPCR modulation (Milligan, 2008; Schlyer and Horuk R, 2006; George et al., 2002).

**Receptor oligomerization – effect on maturation**

Receptor oligomerization is thought to be important for receptor maturation, trafficking, agonist specificity, and signaling (Bulenger et al., 2005; Milligan, 2008). There is evidence that some receptors may be delivered to the cell surface as oligomers. For example, surface expression of α2C- and α1D-adrenergic receptors is greatly enhanced by coexpression of β2-adrenergic receptors (Prinster et al., 2006; Uberti et al., 2005). Other studies using mutant truncated receptors show that some of these mutants exert a dominant-negative effect on wild type receptors, trapping them intracellularly thus preventing them from reaching the cell surface (Zhu and Wess, 1998; Kang et al., 2005). Indeed, it is such mutants that prevent the vasopressin 2 receptor from reaching the cell surface in nephrogenic diabetes insipidus (Ranadive et al., 2008). Wilson et al., (2005) used a different approach when studying the hetero-oligomerization between the chemokine receptors CXCR1 and CXCR2. They created a mutant CXCR1 by adding a strong ER retention motif to its C-terminal tail, resulting in an ER-retained receptor. Coexpressing this mutant with wild-type CXCR1 or CXCR2 functioned to prevent their delivery to the cell surface. Coexpression of the CXCR1 mutant with the α1A-adrenoreceptor did not modulate the surface expression of this receptor, which supports other data that CXCR1 does not interact with this receptor (Wilson et al., 2005). Taken together, these data suggest that receptor oligomers are able to form during the maturation process.

**Receptor oligomerization – effect on trafficking and signaling**

Hetero-oligomerization has been shown to both inhibit and facilitate endocytosis, depending on the pair of receptors that are being expressed. One example is the hetero-oligomerization between the β1-AR (poorly internalized) and the β2-AR (strongly internalized), which results in inhibition of agonist-promoted internalization of the β2-AR (Lavoie et al., 2002). In some cases it has been shown that this change in internalization is caused by a change in β-arrestin preference. The thyrotropin-releasing hormone receptor 1 and 2 (TRHR1 and TRHR2) hetero-oligomer recruits β-arrestin 1, which is different from the TRHR2 homo-oligomer that recruits β-arrestin 2 (Hanyaloglu et al., 2002). This change in β-arrestin preference may cause a normally recycling receptor to enter a degradation
pathway, or vice versa (Bartlett and Whistler, 2005). Hetero-oligomerization also has effect on receptor signaling. For example the δ-opioid (DOP) and μ-opioid (MOP) receptor hetero-oligomerization has been reported to alter G protein-coupling selectivity (George \textit{et al.}, 2000), and D1/D2 dopamine receptor hetero-oligomers are reported to generate novel Ca$^{2+}$ signals in the striatum (Rashid \textit{et al.}, 2007).
Bradykinin receptors

Kinins are potent proinflammatory peptides that are rapidly produced extracellularly following pathological insults and tissue damage. These peptides act through two receptor subtypes, B₁ (B1R) and B₂ (B2R), which share 36% sequence homology and both belong to the rhodopsin class of GPCR (Fig. 4). Through these receptors, kinins elicit numerous inflammatory responses including vasodilatation, increased vascular permeability, and pain (Leeb-Lundberg et al., 2005).

**Figure 4.** Schematic representations of the human B2R and B1R amino acid sequences. EL = extracellular loops, IL = intracellular loops., * = the cluster of serines and threonines phosphorylated by GRK or PKC and important for desensitization. Adapted from Fredrik Leeb-Lundberg with permission.

The components of the kallikrein-kinin system

Kinins are pharmacologically active polypeptides that are released in tissues and body fluids by the enzymatic actions of kallikreins on kininogens. There are two forms of kininogens in humans: high-molecular-weight kininogen (HMWK) and low-molecular-weigh kininogen (LMWK). Kininogens are synthesized in the liver and circulate in the plasma and other body fluids. Kallikreins exist as tissue-kallikrein in various organs, such as kindney, heart and synovial tissue, and as
plasma-kallikrein in the circulation. They are produced as inactive pre-kallikreins, and upon activation they act on kininogens to produce the B2R agonists bradykinin (BK) and kallidin (Lys-BK, or KD). Kininase I acts on BK and KD to remove the C-terminal arginine, thus producing the B1R agonists des-Arg⁹-BK (DABK) and des-Arg¹⁰-KD (DAKD) (Sharma, 2008). The peptide sequences for these kinins are presented in Table 1. Kinins are rapidly (<15 sec) inactivated by peptidases, such as membrane-bound kininase II (also called angiotensin converting enzyme, ACE) and enkaphalinase. These enzymes are present in the plasma, endothelial cells and in the tissues (Sharma, 2008).

Table 1. Peptide sequences of kinins.

<table>
<thead>
<tr>
<th>Name</th>
<th>Peptide sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bradykinin</td>
<td>Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg</td>
</tr>
<tr>
<td>Lys-Bradykinin (kallidin)</td>
<td>Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg</td>
</tr>
<tr>
<td>des-Arg⁹-BK</td>
<td>Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe</td>
</tr>
<tr>
<td>des-Arg¹⁰-kallidin</td>
<td>Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe</td>
</tr>
</tbody>
</table>

Kinin receptor expression and signaling

The B2R is constitutively expressed in most cells and signals in response to BK or KD. Upon activation, B2R rapidly desensitizes and internalizes, and recycles back to the plasma membrane following internalization. In contrast, the B1R is induced in response to inflammatory insult, constitutively active, and further activated by the agonists DABK and DAKD (Marceau et al., 1998; Leeb-Lundberg et al., 2005). B1R also spontaneously internalizes in a manner that is impeded by the agonist, and upon internalization B1R targets lysosomes for degradation (Enquist et al., 2007).

B2R signals primarily through Gαq, even though it has been reported to interact with several other G proteins as well, including Gαi, Gαs, and Gα12/13. Signaling through Gαq involves the activation of PLC, followed by phosphoinositide (PI) hydrolysis, an increase in intracellular free Ca²⁺, and activation of PKC. Depending on the cell-type, B2R has also been shown to activate numerous other effector molecules such as phospholipase D (PLD), phospholipase A₂ (PLA₂), and to trigger the MAPK pathway, the caveolae-associated Janus-activated kinase (JAK)/signal transducers and activation of transcription (STAT) pathway, and nitric oxide (NO) release via activation of endothelial-specific nitric oxide
synthase (eNOS). BK also activates a number of transcription factors that regulate
the induction of cytokines, such as interleukin 1β (IL-1β). B1R signals through
Gαq and Gαi, and activates many of the same signaling pathways as B2R. The
many signaling pathways triggered by B2R and B1R are extensively reviewed
elsewhere (Leeb-Lundberg et al., 2005; Blaukat, 2003).

Post-translational kinin receptor modifications

The B1R and B2R each contain 3 potential sites for N-glycosylation in their
extracellular domains, and both receptors have been reported to be N-glycosylated
(Fortin et al., 2006; Micheneau et al., 2004). The B1R contains several serines and
threonines in the N-terminal domain and extracellular loops that may be subject to
O-glycosylation (Kang et al., 2005), but this has not been investigated. However,
B2R partially purified from rat uterus was found not to be subject to O-
glycosylation (Yaqoob et al., 1995). Bradykinin receptors contain four cysteine
residues in their extracellular domains, but the presence of disulphide bridges is
not clear even though there are indirect evidence suggesting a disulphide bridge
between Cys\textsuperscript{103} and Cys\textsuperscript{184} in the human B2R, which seems to be necessary for
maturation. In the same study, an S-S bridge between Cys20 and Cys277 is
suggested (Herzig et al., 1996). The disulphide bridge between Cys\textsuperscript{103} and Cys\textsuperscript{184}
is homologous to a highly conserved disulphide bridge among GPCRs. Rhodopsin
receptors have a disulphide bridge between Cys\textsuperscript{110} and Cys\textsuperscript{187}, which was shown to
be important for the stabilization of the receptor structure. In addition, many other
reports show similar disulphide bridges in other GPCRs (Stitham et al., 2006). In
another report B2R disulphide formation is shown to be important for the
formation of B2R dimers (Michineau et al., 2006). The same report also shows
that B2R is modified by sialylation, which seems to be important for the
stabilization of a B2R-homodimer.

Mammalian B2Rs contain 2 cysteine residues in their tail regions. Rodent B1Rs
have no cysteines, whereas nonrodent B1Rs contain 1-2 cysteines in their tails.
Palmitoylation of B2R cysteine residues has been shown by mass spectrometric
analyses (Soskic et al., 1999), whereas the extent of B1R palmitoylation has not
yet been investigated. B2R palmitoylation was also reported in transfected COS-7,
CHO-KI, and HEK293T cells, which was suggested to be involved in the
stabilization of the receptor-agonist complex (Pizard et al., 2001). B2R contains
clusters of serine and threonine residues and display agonist-dependent
phosphorylation and desensitization. On the other hand, the B1R is less
phosphorylated and desensitized. Accordingly, B1R-mediated signaling is much

25
more sustained compared to the transient B2R-mediated signaling (Leeb-Lundberg et al., 2005).

**Kinin receptor trafficking**

Membrane rafts are cholesterol and sphingolipid-enriched microdomains in the plasma membrane and are thought to bring signaling molecules together, thus serving as organizing centers for receptor signaling (Lavoie et al., 2009). Caveolae have a lipid composition similar to rafts, consist of 50-100 nm invaginations in the plasma membrane, and are defined by their content of the structural proteins caveolin and cavin (Patel et al., 2008; Pani and Singh, 2009). Most of the B2Rs are located in the plasma membrane. Association between endogeneous B2R and caveolae was shown in DDT1 MF-2 hamster smooth muscle cells. In addition, BK stimulation of these cells resulted in accumulation of Gaq in the caveolae fraction within 5 min (de Weerd and Leeb-Lundberg, 1997). Lamb et al. (2001) reported that some of the plasma membrane receptors were enriched in caveolae upon agonist binding in HEK293 cells transfected with human B1R or B2R. They also showed that agonist binding to these receptors were protected from acid wash treatment and that the enrichment of B2R in caveolae was not dependent on β-arrestin-2 or dynamin (Lamb et al., 2002). The function of B2R caveolae association is not clear, but it may represent a membrane structure that brings signaling components in close proximity with the receptors. B2R and BK also internalize via clathrin-coated pits upon agonist binding, signaling, and desensitization of the receptor. Following internalization, B2R then enters a recycling pathway and return to the plasma membrane. In COS-7 (monkey kidney fibroblasts) cells, β-arrestin-2 was shown to colocalize with internalized B2R and the uncoupling between β-arrestin-2 and B2R was necessary for receptor recycling (Simaan et al., 2005). Another report using HEK293 cells showed that agonist-stimulated B2Rs recruit β-arrestin-2 and internalize in a clathrin-dependent manner (Enquist et al., 2007).

The B1R, on the other hand, exists on the cell surface, but is mostly retained intracellularly. The cell surface-located receptors are not enriched in caveolae following agonist stimulation (Lamb et al., 2002), but internalize spontaneously through clathrin-coated pits in a manner that is impeded by the agonist, and target the lysosomes for degradation in HEK293 cells (Enquist et al., 2007). Sabourin et al. (2001) reported that B1R localized to caveolae upon agonist-stimulation. However, they used a C-terminally tagged B1R-YFP (yellow fluorescent protein) construct that was mainly localized in the plasma membrane. This is contradictory to other reports and it is possible that the relatively large size YFP tag in the C-
terminus, which is an important regulatory part of GPCRs, changes the trafficking features of B1R. The constitutive B1R internalization is not dependent on β-arrestin-2 and may be a mechanism for regulating the constitutive receptor activity that has been reported in these cells (Leeb-Lundberg et al., 2001). The pool of intracellular B1Rs probably represents slowly maturing receptors, which has been proposed to be due to a slow folding in the ER (Fortin et al., 2006; Enquist et al., 2007). Indeed, it has been suggested that some of the intracellular B1Rs never reach the cell surface, but are degraded intracellularly by the proteasome (Fortin et al., 2006). Thus, ER exit appears to be a critical “bottle neck” or regulatory point in the presentation of B1R on the cell surface.

Protein-protein interactions in kinin receptor signaling

Protein-protein interactions such as receptor oligomerization can affect receptor trafficking, agonist specificity and signaling. B2R has been reported to form homo-oligomers in rat pheochromocytoma PC-12 cells, that endogenously express B2R. This report suggests that the N-terminus of the B2R is important for oligomerization and that oligomerization is induced by agonist stimulation (AbdAlla et al., 1999). However, to obtain these results crosslinking was used to trap BK-B2R complexes, and anti-BK antibodies were used for visualization. This means that any receptor monomers or oligomers that were not able to bind agonist, were not detected in this study. Paper I in this thesis shows that B1R forms constitutive homo-oligomers that reach the cell surface, as determined by immunoelectron microscopy and co-immunoprecipitation in HEK293 cells transfected with human B1R. B1R homo-oligomerization was required for receptor surface expression, since coexpression with a truncated B1R mutant trapped the intact B1R intracellularly (Kang et al., 2005). Transfected human B1R and B2R have also been reported to form receptor hetero-oligomers in HEK293 cells. These B1R-B2R hetero-oligomers had enhanced signaling capacity, as determined by PI hydrolysis, compared to B1R and B2R alone (Kang et al., 2004). B2R may also be able to heterodimerize with angiotensin II type 2 receptors (AT₂R). This was shown by using confocal fluorescence resonance energy transfer (FRET) and co-immunoprecipitation in modified PC-12 cells (PC-12W cells) with endogeneous AT₂R and B2R expression (Abadir et al., 2006). B2R hetero-oligomerization with AT₁R enhances AT₁R signaling and alters receptor internalization (AbdAlla et al., 2000, 2001). B2R- AT₁R hetero-oligomers also contribute to angiotensin II hyperresponsiveness of mesangial cells in experimental hypertension (AbdAlla et al., 2005), and in preeclampsia – a pregnancy-specific disease characterized by proteinuria and hypertension (AbdAlla et al., 2001; Quitterer et al., 2004).
However, four independent research groups failed to reproduce the results obtained by AbdAlla et al. regarding the B2R-AT_1R interaction, as presented in a recent report (Hansen et al., 2009). These authors conclude that B2R/AT_1R oligomerization does not occur as a natural consequence of their simultaneous expression and that no enhancement of AT_1R signaling was obtained upon B2R coexpression.

B2R is also able to interact with ACE, a protease that is involved in the proteolysis and inactivation of bradykinin (Marcic et al., 2000). Another report studied the interaction between B2R and ACE in CHO cells, and found that they co-immunoprecipitated and that BK stimulation resulted in co-internalization of B2R and ACE (Chen et al., 2006). The metalloendopeptidase thimet oligopeptidase (EP24.15) is a neuropeptide-metabolizing peptidase that acts on kinin, as well as many other substrates, to produce BK(1-5) and BK(6-9). B2R and EP24.15 may have a more intimate role, since they are colocalized in plasma membrane lipid rafts (Jeske et al., 2006). The interaction between B2R and EP24.15 is studied in paper II in this thesis.

Physiological relevance of kinin receptors

The B2R affects smooth muscle cells of the duodenum, ileum, and cecum, causing either relaxation or contraction. It is also an important mediator in the vasculature, where it triggers either vasodilation or vasoconstriction, and increases vascular permeability. In addition, B2R affects glucose metabolism and has an antiarrhythmic effect on the heart. Another important area of B2R function is the central nervous system (CNS), where it has been implicated to be involved in Alzheimer’s disease. The B1R mediates vasodilatation and protects the heart from arrhythmias. It is also involved in renal functions, leukocyte recruitment, inflammatory responses, and pain. In addition, B1R is important for renal functions such as natriuresis and glomerular filtration. The B1R induces smooth muscle contraction in the vas deferens, uterus and urinary bladder, and is also involved in pain, inflammation, and hyperalgesia. The physiological effects of the bradykinin receptors have been reviewed in detail elsewhere (Leeb-Lundberg et al., 2005; Marceau and Regoli, 2004; Sharma and AL-Sherif, 2006).

Considering the widespread physiological functions of the bradykinin receptors, it is not suprising that several synthetic agonists and antagonists have been developed against these receptors. Treatment with B2R agonists may possibly be used to increase the vascular permeability and delivery of chemotherapeutic agents across the blood-brain barrier, which could be of benefit for treatment of brain
tumours. Treatment with the B2R antagonist CP-0127 in patients suffering from severe focal cerebral contusions was found to reduce intracranial pressure. After 1 month of treatment with icatibant – another B2R antagonist – the mean forced expiratory volume (FEV) was increased by 10% in asthmatic patients. Bradykinin receptor antagonists may also be possible therapeutic agents for cancer, since some cancer forms have an increased expression of these receptors (Sharma and AL-Sherif, 2006). ACE inhibitors are successful drugs for cardiovascular application. Inhibition of ACE also inhibits the major degradation pathway of BK. This means that the cardioprotective effects of these drugs may in part be due to increased stimulation of the bradykinin receptors. In summary, bradykinin receptors have been reported to be potential drug targets for hypertension, airway diseases, cancer, hereditary angioedema, and diabetes (Marceau and Regoli, 2004; Sharma and AL-Sherif, 2006).
The G protein-coupled estrogen receptor 1 (GPER1, previously called GPR30) is a novel putative estrogen receptor that is widely distributed in normal human tissues such as the cardiovascular system, lung, liver, intestine, bone, reproductive system, and brain (including forebrain, midbrain, hindbrain, and spinal cord). In addition, several primary breast cancers and lymphomas also express GPER1 (Prossnitz et al., 2008; Olde and Leeb-Lundberg, 2009). However, the reported expression of GPER1 differ somewhat between different studies, which in part may be explained by the use of polyclonal anti-GPER1 antibodies of varying specificity and the difficulty in obtaining negative control tissues in native systems.

Estrogens are important sex hormones that mainly act through the estrogen receptors ERα and ERβ. These receptors are primarily nuclear, and their activation by 17β-estradiol (E2) triggers a slow genomic response, where ERα and ERβ act as transcription factors. Non-genomic estrogen signaling is less well characterized and thought to involve a rapid mechanism and receptors located at the plasma membrane (Prossnitz 2008, Olde 2009) (Fig. 5). Variants of ERα and ERβ have been identified in the plasma membrane and may in part be responsible for non-genomic signaling (Razandi et al., 1999; Wang et al., 2005). GPER1 is also a candidate receptor for E2-promoted signaling, and studies with GPER1 knockout mice suggest that estrogen promoted pancreatic insulin release (Mårtensson et al., 2009) and closure of the bone growth plate (Windahl et al., 2009) are dependent on GPER1. It is relatively clear that GPER1 is not involved in classical estrogenic responses in vivo such as reproduction, bone mineral density, or fat mass (Olde and Leeb-Lundberg, 2009). GPER1 mRNA and protein are expressed in the hamster ovary. The expression is high in theca cells, appreciable in granulosa cells and low in luteal cells. The level of GPER1 protein was increased upon LH (luteinizing hormone) and FSH (follicle-stimulating hormone) treatment, which suggests that GPER1 is involved in gonadotropin-regulated cell functions (Wang et al., 2007). In addition, Noel et al. (2009) recently showed that GPER1 is expressed in some subsets of primate LH-releasing hormone (LHRH ) neurons and that it is involved in E2-induced intracellular Ca²⁺-mobilization and LHRH release, in a pertussis toxin sensitive manner. Nevertheless, GPER1-deficient mice are fertile indicating that this receptor is not required for reproduction (Olde and Leeb-Lundberg, 2009). GPER1 is also expressed in the rat paraventricular nucleus and supraoptic nuclei of oxytocin neurons, where it was
mostly associated with the Golgi apparatus and could not be detected on the cell surface (Sakamoto et al., 2007).

**Figure 5.** Estrogen receptor signaling. ER = estrogen receptor. ERα and ERβ are primarily nuclear and activation by E2 triggers a slow genomic response, where ERα and ERβ act as transcription factors. Non-genomic estrogenic signaling is thought to involve receptors localted at the plasma membrane. Candidate receptors for this rapid signaling are ER versions in the plasma membrane, GPER1, or some other yet unidentified receptor. Adapted from Fredrik Leeb-Lundberg with permission.

**GPER1 signaling**

GPER1 is activated by E2, which also activates ERα and ERβ. To aid in the study of GPER1, a GPER1-specific agonist, G-1, was developed (Bologa et al., 2006) (Fig. 6). This makes it possible to distinguish between signals triggered by the classical estrogen receptors (ERα and ERβ) and GPER1. GPER1 acts through Gαs, which leads to an elevation of cAMP, and through a pertussis toxin-sensitive G protein, leading to transactivation of the epidermal growth factor (EGF) receptor. GPER1 activation also leads to intracellular Ca^{2+} mobilization, and activation of PI3K (phosphatidylinositol 3-kinase), ERK1/2 and Src (Prossnitz et al., 2008a; Olde and Leeb-Lundberg, 2009). For example, G-1 was reported to cause an increase in intracellular Ca^{2+} in dissociated and cultured rat hypothalamic.
neurons (Brailoiu et al., 2007). However, the definition of GPER1 as an estrogen receptor is not universally accepted since some papers report that E2 acts independently of GPER1 (Otto et al., 2008).

**Figure 6.** The structure of the GPER1 agonists 17β-estradiol and G-1.

G15 is a recently developed GPER1 antagonist, with no affinity for ERα and ERβ, and was shown to prevent both E2- and G1-mediated intracellular Ca^{2+} mobilization in ERα- and ERβ-negative breast cancer cells. By using G-1 and G15, the authors also found that GPER1 contributes to estrogen-mediated proliferation of the uterine endothelium and that GPER1 may have an important role in the antidepressive effects of estrogen (Dennis et al., 2009).

**Subcellular localization of GPER1**

The subcellular localization of GPER1 is under debate. Revankar et al. (2005) expressed a green fluorescence protein (GFP) conjugated GPER1 in COS-7 cells and found that these receptors were localized intracellularly in the endoplasmatic reticulum. They also used a polyclonal antibody directed against the C-terminus of GPER1 to study endogeneous expression in various cell lines. Again, GPER1 was found in the endoplasmatic reticulum, but not in the plasma membrane. In addition, permeabilization of the plasma membrane was required to obtain binding between fluorescent estrogen derivatives and GPER1. The authors thus suggested that GPER1 binds and signals in response to E2 in the endoplasmatic reticulum, (Revankar C., et al, 2005), which is unique for a GPCR. Otto et al. (2008) used transiently transfected cells as well as cells endogeneously expressing GPER1 to explore the subcellular localization of GPER1. They found that GPER1 was localized in the endoplasmatic reticulum but that it was unable to bind radioactively labelled E2. In addition, they were not able to achieve any impact on
intracellular cAMP or calcium levels upon E2 administration (Otto et al., 2008). Others claim that GPER1 also exists on the plasma membrane since it is activated by the membrane-impermeable E2 analogs E2-BSA and E2-HRP (Filardo et al., 2007). By using confocal immunofluorescence microscopy, Filardo and co-workers showed that N-terminally HA-tagged GPER1 (HA-GPER1), transfected in HEK293 cells, colocalized with the plasma membrane marker concavalin A. HA-GPER1 was found in patches on the plasma membrane, and to a small extent colocalized with clathrin in untreated cells. E2 stimulation increased HA-GPER1 sequestration and clathrin colocalization. They also conducted subcellular fractionation of transfected HEK293 cells as well as SKBR3 cells (human breast cancer cells), that endogeneously expresses GPER1, showing that estrogen binding and G protein activation were associated with the plasma membrane fractions (Filardo et al., 2007). GPER1 has also been reported to concentrate within the plasma membrane of pyramidal neuronal cells of the rat hippocampus (Funakoshi et al., 2006). Thus, the results on the subcellular localization of GPER1 are very diverging, and more studies need to be conducted.

GPER1 and breast cancer association

Estrogen is known to elicit proliferative effects on breast cancer cells. Tamoxifen is an ERα antagonist that is used for endocrine treatment of ERα positive tumours. However, this antagonist also works as a GPER1 agonist, which could dramatically influence the outcome of the treatment (Prossnitz et al., 2008a). Filardo et al. (2008) investigated the level of GPER1 expression in 321 cases of human primary breast cancer as well as in normal breast tissue. They found that GPER1 was strongly expressed in normal breast tissue and that the level of receptor expression varied among different breast tumor specimens, where 60% of the tumors expressed GPER1 levels similar to normal breast tissue, and 40% expressed low or undetectable GPER1 levels (Filardo et al., 2008). Expression of GPER1 in breast tumours correlates strongly with tumour size, HER 2 (human epidermal growth factor receptor 2) expression, and distant metastasis (Filardo et al., 2006). Another report used ERα- and ERβ-negative human breast cancer cells to study GPER1 mediated signals triggered by hydroxytamoxifen (OHT). They found that E2 triggered GPER1-mediated activation of a transcription factor network, where the gene coding for the connective tissue growth factor (CTGF) was the most strongly induced. CTGF then promotes proliferation and cell-migration of the SKBR3 breast cancer cells, thus promoting aggressive tumour behaviour and metastasis. In the same report, they showed that CTGF is also induced by OHT in fibroblasts from breast tumour biopsies. Thus, OHT used in endocrine therapy may promote aggressive tumour behaviour and metastasis in a
GPER1 dependent manner (Pandey et al., 2009). Considering the expression and signaling profile of GPER1 in breast cancer cells, it is obvious that this receptor constitutes a target for anti-carcinogenic drug design and emphasizes the importance of evaluating the level of GPER1 expression in an ERα positive tumour before using tamoxifen in endocrine therapy.
Present investigation

Aims

1. Investigate the role of B1R homo-oligomerization in receptor maturation and cell surface expression.  
   (Paper I and III)

2. Analyze the way by which a truncated B1R fragment (B1stop135) disrupts B1R cell-surface expression and signaling.  
   (Paper III)

   (Paper II)

   (Paper II)

5. Study GPER1 localization, membrane trafficking, and signaling.  
   (Paper IV)

Summary

Paper I

**B₁ bradykinin receptor homo-oligomers in receptor cell surface expression and signaling: effects of receptor fragments.**

B1R is an inducible and constitutively active receptor, which is further activated by the agonists DABK and DAKD (Leeb-Lundberg et al., 2001). This receptor is involved in inflammatory and pain responses to injury. Therefore it is of great interest to understand the mechanisms behind B1R expression and regulation in order to identify novel drug targets in this receptor mechanism. The aim of this study was to investigate the role of B1R homo-oligomerization in cell surface receptor expression. This was done by using HEK293 cells transiently transfected with N-terminally HA- or FLAG-tagged B1R (HB1 and FB1). HB1 and FB1 were monitored by using epitope-specific monoclonal antibodies, and antibodies
directed against the human B1R C-terminal residues 317 to 353. FB1 expressed as a 35 kDa species, which is assumed to be the monomeric form of the receptor, as well as other species at higher molecular masses. Interestingly, the relative masses of these species suggest that they may be multiples of the monomeric 35 kDa form and therefore representing some higher order homo-oligomeric receptor forms. To further investigate B1R homo-oligomerization, FB1 and HB1 were expressed in HEK293 cells, either individually or in combination, and then immunoprecipitated and immunoblotted with epitope-specific antibodies. FB1 and HB1 co-precipitated in a cell-dependent manner, since mixing of lysates expressing FB1 or HB1 individually failed to yield any co-precipitation. Immunoelectron microscopy was used as a detergent-free method to provide additional evidence for B1R homo-oligomerization. This was done by incubating intact cells expressing HB1 with 5 nm gold-labelled anti-HA antibodies at 4°C to selectively detect cell surface receptors. The cells were then fixed and prepared for microscopy. Receptors were readily shown on the cell surface and clusters of labelling were clearly visible.

In order to map the B1R homo-oligomerization epitope, several truncated B1R mutants were created. The mutants were coexpressed with intact B1R and their ability to interact was investigated by immunoprecipitation. It was concluded that the B1R homo-oligomerization epitope lies between residue Leu<sup>26</sup> on top of transmembrane helix 1 and Val<sup>71</sup> at the C-terminal end of intracellular loop 1. One of the truncated B1R mutants (B1stop135) was used to investigate its effect on B1R homo-oligomerization. Expression of HB1 and FB1 together with different concentrations of HB1stop135 led to a concentration-dependent decrease in the amount of FB1 that co-precipitated with HB1. This may be caused by the ability of B1stop135 to disrupt B1R homo-oligomers and form hetero-oligomers with the intact receptor. Radioligand binding of intact cells and biotinylation of cell surface receptors revealed that B1stop135 dose-dependently decreased the level of B1R cell surface expression. In addition, B1stop135 also caused a dose-dependent decrease in constitutive B1R-mediated PI hydrolysis. To investigate in more detail whether B1stop135 interfered with the plasma membrane targeting of the B1R or simply reduced the total B1R expression, cells expressing HB1 and FB1stop135, either individually or together, were lysed hypotonically and fractionated on a sucrose density gradient. The fractions were then immunoblotted with either anti-HA or anti-FLAG antibodies. Coexpressing FB1stop135 with HB1 redistributed HB1 from the plasma membrane fractions to fractions of lighter density. Thus, B1stop135 interacts with B1R and forms hetero-oligomers, which are unable to properly target to the cell surface.
**Paper II**

**Kinin B₂ receptor-mediated bradykinin internalization and metalloendopeptidase EP24.15-dependent intracellular bradykinin degradation.**

B₂R is constitutively expressed on most cells and is activated by BK or KD, which are formed upon tissue injury or inflammatory insult (Leeb-Lundberg *et al.*, 2005). BK is degraded by extracellular as well as intracellular peptidases. EP24.15 is a soluble metalloendopeptidase that is known to cleave BK extracellularly to produce BK(1-5) and BK(6-9). To directly address the cellular and functional relationship between B₂R and EP24.15, and to determine the role of EP24.15 in the degradation of cell surface-bound and intracellular BK, a cellular model system was created in which FLAG- or HA-tagged B₂R and EP24.15 were expressed individually or together. B₂R was mostly localized in the plasma membrane, whereas EP24.15 was only detected intracellularly and no obvious colocalization between B₂R and EP24.15 was observed using confocal immunofluorescence microscopy. However, the two proteins co-immunoprecipitated specifically, indicating a direct or indirect interaction. Furthermore, biotinylation of intact cells at 4°C followed by immunoprecipitation and immunoblotting with streptavidin conjugated to horseradish peroxidase (HRP) revealed that EP24.15 was also present on the extracellular side of the plasma membrane, a possible site of B₂R interaction.

EP24.15 attenuated maximal B₂R responsiveness without influencing the potency of BK to stimulate PI hydrolysis and intracellular Ca²⁺ mobilization. The unaffected potency of BK indicates an EP24.15-dependent mechanism involving direct or indirect protein-protein interactions. To determine if EP24.15 has any effect on intracellular degradation of BK, intact cells expressing B₂R alone or together with EP24.15, were incubated with [³H]BK. Intracellular BK-derived material was analyzed by first stripping intact cells with low pH buffer to remove all remaining cell surface-bound BK and then fractionating the cells into high density fraction (HDF) and low density microsomal fraction (LDMF). These fractions were then extracted and subjected reverse-phase high-pressure liquid chromatography. It was found that coexpressing EP24.15 with B₂R clearly resulted in degradation of internalized BK. Thus, EP24.15 is involved in intracellular degradation of BK internalized specifically via B₂R through receptor-mediated endocytosis.
A B₁ kinin receptor fragment exerts dominant-negative behavior by disrupting intact B₁ receptor homo-oligomers in the endoplasmatic reticulum and increasing receptor degradation.

B₁R matures slowly, which has been proposed to be due to a slow folding in the endoplasmatic reticulum (Enquist et al., 2007; Fortin et al., 2006) and may be a regulatory step of this receptor. The aim of this study was to investigate the role of B₁R homo-oligomerization in receptor maturation. In paper I, an N-terminal B₁R fragment terminating in the second intracellular loop of the receptor (B₁stop135) was found to interact with B₁R and dose-dependently decrease B₁R cell surface expression. To analyze B₁R homo-oligomerization, receptor maturation, and the cellular mechanism by which B₁stop135 disrupts this process, a HEK293 cell model system was constructed, in which N-terminally FLAG-tagged B₁R (FB₁) and HA-tagged B₁stop135 (HB₁stop135) were stably expressed either individually or in combination. Using confocal immunofluorescence microscopy FB₁ was shown to be expressed intracellularly, both in a tubular network and in distinct puncta, as well as in the plasma membrane. In contrast, HB₁stop135 was only expressed intracellularly, and coexpression with FB₁ resulted in partial colocalization and complete prevention of FB₁ cell surface expression. HB₁stop135 also exhibited dominant-negative behavior on B₁R agonist binding and signaling, as shown by radioligand binding and agonist-stimulated PI hydrolysis. Coexpression of FB₁ and HB₁stop135 completely prevented agonist binding to FB₁, both in intact cells and in particulate preparations, showing that B₁stop135 not only prevents functional B₁R from reaching the plasma membrane but also disrupts the high affinity agonist binding site in the receptor.

Immunoprecipitation and immunoblotting was used to analyze the molecular structure of B₁R in the absence and presence of B₁stop135. PNGaseF was used to determine if the receptor species were N-glycosylated. FB₁ expressed as multiple species with molecular masses of about 150 kDa, 80 kDa, and 37 kDa, several of which were N-glycosylated. Co-expression of HB₁stop135 and FB₁ dramatically increased the amount of 37 kDa monomeric FB₁ species, at the expense of the multimeric species of higher molecular weight. An additional FB₁ species of approximately 22 kDa also appeared, probably constituting a degradation product. These results provide direct evidence that B₁stop135 prevents B₁R maturation by disrupting B₁R-B₁R homo-oligomers. Coexpression of HB₁stop135 and FB₁ also increased the affinity between the chaperone calnexin and FB₁, which may represent a mechanism by which misfolded receptor hetero-oligomers are retained in the endoplasmatic reticulum.
**Paper IV**

GPER1 in the plasma membrane is subject to rapid agonist-independent receptor endocytosis and recycling.

The G protein-coupled estrogen receptor 1 (GPER1) is proposed to be a membrane estrogen receptor partially responsible for rapid non-genomic estrogen signaling. However, considerable controversy surrounds this receptor both regarding its role as an estrogen receptor and its subcellular localization. Here, we used HEK293 cells stably expressing FLAG-tagged human GPER1 (FGPER1) and mouse myotube C2C12 cells to study GPER1 localization, membrane trafficking, and signaling.

FGPER1 was monitored using monoclonal anti-FLAG M1 or anti-FLAG M2 antibodies. Immunoprecipitation followed by immunoblotting revealed that FGPER1 exists as multiple species in HEK293 cells. Treatment of the anti-FLAG immunoprecipitates with PNGaseF reduced the amounts of the majority of the higher molecular mass species. Thus, GPER1 is N-glycosylated. Confocal immunofluorescence microscopy was used to study the whole cell distribution and the intracellular trafficking of GPER1. GPER1 was mostly localized in the endoplasmatic reticulum at least in part associated with calnexin. Receptors were also present in the plasma membrane. Plasma membrane (PM) GPER1 was subject to rapid and constitutive endocytosis and the internalized receptors co-localized with the early endosomal marker EEA1 and transferrin but not with markers of lysosomes, ER, or Golgi suggesting that they target a PM recycling pathway. Thus, a slow maturation combined with a rapid constitutive endocytosis yields a relatively low steady-state level of PM GPER1. To study GPER1-mediated signalling, C2C12 cells endogenously expressing GPER1 were used. 17β-Estradiol (E2) and G-1, a synthetic GPER1 agonist, both stimulated cAMP production in a GPER1-dependent manner, in these cells. However, the efficacy of E2 is only 11% of that of G-1, indicating that E2 may act as a partial agonist in these cells.
Conclusions

- B1R forms constitutive homo-oligomers in HEK293 cells.

- The epitope for B1R homo-oligomerization is located between Leu\textsuperscript{26} and Val\textsuperscript{71} at the bottom of helix 2.

- B1R homo-oligomerization occurs early in the receptor maturation and is required for B1R cell surface expression and constitutive signaling.

- B1stop135 complexes with B1R and retains B1R intracellularly, probably by forming a misfolded receptor hetero-oligomer with increased affinity for calnexin.

- EP24.15 is involved in intracellular degradation of BK, internalized specifically via B2R through receptor-mediated endocytosis.

- EP24.15 attenuates maximal B2R responsiveness without influencing the potency of BK to stimulate PI hydrolysis and intracellular Ca\textsuperscript{2+} mobilization.

- GPER1 is primarily localized in the endoplasmatic reticulum, but also exists in the plasma membrane where it is subject to rapid, constitutive endocytosis.

- 17β-Estradiol and G-1 both stimulated cAMP production, in a GPER1 dependent manner, in mouse myotube C\textsubscript{2}C\textsubscript{12} cells.

Discussion

G protein-coupled receptors operate in every human cell and regulate multiple physiological events including metabolism, blood pressure regulation, inflammation, reproduction, etc. Not surprisingly, GPCRs have evolved as the most important drug targets for therapeutic intervention with about 50\% of existing clinically useful drugs acting through this family of receptors (Insel \textit{et al.}, 2007; Luttrell, 2008). It is important to understand the dynamic receptor response to pharmacological treatment. Receptor localization and trafficking are critical factors regulating receptor activity. Detailed knowledge about such regulation makes it possible to design novel drugs that control the localization and therefore the activity of the receptors. Receptor homo- and hetero-oligomerization constitutes additional potential drug targets, where unique drugs may be designed
that act on either receptor monomers or oligomers. However, additional evidence is needed to prove the importance of such structures in native tissues.

Kinins are potent proinflammatory peptides that are rapidly produced extracellularly following pathological insults and tissue damage. These peptides act through two receptor subtypes, B1R and B2R, which both belong to the rhodopsin class of GPCR. Through these receptors, kinins elicit numerous inflammatory responses including vasodilation, increased vascular permeability, and pain (Leeb-Lundberg et al., 2005). Bradykinin receptors have been reported to be potential drug targets for hypertension, airway diseases, cancer, hereditary angioedema, and diabetes (Marceau and Regoli, 2004; Sharma and AL-Sherif, 2006). B1R is primarily expressed intracellularly in HEK293 cells. In addition, the surface located B1Rs are subject to constitutive endocytosis. There are many methods investigating receptor-oligomerization. Even though neither of these methods alone is sufficient to prove receptor-oligomerization, together they are consistent with this concept. B1R homo-oligomerization occurs early in the biosynthesis or maturation of the receptor. Disruption of B1R homo-oligomerization by coexpressing B1R with a truncated B1R fragment (B1stop135) results in decreased B1R cell surface expression, agonist binding, and signaling. B1R homo-oligomerization may be a way of regulating B1R maturation and transport to the cell surface. Furthermore, the slow B1R maturation offers a way to carefully control the expression of this constitutively active receptor. B1R is induced in breast cancer and in breast cancer cells. Increased knowledge about the regulation of B1R maturation and cell surface expression will be of great benefit in the development of potential therapeutic agents acting on B1R. In contrast, B2R is efficiently transported to the plasma membrane, has little if any constitutive activity, and internalizes only in response to agonist binding. One regulatory point of B2R signaling is the short lifetime of BK. EP24.15 is a peptidase, which acts on BK to form two inactive kinin fragments. In this thesis, it is shown that EP24.15 also affects the intracellular degradation of BK. EP24.15 decreased B2R-mediated PI hydrolysis without affecting the potency of BK to activate B2R. In addition, B2R and EP24.15 are able to co-immunoprecipitate. Taken together, this indicates a direct or indirect interaction between B2R and EP24.15. Generation of cell-membrane permeable agents acting directly on EP24.15 or modulating the direct or indirect B2R-EP24.15 interaction may represent a novel way of modulating B2R-mediated signaling.

GPER1 is a novel estrogen receptor, which is believed to be involved in estrogen-promoted pancreatic insulin release (Mårtensson et al., 2009), closure of the bone growth plate (Windahl et al., 2009), and also seems to play a role in the antidepressive effects of estrogen (Dennis et al., 2009). It is relatively clear that
GPER1 is not involved in classical estrogenic responses \textit{in vivo} such as reproduction, bone mineral density, or fat mass. GPER1 is widely distributed in normal human tissues such as the cardiovascular system, lung, liver, intestine, bone, reproductive system, and the brain. In addition, several primary breast cancers and lymphomas also express GPER1. There is an ongoing debate about the subcellular distribution of GPER1. Some people claim that GPER1 is an intracellular receptor that primarily exists and signals in the endoplasmatic reticulum, which is unusual for a GPCR (Revankar \textit{et al.}, 2005). Others report that it also exists in the plasma membrane and signals there (Filardo \textit{et al.}, 2007). These contradictory results are investigated in this thesis. We found that GPER1 indeed is primarily localized in the endoplasmatic reticulum, but that it also exists in the plasma membrane where it undergoes rapid, constitutive endocytosis. The low level of plasma membrane expression in combination with the rapid endocytosis may be a reason for the current confusion in this regard and an explanation why some groups have failed to detect GPER1 in the plasma membrane. By lowering the temperature, the rate of receptor endocytosis is decreased, which makes it easier to label and detect GPER1 at the cell surface. The functional significance of the low level of cell surface GPER1 in combination with the large pool of intracellular receptors is not clear at the moment.

\textbf{Future directions}

\textit{Is oligomerization important for B1R maturation in breast cancer cells?}

B1stop135 may constitute a tool to determine if B1R homo-oligomerization is important for receptor maturation and surface expression in breast cancer cells. Mcf-7 is a human breast cancer cell line with endogenous expression of B1R. Radioligand binding of mcf-7 cells, transiently transfected with B1stop135, will answer whether B1stop135 is able to disrupt the formation of intact B1R homo-oligomers and lower B1R surface expression in these cells. Increased knowledge about the regulation of B1R maturation and cell surface expression will be of great benefit in the development of potential therapeutic agents acting on B1R.

\textit{How do B2R and EP24.15 interact?}

Is the interaction between B2R and EP24.15 direct or indirect? Shivakumar \textit{et al.} (2005) used two-hybrid screening to show that the B2R C-terminus interacts with EP24.15. One way to further explore the potential physical interaction between B2R and EP24.15 is to use a mutant B2R truncated at the C-terminal end, and see whether this results in decreased co-immunoprecipitation between B2R and EP24.15. However, it is important to verify that any change in interaction is not
caused by a changed subcellular distribution of the mutant B2R. Another way to study B2R-EP24.15-interaction is to use FRET.

Is E2 the true agonist for GPER1?
The low efficacy of E2 compared to G-1 in cAMP production, as measured in C2C12 cells, indicates that E2 may act as a partial agonist in these cells. To further investigate this, C2C12 cells can be stimulated with a fixed concentration of G-1 in combination with several concentrations of E2, followed by measurement of the cAMP production. If administration of E2 lowers the G-1 response, this would suggest that E2 is not the full agonist for GPER1. This experiment and the evaluation of additional GPER1 ligands is currently in progress. It would also be interesting to further explore the trafficking of GPER1 by using antibodies against Rab 4 or Rab 11. Rab proteins are small GTPases that regulates transport between intracellular vesicles. Rab 4 regulates a fast recycling pathway from early endosomes back to the plasma membrane, whereas Rab11 regulates a slow recycling pathway and directs transport from the perinuclear recycling vesicles back to the cell surface (Seachrist and Ferguson, 2003). Receptor internalization often involves recruitment of specific β-arr subtypes. By transfecting cells expressing GPER1 with fluorescent β-arr constructs it is possible to elucidate which β-arr subtype that is recruited by GPER1.

Caveolins are involved in various events, such as receptor transport to the cell surface, signaling, and endocytosis. Different caveolin isoforms appear necessary to generate distinct functional signaling complexes and alteration of caveolin expression has been implicated in breast cancer. Estrogen itself influences the level of caveolin expression and caveolin seems to play an essential role in membrane ERα and ERβ function, including ERα-dependent NO production (Luoma et al., 2008). It is tempting to speculate that caveolins are also important for GPER1 trafficking and signaling. C2C12 and HEK293 cells express different subsets of caveolins. Is this the reason why we get GPER1-dependent cAMP production in C2C12, but not in HEK293 cells? It would also be interesting to address the level of GPER1 palmitoylation, since this reversible modification is linked to events such as caveolae localization, and receptor trafficking (Claudinon et al., 2009).


Kunskap om hur dessa receptorer regleras är viktig för att kunna hitta nya måltavlor för läkemedelsutveckling.

GPER1 tillhör också GPCR-familjen och har nyligen föreslagits vara en östrogenreceptor. Den uttryckts bland annat i hjärt-kärlsystemet, lungorna, levern, tarmarna, benen, reproduktionssystemet och hjärnan. Dessutom uttrycks GPER1 i många bröstcancertumörer. Ännu vet man inte så mycket om denna receptor, men den föreslås vara inblandad i östrogenstimulerad insulinfrisättning och stängning av tillväxtplattan i ben. Det är mycket diskussion kring denna receptor och åsikterna skiljer sig när det gäller var i cellen receptorn befinner sig och var den binder östrogen. Det är dessutom inte helt klargjort om GPER1 verkligen är en östrogenreceptor.

Syftet med denna avhandling:
- Att undersöka hur B1-receptorn tar sig ut till cellytan.
- Att klargöra var GPER1 uttrycks i cellen och om den aktiveras av östrogen.

Resultat:

När en receptor aktiveras sätter den igång olika signaler inne i cellen. Om denna signalering inte kontrolleras noga kan den skada kroppen. Kontrollen sker på olika

GPER1 finns till största delen inne i cellen och vissa menar att östrogen aktiverar receptorn där, vilket vore unikt för en GPCR. Andra rapporterar att GPER1 även finns på cellytan och att det är där receptorn binder och aktiveras av östrogen. Jag har visat att GPER1 till största delen finns inne i cellen, men även på cellytan. Att den kan vara svår att upptäcka beror dels på att det är relativt få receptorer på ytan, men också på att denna receptor dras in i cellen spontant. För att undersöka om GPER1 aktiveras av östrogen gjordes flera experiment som mäter nivån av ett protein som bildas vid receptoraktivering. Östrogen aktiverade receptorn, men inte så effektivt som ett syntetiskt framställt GPER1-aktiverande ämne (G-1). Det kan tyda på att det är något annat ämne än östrogen som styr receptorns aktivitet. För närvarande pågår ytterligare studier för att klargöra detta.

Acknowledgements

Thank you to all the people that has helped me during my PhD period and contributed to this thesis in different ways. I would especially like to thank…

…FREDRIK, my supervisor. Thank you for teaching me Science and for giving me great freedom to follow my own ideas and curiosity. Thank you for creating an open atmosphere in the lab, where questions are always welcome. I would also like to thank you for keeping the door to your office always open and that you never hesitate to get out into the lab to teach me new techniques or helping out when time is short.

…JOANNA. Thank you for friendship and for always caring! You are a great support, always having time for a smile and kind word. I would also like to thank you for all expert technical assistance and for giving me chocolate when most needed!

…BJÖRN. Thank you for sharing your new ideas with such enthusiasm! I am also greatful for all the times you offered your knowledge to make me learn new techniques or try new ideas.

…TINA. Thank you for friendship and lots of discussions, both scientific in the conference room and less scientific at John Bull. You are a true inspiration with all your creativity and strength, no matter how hard life treats you.

…ULRIKA. Thank you for friendship, for introducing me to Italia, and for offering scientific advice when needed. The lab is empty without you!

…LISE, my officemate. Thank you for friendship, for nice walks and talks!

…DONG-SOO. Thank you for acting as my co-supervisor during my first year at the lab, and for sharing your knowledge and great enthusiasm for Science!

…JOHAN. Thank you for sharing your time, knowledge, and never-ending ideas!

…META. Thank you for making me feel welcome in the lab. You really have the ability to pass on your energy to others. Thank you for all expert technical assistance and for trying to keep the rest of us less chaotic in the lab!
…MARIE. Thank you for friendship and nice chats in the lab. Good luck with your “new” career!

…LOUISE. I am happy that you chose our lab for your project and that I had the opportunity to collaborate with you during the last months. I think we had great fun! Good luck with your research in Malmö!

…Additional past members of Fredrik’s group: MIA, CARL, and KRISTER. Thank you for being part of creating a nice atmosphere in the lab!

...SARA. Thank you for friendship, good collaborations and nice chats!

...CHRISTER OWMAN and additional past members of his lab: ÅKE, ULLA-BRITT, JESPER, NICLAS, AL, ULF, ERIK, and ANNIKA. It was very inspiring to begin my studies surrounded by you all! You created such a nice atmosphere with lots of joy and interesting discussions.

...EVA, KARIN, and BEATA. Thank you for always having time for a kind word, for being helpful, and offering you knowledge.

....EVA-KARIN. Thank you for nice chats early in the morning and for keeping A12 nice and tidy!

…MAMMA, PAPPA, MORMOR and MORFAR. Thank you for your love and support!

...FAMILJEN RASMUSSEN. My sister ISABELL, JOACHIM, and wonderful NICODEMUS. I am happy that you are part of my family! I am always looking forward to the next time I see you!

...ALEXANDER, my LILLEBROR. I am happy to have you :-) You and Anna are always welcome to visit!

... CHRISTER and BIRGITTA SANDÉN. Thank you for being my “bonus-parents” in Skåne, for taking so good care of Safira, and for your help with all kinds of matters!

... The rest of the SANDÉN family. Thank you for making me feel at home in Skåne and for being such a warm and welcoming family!
…OLOF, MALIN, ALEX and PETRA. Thank you for being true friends. You enrich my life!

…JENNY, VBK. I am so happy that you are my friend and for all the memories we share together! You have always been a great support and I couldn’t have done this without you!!

…PETER and SAFIRA. You mean everything to me! I love you!
References


