Functional Analysis of the Signal Peptide of the Protease Activated Receptor 1 (PAR1)

Inaugural-Dissertation
to obtain the academic degree
Doctor rerum naturalium (Dr. rer. nat.)

Submitted to the Department of Biology, Chemistry and Pharmacy
of Freie Universität Berlin

By

Dimitrios E. Zampatis

from Mesolongi-Greece

2011

This thesis was realized at the Leibniz Institut für Molekulare Pharmakologie from September 2008 to November 2011 under the supervision of PD Dr. Ralf Schülein.

1st Referee:

PD Dr. Ralf Schülein

Leibniz-Institut für Molekulare Pharmakologie

Robert Rössle Straße 10

13125, Berlin

2nd Referee:

Prof. Dr. Hartmut Oschkinat

Leibniz-Institut für Molekulare Pharmakologie/ Freie Universität Berlin

Robert Rössle Straße 10

13125, Berlin

Date of defense: **28.08.2012**

Acknowledgements

The present work was realized at the Leibniz-Institut für Molekulare Pharmakologie in Berlin.

I would like to express my sincere and deep thanks to my supervisor, PD Dr. Ralf Shülein, for his daily help, guidance and helpful discussions which contribute lot to the realization of this work.

I would also like to thank Dr. Nikolaos Tsopanoglou, Assistant Professor of the, Medical School, Department of Pharmacology ,University of Patras, for giving me the opportunity to start this study in his lab and for encouraging me to continue studying for my doctorate in Germany.

I thank all my past and present colleagues in the lab for their helpful assistance and discussions and all the technical advice, which helped me a lot.

I thank the LSM unit of the FMP, Dr. Burkhard Wiesner and Mrs. Jenny Eichhorst for their excellent cooperation.

I would like to deeply thank my family, who supported me from the beginning of my studies till today, in particular for their constant moral support.

This PhD Thesis is dedicated to the memory of my beloved mother who was always my source of hope and courage to carry on despite all difficulties.

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INTRODUCTION

Introduction

G protein-coupled receptors (GPCRs) represent the largest protein family in vertebrates. GPCRs are membrane bound receptors and play a key role in signal transduction in cells. In order to act as signal mediators, GPCRs have to be transported to the plasma membrane via the so called secretory pathway.

We focused our study in the protease activated receptor 1 (PAR1) which belongs to a sub-family of the GPCRs namely the protease activated receptors (PARs). Upon activation of the receptor, a peptide fragment is released which is thought to exert biological roles in cells.

The length of this peptide depends on whether the receptor has a cleavable signal peptide or not.

Here we studied, whether the putative signal peptide of the PAR1 is functional, and consequently also addressed the question of the actual released peptide length.

1. The PAR1 receptor

The PAR1 was the first thrombin receptor cloned, and it is the best studied. Its discovery early in 1990s was the beginning for the analysis of a whole family of PAR receptors. PAR1 is encoded by a 3.5 kb DNA fragment which exhibits the following features: A GC-rich 5′ untranslated region, an open reading frame (ORF) which encodes a 425 amino acid protein and a long 3′ untranslated region with several polyadenylation signals and a poly (A) tail (Vu et al., 1991).

PAR1 possesses the typical structure of a GPCR: 7 helical hydrophobic transmembrane domains form three intracellular and three extracellular loops, a C-terminal intracellular domain, and an N-terminal extracellular domain (Vu et al., 1991). The first 21 amino acids of the PAR1 are thought to represent a signal peptide according to a prediction program (see results section). The relatively long mature N

terminus of 101 amino acids contains three consensus sites for asparagine-linked glycosylation (N35, N62, and N75) which are all glycosylated (Xiao et al., 2011).

Two more glycosylation sites are found at the second extracellular loop (N250 and N259) which are also glycosylated (Soto et al., 2010). A disulfide bond was described linking cysteine C175 in the first extracellular loop with cysteine C254 in the second extracellular loop.

The extracellular domains of the receptor are involved mainly in coupling of PAR1 with thrombin during activation of the receptor.

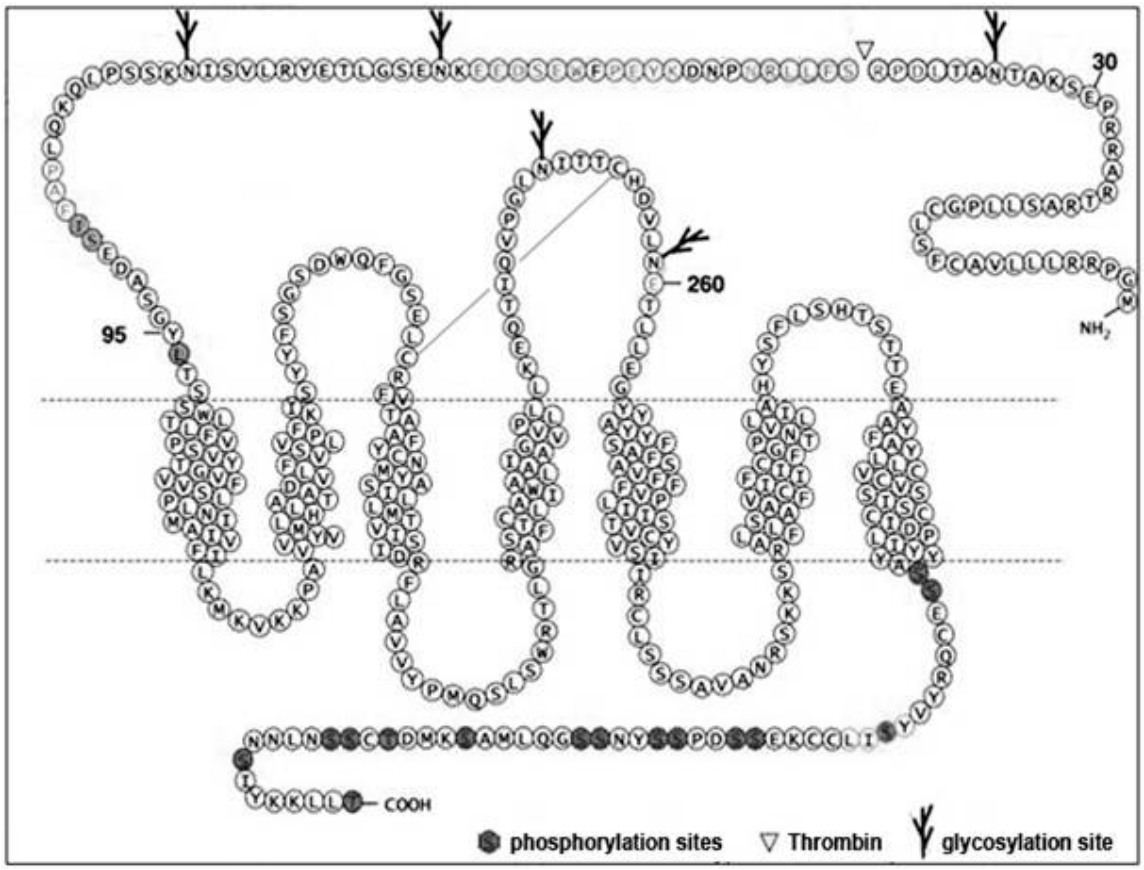

Figure 1. Topological model of the PAR1

The receptor contains a thrombin cleavage site at position S41-R42 (scissors), a hirudin like site (DKYEPF), five (*) glycosylation sites (N35, N62, N75 at the N terminus and N252, N259 at the ECL2 and adisulfide bond (Cys175-Cys254). The C terminus contains the G protein binding sites as well the phosphorylation sites (Adapted from Steinberg, 2005).

The mechanism by which the receptor is activated is unique. Initially, the anion-binding site of thrombin interacts with the DKYEPF (a hirudin-like site) sequence of the receptor (between positions 53 and 64 of the extracellular domain). The

proteolytically cleavage of the receptor in the $LDPR_{41}\downarrow S_{42}FLLRN$ (↓ means cleavage) sequence leads to the formation of a new N terminus in the receptor starting with the sequence SFLLRN (Ossovskaya et al., 2004, Coughlin, 1994).

This terminus functions as a tethered ligand and interacts with regions of the second extracellular loop of the receptor as well as with the N terminus near the first transmembrane domain, thereby activating the receptor (Bahou et al., 1994).

Upon activation, PAR1 is coupled to heterotrimeric G proteins consisting of the subunits α, β and γ. When G proteins are inactive, their α subunit bind GDP. When the receptor is activated, GDP is exchanged for GTP and the G protein is able to bind. PARs couple with members of the Gi, $G_{12/13}$, Gq, leading to biochemical pathway signaling (Figure 2).

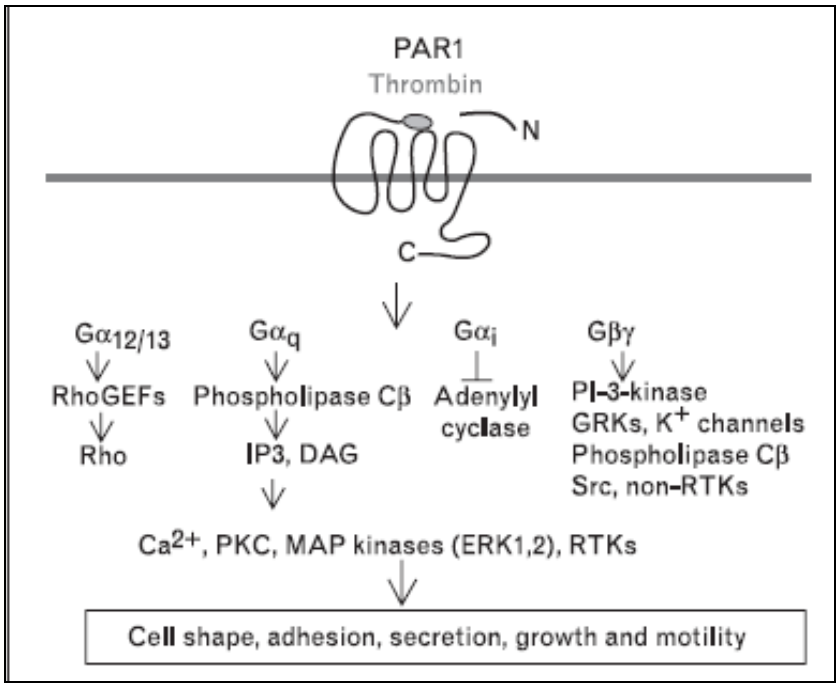

Figure 2. Cellular responses following PAR1 activation

PAR1 couples to Gai, Gαq, Gα12/13 and activates a variety of signaling cascades and cellular responses. Gα12/13 activates small G-proteins such as Rho. Gαq activates phospholipase C-β which triggers phosphoinositide hydrolysis resulting in inositol triphosphate (IP3) and diacylglycerol production, Ca^{2+} mobilization, protein kinase C (PKC) activation, mitogen-activated protein (MAP) kinase activation as well as transactivation of receptor-tyrosine kinases (RTKs). (Traynellis et al 2007)

Thrombin activates in most cell types the phospholipase C (PLC), the protein kinase C (PKC), or the mitogen-activated protein kinase (MAPK), and inhibits adenyl cyclase (AC) (Déry et al., 1998).

PAR1 activation by thrombin leads also to the Gq11α pathway activation. (Baffy et al., 1994, Benka et al., 1995). Gq11α activates the PLC-β_1, which in turn catalyzes the hydrolysis of the diphosphoric inositol (InsP2) in triphosphoric inositol (InsP3) and diacylglycerol (DAG).

The InsP3 enables intracellular Ca^{2+} mobilization, diacylglycerol (DAG) activation of the PKC or produce arachidonic acid (AA), which is used for the production of prostaglandins. Increased intracellular calcium and PKC, activate many follow-up pathways such as the MAPK pathway.

There are four different types of MAP kinases: the Erk1/2 (extracellular regulated kinase 1/2), JNKs (c-Jun NH4-terminal kinases), the p38 proteins (P38 α, β, γ, δ), and ERK5 (Chang et al, 2001).

Proliferation of endothelial cells seems to be mediated by activation of Erk1/2. The activation of the PAR1 is thought to be a necessary condition (Olivot et al., 2001).

The thrombin-mediated activation of PAR1 causes also the activation of mitogenic pathways such as the one of the Janus family of tyrosine kinases (JAKs) (Schäfer et al., 2004). Here, thrombin activates JAK2, resulting thus way in the nuclear translocation of different transcription factors such as the STAT2 and STAT3. Inhibition of JAK2 leads to suppression of the thrombin-induced ERK2 activity and proliferation, suggesting that JAK2 acts upstream of the Ras/Raf/MEK/ERK pathway (Ossovskaya et al., 2004).

Thrombin activates PAR1 by an irreversible mechanism: cleavage exposes the tethered ligand domain which is then able to interact with the cleaved receptor. Such an activation of the receptor would result in prolonged signaling unless there were efficient mechanisms to attenuate the response (Ossovskaya et al. 2004).

In the case of the PAR1, G protein-coupled receptor kinases (GRKs, GRK3 and GRK5) can cause a rapid phosphorylation of the receptor (Vouret-Craviari et al., 1995 a, b, Tirrupathi et al., 2000). Over-expression of the GRK3 enhances agonist-induced PAR1 phosphorylation while suppressing thrombin signaling (Vouret-Craviari et al., 1995b).

GRK5 mediates desensitization of PAR1 while suppressing thrombin signaling (Tiruppathi et al., 2000).

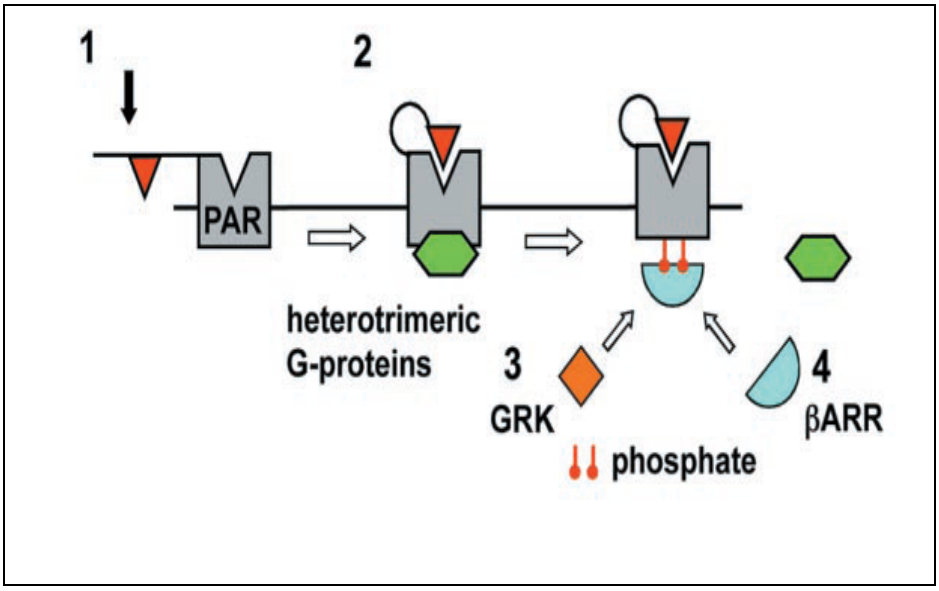

Figure 3. Activation and desensitization of the PAR receptors.

Receptor cleavage (1) activates PARs, which couple to heterotrimeric G proteins (2). Activation induces membrane translocation of GRKs (GRK3 and 5 for PAR1), which phosphorylate the receptor (3) and promote membrane translocation and interaction with arrestins which mediate uncoupling and desensitization (4) (Ossovskaya et al, 2004).

β-Arrestins also mediate PAR1 desensitization (Paing et al., 2002) since they are co-factors of the GRKs, interact with GRK-phosphorylated PAR1 at the plasma membrane and disrupt association with the heterotrimeric G-proteins to terminate the signal.

The β-arrestins play a key role in endocytosis of many receptors. They function as protein adaptors that couple GRK phosphorylated receptors to clathrin and the clathrin adaptor protein complex AP2. However, in the case of PAR1, β-arrestins seem not to play a role in stimulated endocytosis, since this process proceeds with

normal kinetics in mouse embryonic fibroblasts (MEFs) lacking β-arrestin-1 and -2 (Paing et al., 2002).

A constitutive PAR1 endocytosis is proceeds normally under these conditions suggesting that β-arrestins are also not required here (Shapiro et al., 1998).

A prerequisite for the PAR1 to mediate signal transduction is the correct biogenesis and intracellular transport of the receptor to the plasma membrane.

One aim of this study was to determine of whether the PAR1 possesses a putative signal peptide which is able to initiate its intracellular transport.

The following chapter thus summarizes knowledge about the early steps of the intracellular transport of membrane proteins and GPCRs.

2. Intracellular transport of membrane proteins

Integral membrane proteins, such as GPCRs, as well as secretory proteins use the secretory pathway to get to their correct cellular location. Both membrane and secretory proteins are initially synthesized at cytosolic ribosomes and are then transferred to the endoplasmic reticulum (ER) membrane.

Thereafter, membrane proteins are integrated into the ER membrane whereas the secretory proteins are translocated across it. Proteins are then directed in the membrane of vesicles (membrane proteins) or in the lumen of vesicles (secretory proteins) via the ER and the Golgi apparatus to their final destination.

Secretory proteins are delivered finally extracellularly by fusion of the transport vesicles with the plasma membrane. Membrane proteins become part of the plasma membrane or of the membrane of subcellular compartments. The initial step of the intracellular transport, the targeting of the proteins to the ER membrane, is mediated by signal sequences of the proteins.

Secretory proteins and some membrane proteins contain signal peptides for this purpose, which are cleaved-off following ER insertion. Most membrane proteins,

however, do not possess signal peptides. Instead, a hydrophobic domain of the mature protein takes over signaling functions as a signal anchor sequence.

Cleavable signal peptides are found at the N-terminal of a protein. The signal peptides of different secretory and membrane proteins contain one or more positively-charged amino acids adjacent to a continuous stretch of 6–12 hydrophobic residues which form the so called "core"; but otherwise they have little sequence homology.

The "core" constitutes an important element for the function of the signal peptide, since mutations in these sites lead to loss of function.

In case of secretory proteins, the signal sequences are cleaved from the proteins while they are translated by the ribosome. Thus, signal sequences are not present in the mature proteins (Dalbey et al., 1992).

The translocation mechanism of secretory proteins across the ER membrane was intensively studied and served as a model for the integration of proteins into the ER membrane which is far less analyzed.

The transport mechanism of most secretory or membrane proteins occurs while the nascent protein is still bound to the ribosome and being elongated, a process called co-translational translocation**.**

2.1 Co-translational translocation of proteins

Since secretory proteins are synthesized in association with the endoplasmic reticulum (ER) membrane and not with any other cellular membrane, a signal-sequence recognition mechanism must target them to the ER membrane. The two main key components in this targeting process are the signal-recognition particle (SRP) and its receptor which is located in the ER membrane (Rapoport et al., 1999).

The SRP is a cytosolic ribonucleoprotein particle that transiently binds to the signal sequence of a nascent protein, to the ribosome, and to the SRP receptor at the

ER membrane. The SRP is constituted by six polypeptides (P9, P14, P19, P54, P68, and P72 according to their molecular weight) and a 300-nucleotide RNA (Strub et al., 1991, High et al., 1991).

The hydrophobic region of P54 interacts with the signal sequences of the nascent protein and targets them selectively to the ER membrane. The SRP proteins, P9 and P14, interact with the ribosome, while P68 and P72 are required for protein translocation (Rapoport et al., 1999).

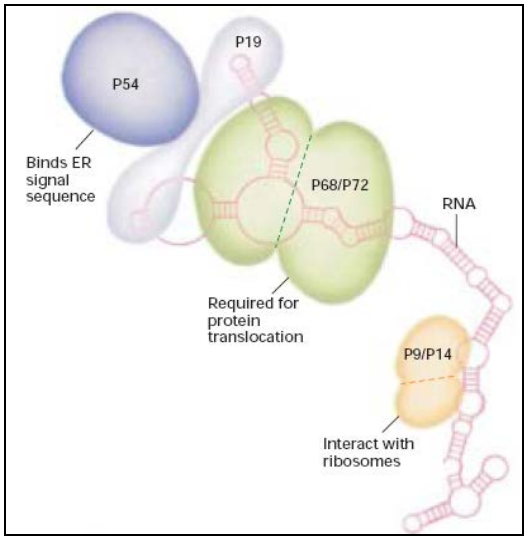

Figure 4. SRP structure

The SRP comprises one 300 nucleotide RNA and six proteins designated P9, P14, P19, P54, P68, and P72 (Molecular Cell Biology, Lodish et al, 6th edition, Freeman)

The SRP receptor is an integral membrane protein made up of two subunits: a α subunit and a smaller β subunit. SRP and SRP receptor function to gate ribosomes that are synthesizing secretory proteins to the translocon complex at the ER membrane.

The coupling of GTP hydrolysis to this targeting process is thought to contribute to the fidelity by which signal sequences are recognized. Probably, the energy from GTP hydrolysis is used to release proteins lacking proper signal sequences from the SRP and SRP receptor complex, thereby preventing their mistargeting to the ER membrane.

Interaction of the SRP/nascent chain/ribosome complex with the SRP receptor is promoted when GTP is bound by both the P54 subunit of SRP and the α subunit of the SRP receptor. Subsequent transfer of the nascent chain and ribosome to a site on the ER membrane where translocation can take place allows hydrolysis of the bound GTP. After dissociation, SRP and its receptor release the bound GDP and recycle to the cytosol.

Once the SRP and its receptor have targeted a ribosome synthesizing a secretory protein to the ER membrane, the ribosome and nascent chain are rapidly transferred to the translocon, a protein-lined channel within the membrane. As translation continues, the elongating chain passes directly from the large ribosomal subunit into the central pore of the translocon (Rapoport et al., 1999).

The 60S ribosomal subunit is aligned with the pore of the translocon in such a way that the nascent protein is never exposed to the cytoplasm and does not unfold until it reaches the ER lumen.

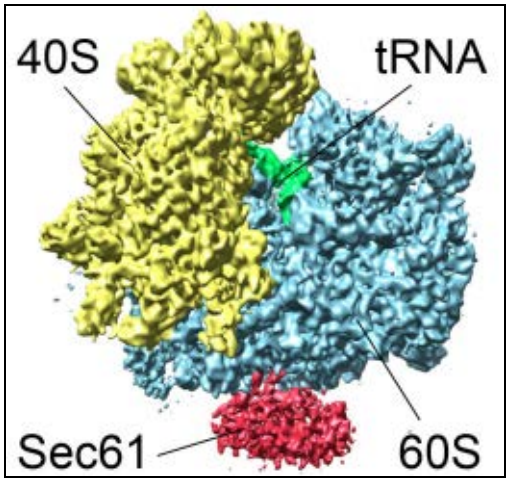

Figure 5. A Cross linking between the translocon and the 60s ribosomal subunit

An mRNA encoding the N terminus of a secreted protein is translated and the nascent protein is pushed through the translocon system. The Sec61 complex is shown in red (Adapted from Becker et al. 2009)

The mammalian ER translocon consists of the core heterotrimeric Sec61 complex (Sec61$\alpha\beta\gamma$) and associated proteins such as the translocating chain-associated membrane protein (TRAM). The mammalian translocon forms an aqueous pore that

spans the ER membrane; the walls of the pore are formed primarily by Sec61α (Johnson et al., 1999).

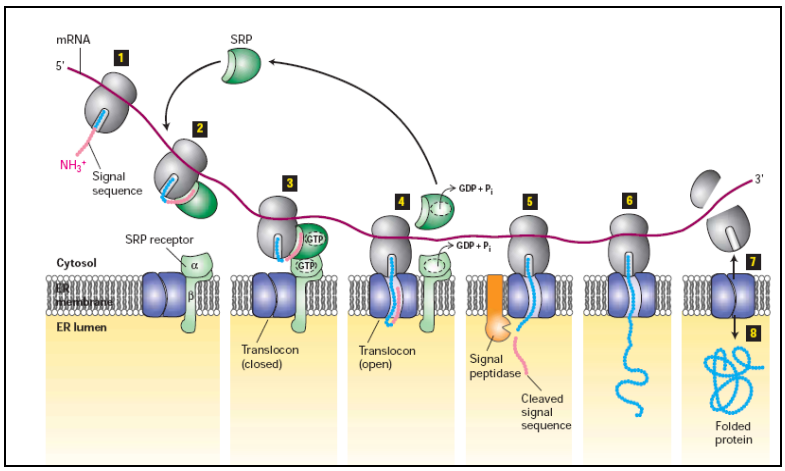

Figure 5B. Cotranslational translocation of proteins into the ER lumen.

Steps 1, 2. ER signal sequences emerge from the Ribosome and bind to the signal-recognition particle (SRP). Step 3: The SRP binds to the SRP receptor in the ER membrane. Step 4: Transfer of the ribosome/nascent polypeptide to the translocon. Step 5: the signal peptide is cleaved by a signal peptidase and is rapidly degraded. Step 6: The peptide chain continues to elongate. Steps 7, 8: Once translation is complete, the ribosome is released, the remainder of the protein is pulled into the ER lumen, the translocon closes, and the protein develops its native folded conformation. (Molecular Cell Biology, Lodish et al, 6th edition, Freeman)

As the growing polypeptide chain enters the lumen of the ER, the signal peptide is cleaved off by the signal peptidase, which is a transmembrane ER protein associated with the translocon. This protease recognizes a sequence on the C terminus side of the hydrophobic core of the signal peptide and cleaves the chain specifically at this sequence once it has emerged into the luminal space of the ER.

After the signal sequence has been cleaved, the growing polypeptide moves through the translocon into the ER lumen. The translocon remains open until translation is completed and the entire polypeptide chain has moved into the ER lumen.

Whereas, secretory proteins are completely translocated across the ER membrane by the translocon, membrane proteins possessing either signal peptides or signal anchor sequences leave the Sec61 channel laterally and become part of the ER membrane (Mothes et al, 1997).

2.2 Insertion of membrane proteins into the ER membrane

Integral membrane proteins located in ER, Golgi, and lysosomal membranes and in the plasma membrane, are first embedded via the translocon in the ER membrane and move then in the membrane of vesicles to their final destinations. During this transport, the orientation in the membrane protein is preserved (Goder et al., 2001, von Heijne, 1999).

The topology of a membrane protein refers to the number of times that its polypeptide chain spans the membrane and the orientation of these membrane-spanning segments within the membrane. Protein topology is established in the ER membrane during the translocon-mediated ER insertion process.

The key elements of a protein that determine its topology are the membrane-spanning segments themselves, which usually contain 20–25 hydrophobic amino acids. Each such segment forms an α helix spanning the membrane, with the hydrophobic amino acid residues anchored to the hydrophobic interior of the phospholipids bilayer.

Integral membrane proteins are categorized into four topological classes (Goder et al., 2001). Topological classes I, II, and III comprise single-pass proteins, which have only one membrane spanning α-helical segment.

Type I proteins have cleaved N-terminal signal peptides and are anchored in the membrane with their hydrophilic N-terminal regions on the luminal face (later exoplasmic face) and their hydrophilic C-terminal regions on the cytosolic face.

Type II proteins do not contain cleavable signal peptides and are oriented with their hydrophilic N-terminal regions on the cytosolic face and their hydrophilic C-terminal regions on the exoplasmic face (i.e., the opposite of type I proteins). Type III proteins have the same orientation as type I proteins, but do not contain cleavable signal peptides. These different topologies reflect distinct mechanisms used by the cell to integrate them into the ER membrane and to establish the membrane orientation of the transmembrane segments.

The proteins forming topological class IV contain multiple membrane-spanning segments. Many of the membrane transport proteins and the numerous G protein–coupled receptors belong to this class; these proteins are sometimes also called multipass proteins.

A final type of membrane protein lacks a hydrophobic membrane-spanning segment. Instead, these proteins are linked via an amphipathic phospholipids anchor to the membrane.

Multipass proteins fall into one of two types, depending on whether their N terminus extends into the cytosol or the ER lumen (later exoplasmic space). This N terminus topology usually is determined by the hydrophobic segment closest to the N terminus and the charge of the sequences flanking it (Englund, 1993).

2.3 Proteins with N terminus in the exoplasmic space

The large family of G protein–coupled receptors, all of which contain seven transmembrane α-helices, constitute the majority of type IV-B proteins.

For membrane proteins with extracellular N termini such as GPCRs and PARs, two different types of signal sequences are described. The members of this group contain signal peptides which are capable for the ER targeting/insertion and which are later removed by signal peptidases of the ER membrane as described by Spiess (Spiess, 1995).

The second group uses the first transmembrane domain (TM1) of the mature protein as signal anchor sequences (Spiess, 1995). The ER targeting and insertion of these GPCRs is mediated by these two types of signal sequences.

For GPCRs with and without signal peptides, the ER insertion mechanism differs substantially. For a membrane protein without signal peptide (Figure 6A), the N terminus is completely translated in the cytoplasm, because the translation does not stop until the signal anchor sequence (TM1) is presented.

In this case, the N terminus has to be transported post-translationally by the translocon complex through the ER the membrane.

On the other side, for a GPCR or an integral membrane protein with an additional signal peptide (Figure 6B), the N-terminal is not translated in the cytoplasm because the signal peptide is located in front of the N terminus and stops the elongation by SRP binding. Here, the N terminus can be transported co-translationally through the translocon complex.

From all the above, it was reasonable to think that signal peptides are essential for those membrane proteins and GPCRs which have difficulties to translocate their N termini post-translationally. Alternatively, they may be necessary for the receptor to be proper expressed at the plasma membrane. Recent data show that signal peptides of GPCRs may indeed serve different functions.

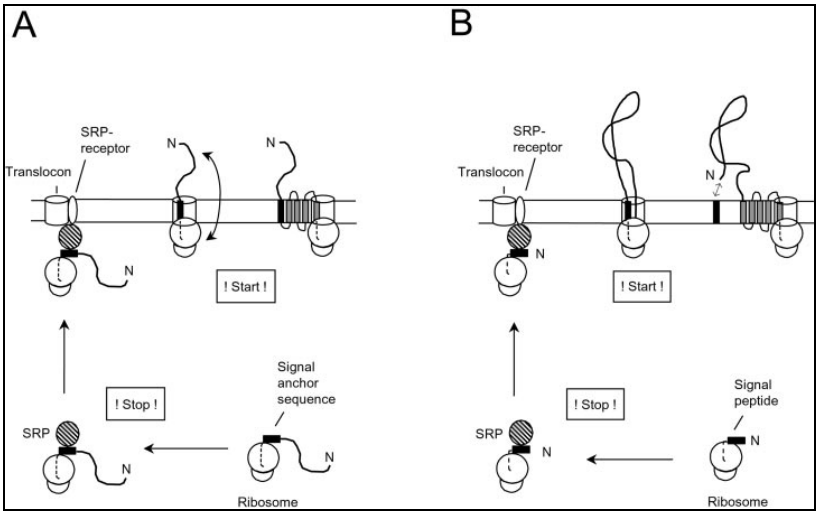

Figure 6. Schematic representation of the GPCR targeting/insertion mechanisms

A) Without and B) with signal peptide. The ER signal sequences (signal anchor sequence or signal peptide) are marked by black boxes (Köchl et al., 2002)

2.4 Signal peptide functions of GPCRs

As described above, signal peptides normally mediate the early steps of protein biosynthesis, the ER membrane targeting and insertion of the nascent protein.

Cleavable signal peptides of membrane proteins share characteristic features with the signal peptides of the secretory proteins such as their length (20 to 30 amino

acids,) the N-terminal region (n region), the central hydrophobic core (h region) and the polar C-terminal region containing the signal peptidase cleavage site (von Heijne G, 1990).

For GPCRs, it is known that only 5-10% of the receptors contain signal peptides; 90-95% of the GPCRs instead possess signal anchor sequences. The functional significance of the signal peptides of GPCRs was studied recently and it was shown that they have surprisingly different functions contributing to their role in ER targeting and insertion.

In the case of the endothelin B (ETB) receptor for example, it was shown that the signal peptide is necessary for the N terminus translocation of the receptor and consequently for establishing a functional receptor (Köchl et al., 2002). Interestingly, the signal peptide was unable to open the Sec61 channel alone. Efficient translocon gating was depended on an additional N-terminal sequence of the mature receptor (gating domain) (Alken et al., 2005).

In the case of the receptor for the vasoactive intestinal peptide (VPAC1), the signal peptide was also essential for the N terminus translocation (Couvineau et al., 2004).

In the case of the corticotropin-releasing factor receptor type 1 (CRF1R) this was, however, not the case. The signal peptide was not a crucial requirement for N terminus translocation and its deletion led to a fully functional receptor. However, receptor's expression was reduced suggesting that the signal peptide contributes to an efficient cell surface expression (Alken et al., 2005)

In the case of the glucagon-like peptide-1 (GLP1) receptor, it was recently demonstrated that deletion of the signal peptide led to a complete loss of biosynthesis suggesting that the transmembrane domains cannot function as signal anchor sequences (Huang et al.,2010).

Moreover, yet another different class of GPCR signal peptides was described lately. The corticotropin-releasing factor type 2a receptor (CRF2aR) was found to possess a "pseudo"" signal peptide which had all the necessary features to act as a

signal peptide. However, it was unable to mediate ER targeting, was uncleaved and remained as an additional hydrophobic domain at the N terminus of the mature receptor (Rutz et al., 2006).

To date, this is a unique case within the GPCR family. A simple mutation in the "pseudo" signal peptide was able to convert it into a fully functional signal peptide. Moreover, it was shown that the presence of the "pseudo" signal peptide influences signal transduction by impairing the coupling of the CRF2aR to the Gi protein (Schulz et al., 2010).

In the case of the PAR1, it is known that a physiologically-relevant N terminus peptide consisting of 41 amino acid residues, namely ''parstatin'', is released upon activation with thrombin.

Since the PAR1 also possesses a putative signal peptide, the actual parstatin length is dependent on whether a functional signal peptide is present or not.

3. Parstatin, a peptide which is cleaved off from PAR1 following receptor activationn

Parstatin possesses 41 amino acids, has a molecular weight of 4.47 kDa and the following sequence:

NH_{2}-$_{1}$MGPRRLLLVAACFSLCGPLLSARTRARRPESKATNATLDPR$_{41}$-OH

The peptide contains two domains with different hydrophobicity. The first part until the amino acid Ser^{21} (Met^{1}-Ser^{21}) is hydrophobic, while the C terminal (Ala^{22}-Arg^{41}) part is hydrophilic.

3.1 Functions

Previous and recent experiments with parstatin led to important results and conclusions about the putative biological role of the peptide:

3.1.1. Parstatin causes platelet activation

Early studies have shown that parstatin promotes platelet aggregation and platelet endothelial cell adhesion in vitro and is a potent stimulator of platelets (Furman et al., 1998, Furman et al., 2000, Claytor et al., 2003). However, for these studies, washed platelets were used in the absence of serum, which raises some concerns about the role of parstatin in platelet functions *in vivo*.

More specifically, it was shown that the cleaved peptide is a strong agonist for platelet function. Parstatin was more potent than the TR42–55 (thrombin receptor activating peptide, TRAP) and almost as potent as thrombin in activating platelets. Treatment of platelets with parstatin was followed by an increased surface expression of P-selectin of the platelets, exposure of the fibrinogen binding site on the GPIIb-IIIa complex and fibrinogen binding to the activated GPIIb-IIIa complex (Furman et al., 2000).

It could also be shown that parstatin is in comparison to thrombin a potent stimulus for platelet–endothelial cell adhesion. It induced a threefold increase in platelet–endothelial cell adhesion and a 10-fold increase in the levels of fibrinogen bound to the platelet surface (Claytor et al., 2003).

3.1.2. Parstatin is a potent inhibitor of endothelial cell functions and angiogenesis.

Parstatin suppressed both basic angiogenesis and bFGF (basic fibroblast growth factor) and VEGF (vascular endothelial growth factor)-stimulated angiogenesis in the chicken chorioallantoic membrane (CAM) model and the rat aortic ring model.

Parstatin also inhibited endothelial cell migration and capillary-like network formation in the Matrigel and fibrin angiogenesis models in vitro (Zania et al., 2009). Treatment of endothelial cells with parstatin resulted in inhibition of cell growth by inhibiting ERK1/2 phosphorylation in a specific and reversible fashion and by promoting cell cycle arrest and apoptosis through a mechanism involving activation of caspases. The molecular mechanism by which parstatin could exert these effects is not clear.

It is of interest that parstatin can cross the plasma membrane, indicating a crucial role of the hydrophobic domain of the peptide to exert its biological functions in endothelial cells (Zania et al., 2009). These results support the notion that parstatin may represent an important negative regulator of angiogenesis with potential therapeutic applications.

Indeed, recent studies provided the first strong evidence that parstatin has a therapeutic potential in the treatment of neovascular ocular diseases; parstatin was demonstrated to prevent corneal, choriodal and retinal neovascularization (Huang et al., 2010)

Current protein-based therapies for ocular angiogenesis inhibit only VEGF, and, because of their large size, they have to be administered by repeated intraocular injections.

Parstatin, which blocks angiogenic activity of both VEGF and bFGF, may provide a better efficacy for the treatment of ocular neovascularization than does targeting VEGF alone. In addition, agents that can be delivered by topical administration to the cornea offer substantial advantages such as: less invasive delivery mode, a potential for a superior safety profile, and a reduced systemic exposure.

The anti-angiogenic effects of parstatin in these experimental models of ocular neovascularization are comparable to those of the most effective treatments currently known, such as treatment with anti-VEGF, anti-VEGF receptor-2, and anti-PIGF antibodies. The parstatin peptide used in the above-mentioned rodent models corresponds to the cleaved fragment of human PAR1, which shares only 63% and 67% homology with the mouse and rat parstatin, respectively.

3.1.3. Parstatin protects myocardium from ischemia and reperfusion injury

Furthermore, it was recently demonstrated that parstatin is an effective agent for cardioprotection during ischemia and reperfusion (Strande et al, 2009).

The protective role of parstatin was assessed in an *in vivo* and *in vitro* rat model of myocardial ischemia–reperfusion injury. In *in vivo* experiments parstatin treatment before, during, and after ischemia decreased infarct size by 26%, 23% and 18%, respectively. Parstatin treatment immediately before ischemia decreased infarct size by 65% and increased recovery in ventricular function by 23% in the *in vitro* model.

The cardioprotective effects of parstatin were abolished by inhibition of nitric oxide synthase (NOS), ERK1/2, p38 MAPK, and K^+ATP channels *in vitro*. Furthermore, parstatin increased coronary flow and decreased perfusion pressure in the isolated heart. The vasodilatory properties of parstatin were confirmed in rat coronary arterioles.

These results provide the first strong evidence for a therapeutic potential of parstatin in the treatment of cardiac injury resulting from ischemia and reperfusion. Interestingly, in an *in vivo* rat model of myocardial regional ischemia/reperfusion injury, the parstatin fragment 1-26 containing the putative signal peptide has turned out to be more potent in cardioprotection than the full-length parstatin (Routhu et al., 2010). Parstatin (1-26) fragment was able to reduce infarct size by 78% and 62% when applied before or after reperfusion, respectively.

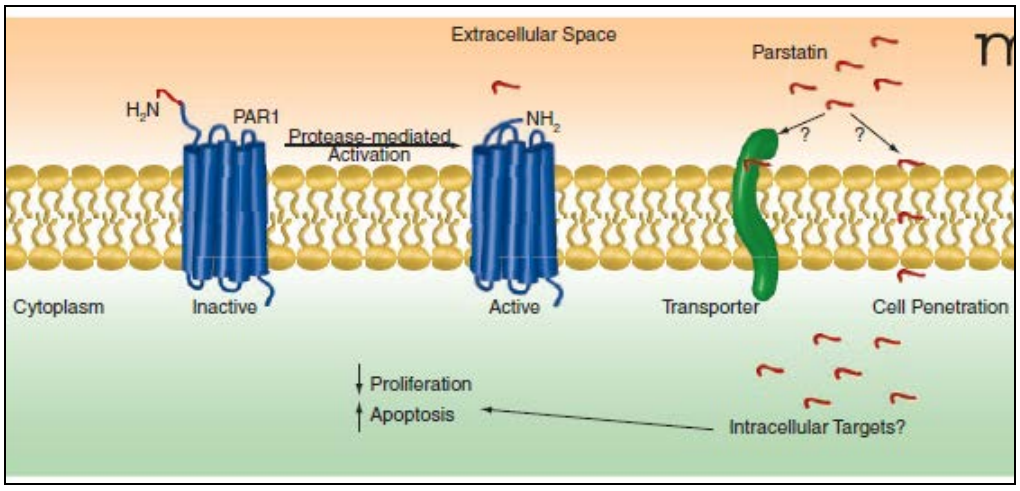

Figure 7. Processing of parstatin from the protease-activated receptor (PAR1) and possible intracellular uptake mechanisms.

Thrombin activates PAR1 by cleaving a forty-one amino-acid fragment and inducing activation of a tethered ligand. The cleaved peptide, parstatin, is located at the cell surface and is internalized perhaps by transporter- or lipid-mediated cell penetration. Parstatin subsequently inhibits cell proliferation and induces apoptosis (Dunkan et al., 2009)

Aim of the study

The first 21 amino acids of the PAR1 form a putative signal peptide according to prediction programs. The fact that a functional signal peptide would be cleaved off in the early secretory pathway raises the question of the actual length of the parstatin peptide released by thrombin cleavage, extracellularly-released at the cell surface. In the case of a signal peptide cleavage parstatin would be significantly shorter (i.e. only 19 residues instead of 41 residues).

The aim of this study was thus to demonstrate whether the putative signal peptide of the PAR1 is indeed functional. If so, another aim was to determine of whether the signal peptide of the PAR1 has any additional functional significance beyond its ER targeting/insertion functions.

METHODS

Methods

1.1. Molecular biology techniques

1.1.1. Primers design

Manipulations of the plasmids were carried out according to the handbooks of Sambrook and Russel (Sambrook and Rusell, 2001). All primers were designed with the program Sci Ed Central and were synthesized by the Biotez Company (Berlin).

Table 1. Recombinant plasmids

Recombinant	Vector	Primers (5′->3′)
pPAR.NT.GFP	pSecTag2A	CGCCCAAGCTTATGGGGCCG GGGATCCAGCCAGGAGCTGGTC
pPAR1.TM1	pSecTag2A	GCCACCCGAAGCTTCACCATGGGGCCG GACCGGTGGATCCGCCGGCTTCTTGAC
pΔSP.PAR1.TM1	pSecTag2A	GGCCGGAAGCTTATGGCCCGCACCCGGGCC GACCGGTGGATCCGCCGGCTTCTTGAC
pPAR1.GFP	pEGFP.N1	GGAATTCGAAGCTTCACCATG GACTCGGGATCCGTTAACAG
pΔSP.PAR1.GFP	pEGFP.N1	CTGTGCGGCCCAAGCTTGATGGCCCGCACCCGGGCC GACTCGGGATCCGTTAACAG
pCRF1.GFP	pEGFP.N1	Rutz et al, 2006
pCRF2a.GFP	pEGFP.N1	Rutz et al., 2006
FLAG CFR1.GFP	pEGFP.N1	CTAGCCATATGGACTATAAGGACGATGACGATAAGA
FLAG PAR1.GFP	pEGFP.N1	CTAGCCATATGGACTATAAGGACGATGACGATAAGA
FLAG V2R.GFP	pFLAG.V2	CTAGCCATATGGACTATAAGGACGATGACGATAAGA

For construct PAR1.NT.GFP.His, the following primers were used:

PRIMER A forward

5′- CGCCC*AAGCTT*ATGGGGCCG-3′

PRIMER B reverse

5′-GG*GATCCAG*CCAGGAGCTGGTC-3′

-For construct PAR1.GFP.His:

PRIMER A forward

5′-GGAATTCG*AAGCTT*CACCATG

PRIMER B reverse

5′- GACTCG*GGATCC*GTTAACAG -3′

-For construct ΔSP.PAR1.GFP:

PRIMER A forward

5′-CTGTGCGGCCC*AAGCTT*GATGGCCCGCACCCGGGCC-3′

PRIMER B reverse

5′-GACTCG*GGATCC*GTTAACAG-3′

-For construct PAR1.TM1:

PRIMER A forward

5′-GCCACCCG*AAGCTT*CACCATGGGGCCG -3′

PRIMER B reverse

5′-GACCGGT*GGATCC*GCCGGCTTCTTGAC -3′

-For construct ΔSP.PAR1.TM1:

PRIMER A forward

5′-GGCCGG*AAGCTTA*TGGCCCGCACCCGGGCC -3′

PRIMER B reverse

5′-GACCGGT*GGATCC*GCCGGCTTCTTGAC -3′

-For FLAG constructs:

PRIMER A forward

5′-CTAGCCATATGGACTATAAGGACGATGACGATAAGA-3′

PRIMER B reverse

5′-AGCTTCTTATCGTCATCGTCCTTATAGTCCATATGG-3′

For the construction of the plasmids used in this study (Table 1), the following cloning strategy was used:

In the case of the FLAG constructs, the primer annealing cloning technique was used to introduce the FLAG epitope into the pPAR1.GFP (see below, 1.1.4. oligo cloning). Plasmids pCRF1.NT and pCRF2a.NT encode GFP fusions to the N terminus of the rat corticotropin-releasing factor receptor type 1 (CRF1R, position Ala^{119}) and type 2a (CRF2aR, position Ala^{121}) in the vector pSecTag2A plasmid, respectively.

An additional C-terminal His6-sequence allowed the purification of all GFP fusion proteins. Plasmid pPAR1.NT was constructed by fusing C-terminally His-Tagged GFP to the N terminus of the human PAR1 accordingly (position Leu^{101}). Full length receptor constructs: plasmids pCRF1 and pCRF2a encode C-terminal GFP fusions to the full length CRF1R (position Thr^{413}) and CRF2aR (position Val^{411}) in the vector plasmid pEGFP.N1 respectively.

The stop codons of the respective receptors were deleted by the GPF fusions. Plasmid pPAR1 was constructed by fusing GFP to the full length PAR1 accordingly (position Thr^{425}). Plasmid ΔSP.PAR1 encodes the signal peptide mutant of the latter construct (deletion of residues 1–21). Plasmids pFLAG.CRF1 and pFLAG.PAR1 encode C-terminally GFP-tagged full length receptors, possessing an additional N-terminal FLAG tag (sequence DYKDDDDK). Plasmid pFLAG.V2, a corresponding construct of the human vasopressin V2 receptor (V2R) encodes the C-terminally GFP-tagged full length receptor.

1.1.2. Primers for RT PCR and qRT PCR

GAPDH sense

5′-TGAAGGTCGGAGTCAACGGATTTG-3′

GAPDH antisense

5′-CATGTGGGCCATGAGGTCCACCAC-3′

CRF1 sense

5′-CTACGGTGTCCGCTACAACACGAC-3′

CRF1 antisense

5′- CCCTCACCGAACATCCAGAAGAAG-3′

PAR1 sense

5′-GCAACAAATGCCACCTTAGATCCC-3′

PAR1 antisense

5′-CACAGACACAAACAGCACATCTGC-3′

1.1.3. Polymerase chain reaction (PCR)

Polymerase Chain Reaction was performed using the above primer pairs. As a DNA template, PAR1 in pcDNA3.1 was used.

The primers were designed to contain 15 to 30 nucleotides with high GC content (GC> 50%) and a G or C nucleotide at the 5′ and 3′ –end of the primer. Melting temperature (Tm) of primers was approximately 5 ^{o}C lower than the melting point. The annealing and melting temperatures were estimated using the following formula:

Tm= 4 x (G+C) + 2 x (A+T), where A, T, G, C represent the number of nucleotides in the primer

The PCR reaction was performed using the reaction buffer B (buffer B 10X) by Roboklon:

100 mM Tris-HCl (pH 9.0 at 20 ^{o}C)	15 mM $MgCl_2$
15 mM NH_4SO_4	0.1% Triton X-100
500 mM KCl	

The PCR reaction sample was:

10 μM dNTPs

~100 ng plasmid DNA

10 μM of each primer

5U OptiTaq DNA polymerase (Roboklon)

in final volume of 50 μl.

The PCR program used was the following:

PCR program

Genomic DNA template			
	Temperature	***Duration***	***Cycles***
Initial denaturation	94 °C	2 min	1
Denaturation	94 °C	15 sec	35
Annealing	58 °C	30 sec	
Elongation	72 °C	1 min/kb	
Final elongation	72 °C	7 min	1

For the elongation time, the rule 1 min per 1 kb of produced DNA was followed. After the 35 circles, samples were incubated at 72 °C for 7 min. This temperature was used because of the high GC percentage in the PAR1 gene.

The PCR was realized in a DNA GeneAmp®PCR-System 9700 Applied Biosystems Thermic cycler. The products of the reaction were separated by agarose gel electrophoresis (1.5%) and detected under a UV lamp.

1.1.4. Primer annealing (Oligo cloning)

Using this technique, primers of 25 to 200 nucleotides are introduced into the target sequence. During annealing, the primers bind to the template DNA after the strands of the template have been separated by increasing the temperature of the reaction.

The protocol was the following:

Buffer (1x): 80 µl 1M Tris-HCl (pH 8.0)	67 µl 3M NaCl
1853 µl H_2O	Buffer (5x):400 µl 1M Tris-HCl (pH 8.0)
333 µl 3M NaCl	1267 µl H_2O

	Sample	Negative 1	Negative 2
Primer Fw (50 µM)	30 µl	30 µl	-
Primer Rev(50 µM)	30 µl	-	-
False primer	-	30 µl	-
Buffer 1x	15 µl	15 µl	15 µl
H_2O	-	-	60 µl
Total	75 µl	75 µl	75 µl

The sample was incubated in a PCR machine for 2 min at 95 °C and cooled down to 25 °C for about 4-5 h. Thereafter, the DNA was digested with the appropriate restriction enzymes and ligated.

1.1.5. Digestion with restriction enzymes and ligation

To create a plasmid with cohesive 5′ and 3′ ends, digestion of the plasmid was realised with the restriction enzymes of choice according to manufacturer's recommendations (New England Biolabs) at 37 °C for 1 h according to the following protocol:

Buffer 2 (NEB buffer 2)	2 µl
Enzyme A 1 U/µl	1 µl
Enzyme B 1U/µl	1 µl
plasmid DNA	1.5 µg
ddH_2O to final volume	20 µl

Samples were purified by agarose gel electrophoresis and used for ligation. The T4 DNA ligase catalyses the formation of a phosphodiester bond between 5' phosphate and 3' hydroxyl ends in duplex DNA. Plasmid DNA and insert from the above reaction were mixed in a ligation reaction to create the plasmid of interest:

Insert	20 ng/µl	5 µl
Plasmid	40 ng/µl	1 µl
T4 DNA Ligase (3 U/µl)		1 µl
10x T4 DNA Ligase Buffer		1 µl
ddH_2O to final volume		20 µl

10x T4 DNA Ligase Buffer

100 mM $MgCl_2$
100 mM DTT
10 mM ATP
500 mM Tris-HCl
5 µg/ml BSA, (pH 7.5)

Thereafter, the sample was incubated in 4 ^oC for 16 h.

1.1.6. Total RNA extraction, cDNA synthesis, and qRT PCR

For total RNA extraction assay, the protocol used from Huang et al. (2010) was adapted with modifications. HEK 293 cells, $4x10^6$ grown on 100 mm diameter dishes were transiently transfected as described above. The Trizol® reagent protocol was used with slight modifications to the manufacture's recommendations.

After adding Trizol reagent (1 ml), the resulting suspensions were transferred to microfuge tubes and cells fully lysed by titration (10 times) through a 24-gauge needle. Samples were incubated at room temperature for 5 min to allow complete dissociation of the nucleoprotein complexes. Chloroform (0.2 ml) was added. The tubes were shaken vigorously for 15 s, incubated at room temperature for 2–3 min and centrifuged (12 000 *g*, 15 min, 4 °C) (Huang et al, 2010).

The upper aqueous phase was transferred to a clean microfuge tube and the RNA was precipitated by the addition of isopropyl alcohol (0.5 ml), incubation at room temperature for 10 min and centrifugation (12 000 *g*, 10 min, 4 °C). The RNA pellet was washed with 1 ml 75% (v/v) ethanol and collected by centrifugation (7500 *g*, 5 min, 4 °C). After brief drying by air, the RNA was dissolved in water (100 µl) and treated with DNase I (1000 U/ml, 30 min, 37 °C). The RNA was purified by addition

of Trizol (1 ml), and repeating the procedure outlined above. Finally, the RNA was resuspended in water (100 μl).

After drying the pellet, we used the Rneasy kit from Qiagen for further purification of the samples. At the end, the pellet was dissolved in 35 μl of RNAse free water. For the cDNA synthesis, the SuperScript III First-Strand Synthesis Super Mix kit from Invitrogen was used according to the manufacturer's protocol.

For the qRT PCR, the TaqMan Gene Expression Assays technology and primers from Applied Biosystems were used. The qRT PCR was performed according to supplier's protocol, and the results were normalized to the endogenous control of glycerinaldehyd-3-phosphat-dehydrogenase (GAPDH) and compared to a reference sample (untreated HEK 293 cells). Statistical analysis of the results was performed with the DataAssist v2.0 Software provided by Applied Biosystems.

The protocol for the RT PCR was the following:

Temperature	Time	Cycles
Initial denaturation 95 ^{o}C	3 min	
Denaturation 95 ^{o}C	30 sec	35
Primer annealing 63 ^{o}C	30 min	35
Elongation 72 ^{o}C	20 sec	35
Final elongation 72 ^{o}C	7 min	
4 ^{o}C	∞	

For the mRNA degradation assay, HEK 293 cells ($2x10^{6}$) were transfected with 2 μg of DNA in 60 mm diameter dishes. After 24 h, cells were treated with 5 μg/ml Actinomycin D (Sigma Aldrich) for different time points. For the total RNA extraction, cDNA synthesis, and qPCR, the above kits and protocol were used.

1.1.7. In vitro transcription

The EasyXpress Insect kit II (Qiagen, Hilden, Germany) was used for the *in vitro* transcription according to the manufacturer's protocol.

1.1.8. Agarose gel electrophoresis for DNA analysis and purification

Agarose gel electrophoresis was used to separate, identify and purify DNA fragments. For DNA analysis, agarose gel electrophoresis gel, 1 to 1.5 % depending on the size of the DNA fragment was performed in 1x TAE buffer. Ethidium bromide was added to a final concentration of 0.5 μg/ml after heating the solution in a microwave oven. The solution was cooled down and then poured into the apparatus. Samples were mixed with 6x loading Dye and electrophoresis was carried out at 100 V for 45 min.

1x TAE buffer	40 mM Tris-HCl, (pH 8.0), 1 mM EDTA
6x DNA loading Dye	50% Glycerol (% v/v), 1% bromophenol blue (w/v) in 1x TAE buffer

DNA molecular weight standard (GeneRuler™ 1kb DNA ladder)

1.1.9. Transformation of *E.coli* DH5α with plasmid DNA

Plasmid DNA was inserted in *E. coli* DH5α competent cells using electroporation and an electric field of 300-400 mV (T <1 msec). The application of an electric field leads to an opening of the lipid bilayer of the cells, thus allowing entry of the plasmid DNA.

For the transformation of the bacteria, we used a BioRad apparatus adjusted at 1250 V, 2 μl of the DNA plasmid and 40 μl *E. coli* DH5α. After transformation, the sample was dispersed with 500 μl LB medium (Lysogenic Broth) preheated to 37 °C. Samples were incubated at 37 °C for 1 h, at 350 rpm in a shaker. The sample was centrifuged at 5500 rpm for 4 min. Finally, 200 μl from the sample were plated on agar plates containing ampicillin. Plates were incubated for 16 hours at 37 °C.

Reagents:

LB (Lysogenic Broth) medium

1% Tryptone,

1% NaCl,

0.5% Yeast extract

The medium was autoclaved for 20 min at 120 °C. Antibiotics were added in a final concentration of 100 μg/ml (ampicillin) and 30 μg/ml (kanamycin) after cooling the medium to 45 °C.

LB agar/ampicillin plates

1% tryptone

1% NaCl

0.5% Yeast extract

12 g/l agar

100 μg/ml ampicillin

To prepare agar plates, the medium was poured into sterile petri dishes and cooled down until it reached a solid state. Plates were dried by leaving them for about 30 min in a laminar flow hood. Plates were finally stored at 4 °C.

1.1.10. Plasmid DNA extraction from *E.coli*

The plasmid DNA isolation in small (mini prep) or large scale (midi prep) was performed using the NucleoSpin Plasmid DNA quick pure kit from Macherey Nagel and the QIAGEN Plasmid Midi Kit, respectively.For the small scale isolation, a single colony was inoculated in 5 ml of LB containing the appropriate antibiotic. The sample was incubated for 16 hours at 37 °C at 190 rpm in a shaker.

Plasmid DNA isolation was carried out using the protocol provided by the NucleoSpin Plasmid quick pure kit. For the large scale isolation (up to 500 μg) a 5 ml

pre-culture of a single colony was inoculated in 100 ml of the LB medium and the sample was incubated for 16 h at 37 °C.

Plasmid extraction was performed with the QIAGEN Plasmid Midi Kit according to the supplier's recommendations.

1.1.11. Plasmid DNA extraction in small scale (mini prep)

Bacteria were centrifuged at 13.000 rpm for 1 min in a table centrifuge, the supernatant was removed and pellet was resuspended in 250 µl of the RNA containing solution A1. Thereafter, 250 µl of the SDS containing solution A2 was added to lyse the cells.

The mix was gently mixed to avoid release of the chromosomal DNA, and the sample was left for 5 min at RT until the solution became clear. 300 µl of solution A3 was added to neutralize the solution.

Proteins and chromosomal DNA were precipitated by centrifugation at 13.000 rpm for 5 min in a table centrifuge.

The supernatant containing the dissolved plasmid DNA was transferred to a 2 ml eppendorf tube. Plasmid DNA was loaded on a column by centrifugation at 13.000 rpm for 1 min.

450 µl of the alcoholic solution AQ was added. Columns were centrifuged at 13.000 rpm for 3 min to wash the plasmid DNA. Plasmid DNA was eluted by the addition of 25 µl of ddH2O for 1 min. Elution was completed by centrifugation at 13.000 rpm for 1 min.

The precise compositions of solutions A1, A2, A3 and AQ are not provided by the manufacturer.

1.1.12. Plasmid DNA extraction in large scale (midi prep)

This method is based on the same protocol used for the small-scale plasmid DNA extraction (see above).

1.1.13. Photometric quantification of nucleic acids

Prior to the measurement, the sample of the nucleic acid was diluted (1:40, 2 µl DNA + 78 µl ddH_20) in water and then transferred into a cuvette. One unit of absorption (optical density, OD) at 260 nm wavelength corresponds to ~50 µg/ml dsDNA, 40 µg/ml ssDNA and RNA, and 20 µg/ml oligonucleotides. Purity of the sample was determined by measuring the absorption at 260nm and 280nm. Clean samples of DNA and RNA are characterized by a A260/A280 ratio of 1.8 and 2.0, respectively.

1.1.14. DNA sequencing

DNA sequencings were carried out according to the dideoxy method of Sanger et al., (1977) using the ABI PRISM™ Dye Terminator cycle sequencing ready reaction kit:

	1 sample	N samples, N=χ
Big Dye	0.5 µl	X 0.5 µl
5x sequencing buffer	1.75 µl	X 1.75 µl
Primer	1 µl	X 1 µl
DNA	1 µl	X 1 µl
H_2O	5.75 µl	X 5.75 µl
Total	10 µl	

The PCR program for sequencing was the following:

Temperature	Time	Cycles
Initial denaturation 96 °C	30 sec	
Denaturation 96 °C	30 sec	25
Primer annealing 52 °C	1 min	25
Elongation 60 °C	2 min/kb	25
Final elongation 4 °C	∞	

DNA fragments were precipitated according to the following protocol:

For 10 µl of reaction, 1 µl sodium acetate/EDTA and 40 µl 95% ethanol were added. After short vortexing, samples were put on ice for 15-20 min. Samples were centrifuged for 15 min at 15000 g (RT) and the supernatant was discarded. Samples

were washed with 250 µl 70% ethanol and centrifuged again for 15 min at 12000 g (RT). The supernatant was discarded and the pellet was dried by centrifugation for 15-20 min in vacuum. Thereafter, the sample was analyzed.

2. Cell culture techniques

2.1.1. General

HEK 293 cells were used exclusively in this study. These cells can be easily grown and transfected and are ideally suited for microscopy.

Cell line	Description	Origin
HEK 293	Human embryonic kidney cells, adenovirus type 5 transformed.	DSMZ GmbH, Germany

2.1.2. Cell dish preparation (35 mm diameter).

Plates were covered with 250 µl Poly-L-Lysine for 30 min. After removal of the solution, plates were dried under a clean bench. Plates were wrapped in alu-foil and placed in the refrigerator at 4 °C.

Reagents:

Poly-L-Lysine solution: 15 ml dH_2O, 5 ml L-Lysine**.** Prior to use, the stock solution was diluted 1:4.

-Seeding of cells (35 and 100 mm diameter dishes)

DMEM® (Dulbecco's Modified Eagle's Medium) from Sigma Aldrich 37 °C and the T/E (Trypsin/EDTA) were heated to 37 °C to prevent a temperature shock for the cells. The medium was removed from the T75 flask and the cells were resuspended in 1 ml trypsin T/E. Cells were resuspended for 30-60 sec and the trypsin solution was discarded. Cells were placed at 37 °C for 2-5 min. Cells were supplemented with 5 ml DMEM and put into a glass vial. 25 µl of the cell suspension was added into a special plastic tube containing 10 ml Casy®ton (Casy® Technology Roche).

Cell concentration was measured according to the following formula:

$$C_{measured\ cells}/C^*_{cells\ need\ to\ seed} = 1000/\chi$$

For 35 mm diameter plates, the number of cells was adjusted to 200.000 cells/plate in a final volume of 2 ml.

For a 100 mm diameter plate, we have used the same procedure as for the 35 mm dishes but using this time $4x10^6$ cells/plate (see text above).

2.1.3. Transient transfection of HEK 293 cells (35 and 100 mm dishes)

Transient transfection was performed using Lipofectamine 2000 (Invitrogen) reagent containing liposomes of cationic and neutral lipids. Negatively charged phosphate groups of the DNA bind to the positively charged surface of the liposomes and the residual positive charge then mediates binding to sialic acid residues at the cell membrane.

The DNA/liposome complex is formed in serum free medium (DMEM). The fusion of the lipid/DNA complex with the cells results in the efficient uptake and expression of the DNA.

Before transfection, cells were seeded (see above) in culture plates and grown in DMEM medium.

-Protocol for 35 mm dishes:

In a glass vial (A), 120 μl SFM (serum-free medium) and 1.2 μg of plasmid DNA were mixed.

In a separate tube (B), 1 ml SFM and 5 μl Lipofectamine 2000® (Invitrogen) were mixed. Vials were incubated for 5 min at RT. Vial A was supplemented with 125 μl from the vial B and incubated for another 20 min.

250 μl of the mixture were transfected to the cell dishes and the samples were incubated for 24 hours at 37 ^oC.

-For 100 mm dishes, the above protocol was used.

The precise composition of Casy®ton is not provided by the manufacturer.

2.1.4. Confocal laser scanning microscopy

Confocal laser scanning microscopy (CLSM) is a novel technique with which we are able to obtain high-resolution images with depth selectivity. Its key component is the ability to acquire focused images from the selected depths, a process known as optical sectioning (Pawley, 2006).

Then, images are recorded and reconstructed using a computer allowing three-dimensional reconstructions of topologically complex objects. In a laser scanning microscope, a laser beam passes through a light source aperture and is then focused by an objective lens into a small (ideally, diffraction limited) focal volume within or on the surface of a specimen. In biological applications the specimen may also be fluorescent (Fellers et al., 2007).

After passing a *pinhole*, the light intensity is detected by a photodetection device (usually a photomultiplier tube (PMT) or avalanche photodiode), transforming the light into an electrical signal which is recorded by a computer (Figure 8).

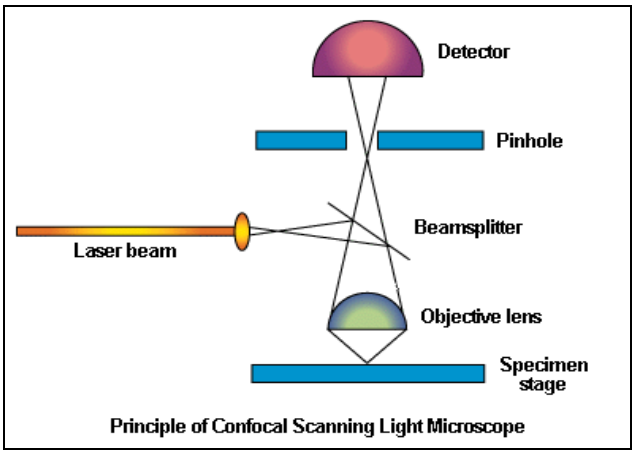

Figure 8. Principle of confocal laser scanning microscopy

(Electron Microscopy Unit, University of Cape Town)

CLSM provides the ability for direct, noninvasive, optical sectioning of intact living specimens with a minimum of sample preparation. (Fellers et al., 2007).

In the case of biological samples, these are tagged with fluorescent dyes such as GFP (green fluorescent protein) to make the samples visible.

2.1.5. Localization of GFP tagged receptors in transfected HEK 293 cells

Cells ($2x10^5$) grown on glass coverslips (pre-treated with 25 μg/ml poly-L-lysine) in 35 mm diameter dishes, were transiently transfected with plasmid DNA (1.2 μg) and Lipofectamine 2000 according to the supplier's recommendations.

Cells were incubated overnight, washed once with PBS, and transferred immediately into a self-made chamber.

Cells were covered with 1 ml of PBS, and the GFP receptor's fluorescence signals were visualized at room temperature on a Zeiss LSM510-META invert confocal laser scanning microscope (objective lens: x100/1.3 oil; optical section, <0.8 nm; multitrack mode; λexc, 488 nm, Argon laser, BP filter: 500–530 nm).

Images were imported into the Photoshop software (Adobe Systems, Inc.) and the contrast was adjusted to the original image.

The receptor's GFP signals and the plasma-membrane Trypan blue signals (λex=543 nm, λεm= >590 nm) were analysed by confocal laser scanning microscopy. The quantification of the fluorescence signals was analyzed using the software version 3.2 SP2 of this microscope.

2.1.6. Enzyme-linked immunosorbent assay (ELISA)

HEK 293 cells ($20x10^3$) grown in a 96 well plate pretreated with 25 μg/ml poly-L-lysine were transiently transfected with plasmid DNA (200 ng/well) and cultivated for 24 h at 37 °C. Cells were washed three times with PBS (containing 0.5 mM $MgCl_2$ and 0.5 mM $CaCl_2$) and fixed for 5 min at 4 °C using 4% paraformaldehyde (PFA).

Cells were washed three times with PBS at room temperature. After washing three times with ELISA-buffer (PBS-buffer containing 0.05% Tween 20 and 0.5% BSA) monoclonal, anti-FLAG M2 antibodies were added (1:1000 in DMEM 1mg /ml, BSA-10mM HEPES, pH 7.4) and samples were incubated for 1 h at 37 °C.

Cells were washed three times with PBS and incubated with the anti-mouse HRP conjugated Ab for 1h in RT (1:2000 in DMEM/BSA/HEPES). Sequently, cells were

incubated with the 3, 3', 5, 5'-tetramethylbenzidine substrate (TMB) for 30 min at RT in the dark.

The reaction was stopped by adding H_2SO_4 at a final concentration of 0.5 N. Optical density (OD) at 450 nm (with correction at 630 nm to eliminate plate impurities) was measured using a Tecan Safire multi-detection monochrometer microplate reader (Männedorf, Switzerland).

Assay reliability was verified by blocking antibody binding using a soluble FLAG epitope peptide.

2.1.7. Immunofluorescence assay

HEK 293 cells ($1.5x10^5$) grown on 24 well plates with cover slips pretreated with 25 μg/ml poly-L-lysine were transiently transfected with plasmid DNA (1.2 μg). Cells were cultured for 24 h, washed twice in serum free DMEM and incubated with a monoclonal anti-FLAG M2 antibody (dilution 1:1000) for 1 h at 4 ^oC.

Cells were washed three times with PBS containing 0.5 mM $MgCl_2$ and 0.9 mM $CaCl_2$ and then fixed for 5 min at 4 ^oC with 4% (w/v) PFA.

Cells were washed with PBS containing 1% non-fat dry milk and 150 mM sodium acetate (pH 7.0) and incubated for 15 min at RT in the same buffer without sodium acetate.

Cells were incubated with Cy3-conjugated goat anti-mouse IgG (dilution 1:500) for 1 h at RT, washed and transferred to LSM analysis (λ_{exc} = 488 nm, λ_{em} = 507 nm).

3. Biochemical techniques

3.1.1. Protein Purification of His-tagged proteins using affinity chromatography

His-tagged proteins were purified using Ni-metal affinity columns (Ni-Nta system).

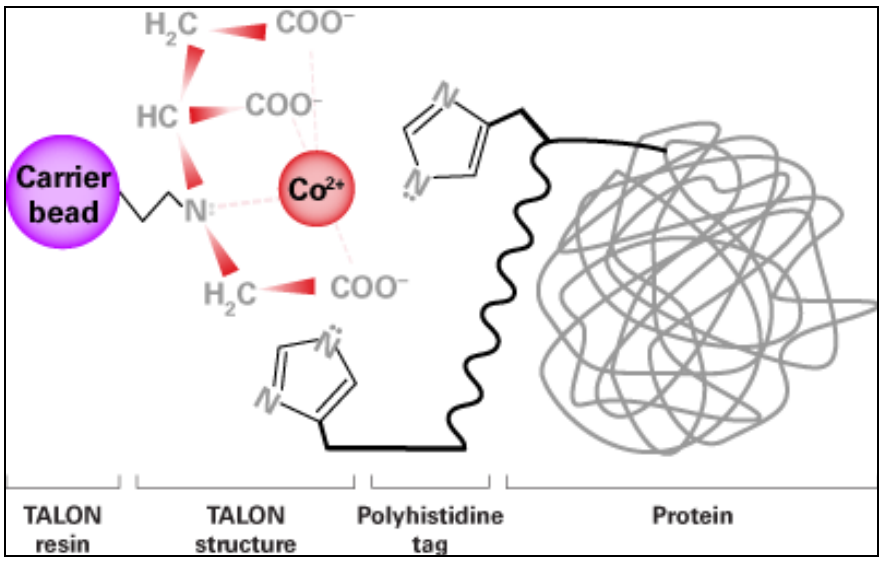

Figure 9. Schematic diagram of the Ni-Nta affinity System (Clontech, Germany)

Here, six histidine residues of the tag interact with high affinity through their imidazole rings with nickel provided by the chromatographic material (Figure 9).

The interaction remains very strong during the various eluation steps and is weakened only at low pH (4.5 - 5.9) or by high imidazole concentrations (250-1.000 mM) competing with the 6xHis tag at the column.

3.1.2. Quantitative detection of secreted GFP fusion proteins

HEK 293 cells (4×10^6) grown on 100 mm diameter dishes, were transiently transfected with 6 µg of plasmid DNA. Cells were grown for 24 h, and the cell-culture medium (10 ml) was collected. After cell debris removal by centrifugation (5 min, 200 g), cells were washed (washing buffer) and TALON metal-affinity resin beads (500 µl bed volume) were added to the cell-culture medium.

The sample was incubated overnight to allow coupling of the His-tagged GFP fusion proteins to the beads. Resin beads were collected (2 min, 700 g), washed, and resuspended in 200 µl of elution buffer. After 15 min of incubation, resin beads were separated by centrifugation.

Reagents:

Washing buffer

37 mM Na_2HPO_4
11 mM NaH_2PO_4
300 mM NaCl, (pH 7.0)

Elution buffer

37 mM Na_2HPO_4
11 mM NaH_2PO_4
300 mM NaCl , (pH 7.0)
350 mM imidazole

3.1.3. Fluorimetric quantification of proteins

For measuring GFP fluorescence signals, 170 µl of the elution solution from the column were centrifuged (2 min, 13000 *g*), and transferred in a special cuvette (3 mm). Fluorimetric analysis was performed using the following settings: λ_{exc} = 488 nm and λ_{em} = 507 nm in a Spectrofluorometer Jasco® FP 6500 (Jasco).

3.1.4. Deglycosylation of proteins

Glycosylation is the enzymatic process that links saccharides to produce glycans, attached to proteins, lipids, or other organic molecules. This enzymatic process produces one of the fundamental biopolymers found in cells (along with DNA, RNA, and proteins). In the case of proteins, glycans can be added co- and post-translationally.

Glycans serve a variety of structural and functional roles in membrane and secreted proteins. The majority of proteins synthesized in the rough ER undergo N-glycosylation, an enzyme-directed site-specific process. N-linked glycosylation is important for folding of many eukaryotic proteins. To digest N-glycosylation, N-Glycosidase F was used.

N-Glycosidase F (or PNGase F), is an amidase that cleaves between the innermost GlcNAc and asparagine residues of high mannose, hybrid, and complex oligosaccharides from N-linked glycoproteins.

The mechanism by which PNGase F acts is the transformation of asparagine (N) in aspartic acid (D) after cutting off the oligosacharic residue. The minimal length to act requires a tripeptide with a coupled oligosaccharide to an asparagine residue.

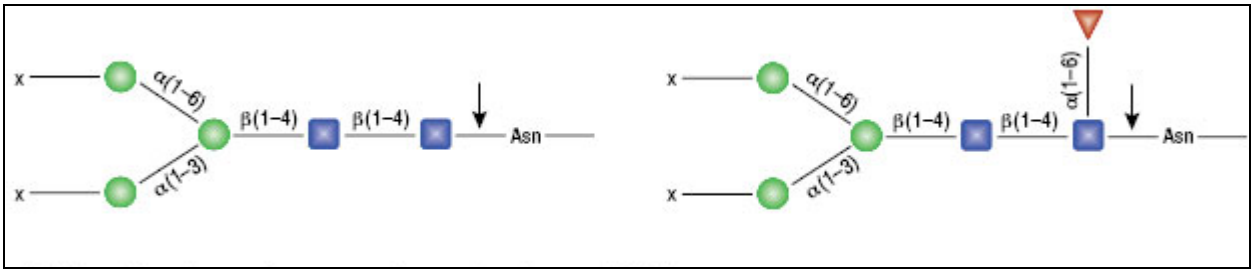

Figure 10. PNGase F: mechanism of action (Sigma Aldrich)

In this study, the following protocol was followed:

The sample was supplemented with 5. 5 µl of denaturing buffer and incubated for 30 min at 42 °C. Reaction buffer G7 (6. 05 µl), PNGase F (1 µl) and Nonidet P40 (6. 05 µl) were added, and proteins were digested for 1.5 h at 37 °C.

Reagents:

Denaturation buffer

5%, w/v, SDS
10% v/v mercaptoethanol

G7 (Reaction buffer)

0.5 M Na_3PO_4 , (pH 7.5)

Nonidet

NP40 10%, (v/v)

PNGase F

500 units/µl

3.1.5. Immunoprecipitation of proteins

For this technique we have used the already well established protocol (see Köchl et al., 2002, Alken et al., 2005, Rutz et al., 2006).

Cells (HEK 293, $4x10^6$) grown on 100 mm diameter dishes were transiently transfected with plasmid DNA (6 μg) and Lipofectamine 2000 according to the supplier's recommendations. Cells were cultivated for 24 h, washed twice with PBS (pH 7.4), and lysed for 1 h with 1 ml of lysis buffer. Insoluble debris was removed by centrifugation (15 min, 20,000 *g*). The supernatant was supplemented with monoclonal mouse anti-GFP antiserum 02 coupled to protein A-Sepharose CL-4B beads, and the sample was incubated overnight (beads were prepared by equilibrating 10 mg of the beads with lysis buffer and subsequent overnight incubation with 2 μl/plate of monoclonal mouse anti-GFP antiserum 02). GFP-tagged receptors were precipitated (2 min, 700 *g*), and the beads were washed twice with 2 ml of washing buffer 1 and once with 2 ml of washing buffer 2.

Reagents:

Washing buffer 1

Tris HCl (pH 8.0) 50 mM
NaCl 500 mM
EDTA (pH 8.0) 1 mM
Triton X-100 , 0.5% (v/v)
SDS 0.1% (w/v)

Washing buffer 2

Tris HCl (pH 7.4) 50 mM,
EDTA (pH 8.0) 1 mM
Triton X-100 0.5 % (v/v)
SDS 0.1% (w/v)

Lysis buffer

Buffer A 10 ml
PMSF 125 μl
Mix 80 μl

Buffer A

Tris HCl (pH 8.0) 50 mM
NaCl 150 mM
EDTA (pH 8.0) 1 mM
Triton X-100 1% (v/v)
SDS 0.1%(w/v)

PBS buffer (pH 7.4)

Na_3PO_4 8 mM,
K_3PO_4 2 mM,
NaCl 0.14 M,
KCl 0.01 M

PMSF: 0.5 mM **p**henyl**m**ethyl**s**ulfonic **f**luoride and protease inhibitors **m**ix

0.5 mM benzamidine
1.4 μg/ml aprotinine
1.4 μg/ml trypsin inhibitor

3.1.6. SDS Polyacrylamide gel electrophoresis (SDS-PAGE)

Precipitated proteins from the previous step were supplemented with 30 μl Rotiload buffer. Samples were boiled at 95 °C for 3 min and subjected to SDS-PAGE using a 10% or 12% acrylamide gel depending on the molecular size of the protein to be separated.

The separating gel solution was prepared according to the following table:

Separating gel solution

10%		**12%**	
H_2O	2.75 ml	H_2O	3.3 ml
30% acrylamide	3.3 ml	30% acrylamide	4 ml
1.5M Tris (pH 8.8)	2.08 ml	1.5M Tris-HCl (pH 8.8)	2.5 ml
10% SDS	0.1 ml	10% SDS	0.1 ml
10% APS	0.1 ml	10% APS	0.1 ml
TEMED	4 μl	TEMED	4 μl

After adding 10% APS and TEMED, the solution was vortexed briefly and the gel was immediately prepared. The top of the gel was covered with a layer of isopropyl alcohol and the gel was allowed to polymerize at room temperature. Thereafter, the isopropyl alcohol layer was removed off and rinsed with H_2O. The stacking gel solution was prepared according to the following table and transferred on top of the polymerized separation gel.

Stacking gel solution

H_2O	3.4 ml
30% acrylamide	0.83 ml
1.0 M Tris-HCl (pH 6.8)	0.63 ml
10% SDS	0.05 ml
10% APS	0.05 ml
TEMED	5 μl

A comb was inserted into the layer of the stacking gel solution to form wells and the stacking gel was allowed to polymerize at room temperature. The polymerized gels was put into the electrophoresis apparatus and covered with running buffer.

Prior loading on the gel, protein samples were supplemented with 4x Rotiload buffer. Samples were denaturated at 42 °C for 10 min, briefly centrifuged and applied to the wells using Hamilton syringe.

The dimensions of the gel were the following: height 7 cm, width 9 cm, thickness 1 mm. The electrophoresis was carried out using 25 mV for 1 h, 15 min. Glass plates and gel electrophoresis apparatus were assembled according to manufacturer's recommendations (Biorad).

Reagents:

Rotiload buffer

60 mM Tris/HCl,
2%, (w/v), SDS,
10%, (v/v), glycerol,
5%, (v/v), 2-mercaptoethanol
0.1%, (w/v), bromophenol blue, (pH 6.8)

SDS electrophoresis buffer (Running buffer)

192 mM Glycine,
25 mM Tris-HCl ,
0.1% SDS.

3.1.7. Western blot detection

During Western blot analysis, proteins been separated by SDS-PAGE are transferred to a membrane (nitro-cellulose, PVDF) using an electric field.

Prior to blotting transfer, membranes were cut in the dimensions of the gel and made hydrophilic using absolute methanol for 1 min. The membrane and the gel were transferred into transport buffer and incubated for 5 min. Six pieces of Whatman 3MM paper were cut in gel dimensions and incubated in transport buffer.

The transfer stock had the following composition: 3 pieces of Whatman, the gel, transfer membrane (PVDF), and 3 pieces of Whatman. The stock was carefully placed on the blot apparatus according to manufacturer's recommendations (Biorad). The electrotransport was carried out at 20 V for 1 h.

Thereafter, the membrane was blocked in a TBS blocking solution (4%) at room temperature. Subsequently, the membrane was rinsed with TBS-Tween/Triton two times for 10 min and with TBS solution for 10 min.

After washing, the membrane was incubated with the monoclonal mouse anti-GFP antibody (Bionova), (1:3000 in TBS containing 2% BSA) for 1 h at room temperature (RT) or overnight at 4 ^{o}C.

The membrane was incubated for 1 hour at RT with horse radish peroxidase (HRP) -conjugated anti-mouse IgG (BD Biosciences, USA), 1:5000 dilution in TBS-Tween/Triton 0.1%. The membrane was washed three times with TBS-Tween/Triton for 10 min at RT. Subsequently, the membrane was incubated with HRP detection solution for 1 min.

The immunoreactive protein bands were detected using with a Lumi-Imager F1™.

Reagents:

Transport buffer

150 mM Glycine
20% methanol
25 mM Tris-HCl , (pH 8.3)

TBS buffer

150 mM NaCl
10 mM Tris-HCl, (pH 7.5)

TBS-Tween/Triton buffer

500 mM NaCl
0.05% (v/v) Tween 20, 0.2% (v/v)
Triton X-100
20 mM Tris-HCl , (pH 7.5)

RESULTS

1. The PAR1 meets all sequence criteria for the presence of a cleavable signal peptide

Cleavable signal peptides of eukaryotic membrane proteins share characteristic features with signal peptides of eukaryotic secretory proteins (von Heijne G, 1990): a polar and often charged N-terminal (n) region, a central hydrophobic (h) region, and a polar C-terminal (c) region.

The C-terminal end contains helix-breaking proline and glycine residues and small uncharged residues at positions -1 and -3 of the cleavage site. Analysis of the N-terminal sequence of the PAR1 with the "SignalP3.0" bioinformatics software (Nielsen et al., 1997, Bendtsen et al., 2004) revealed that the N-terminal sequence Met^{1}-Arg^{23} meets all criteria for the presence of a functional signal peptide which is cleaved by the signal peptidases of the ER (Figure 11). Signal peptide probability reached a maximal 1.0 value and the cleavage site probability is 0.513 between residues Ser^{21} and Ala^{22}.

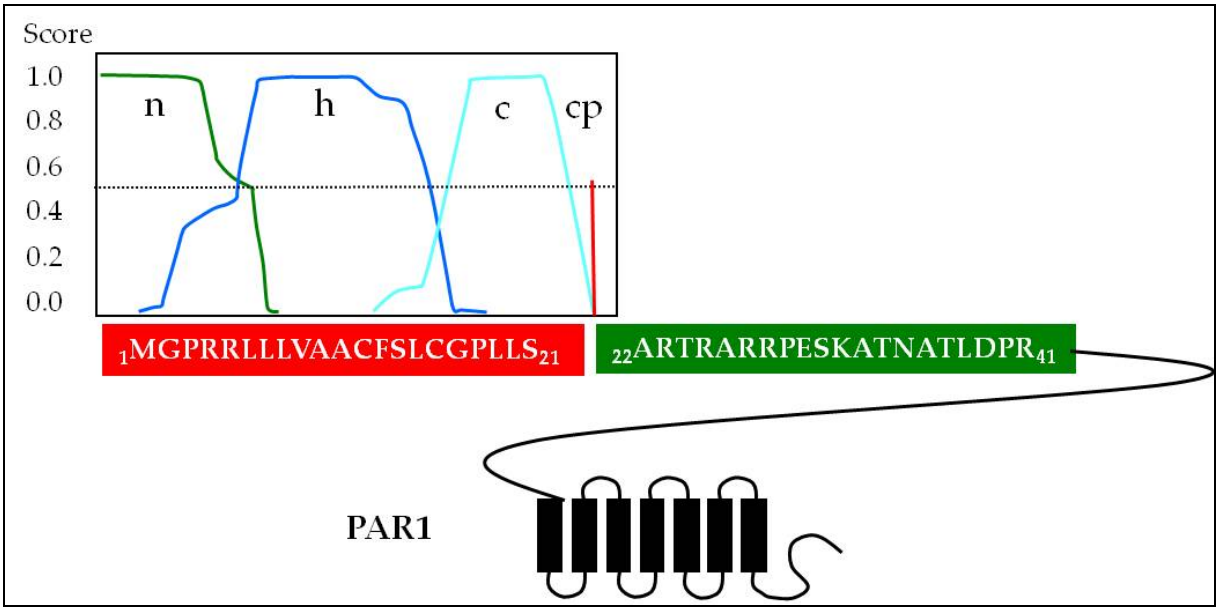

Figure 11. Depiction of the N terminal sequence of the PAR1.

The sequence of the putative signal peptide (Met^{1} to Ser^{21}) and the sequence up to the thrombin cleavage site (Ala^{22} to Arg^{41}) are included. The sequence Met^{1} to Arg^{41} was originally described as parstatin. For the signal peptide, the probabilities of the presence of n (*green*), h (*blue*), and c (*light blue*) regions and the cleavage probabilities (cp, *red*) are indicated in a score ranging from 0 to 1.

These data raise the question whether the thrombin-released peptide at the plasma membrane is indeed 41 amino acid residues long. If the signal peptide is functional, the released peptide would only consist of 19 amino acid residues (Ala^{22}-Arg^{41}) taking into account that the signal peptide is removed during ER translocation.

2. The putative signal peptide of the PAR1 is able to mediate ER targeting of heterologous proteins

To assess whether the predicted signal peptide of the PAR1 receptor is functional or not, fusion proteins containing the N terminus of PAR1 (Met^{1}-Leu^{101}) and His-tagged GFP were constructed (Figure 12)

If the putative signal peptide is functional, the normally cytosolic GFP protein should be converted to a secreted protein (Figure 13). As a secretory protein, GFP should appear initially in the ER and, following transport through the secretory pathway, in the cell culture medium. However, if the putative signal peptide is not functional and uncleaved like e.g. the recently described "pseudo" signal peptide of the CRF2(a)R (Rutz et al., 2006, Schultz et al., 2010, Pal et al., 2010), the construct should remain in the cytosol.

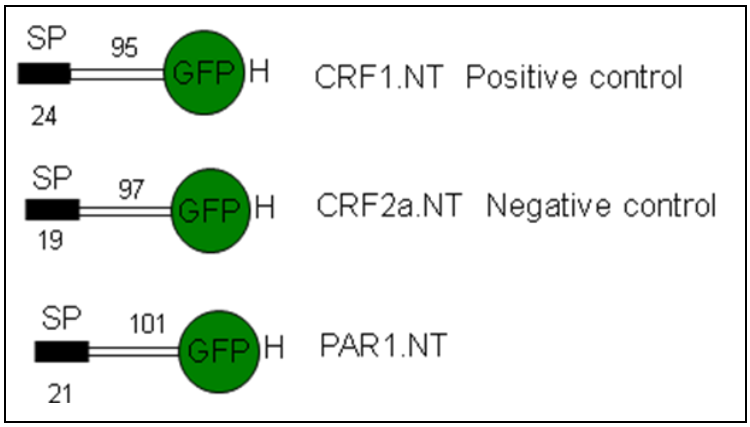

Figure 12. Schematic representation of the constructs used

The indicated N-tails were fused C-terminally with GFP to quantify their expression by confocal LSM or fluorimetric measurements. Signal peptides are indicated by black boxes, the N terminus sequences are depicted by open boxes and GFP by green color. The numbers above each construct indicate the number of N terminus amino acid residues present (without signal peptide); the numerals below the constructs indicate signal peptide length.

As a control for the secretion experiments, the previously described equivalent construct containing the N terminus sequence of the corticotropin-releasing factor receptor 1 (CRF1.NT) which possesses a functional and cleavable signal peptide (Alken et al., 2005) was used (Figure 12). Moreover, the N terminus sequence of the corticotropin-releasing factor receptor 2a (CRF2a.NT) was used, which does not contain a signal peptide but a "pseudo" signal peptide that is not cleaved and forms part of the mature receptor (Rutz et al., 2006), (Figure 12).

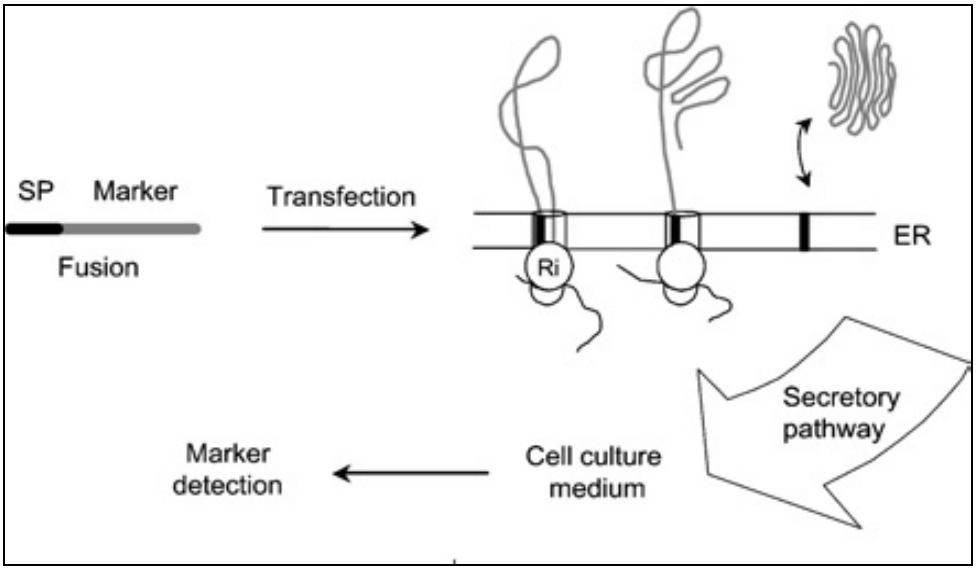

Figure 13. Schematic representation of the method to detect cleavable signal peptides of GPCRs.

Putative signal peptides are fused to marker proteins like GFP. In the presence of a functional signal peptide, the marker protein is directed via the secretory pathway to the extracellular space where it can be detected. Ri, ribosome, ER, endoplasmic reticulum (Alken et al, 2005)

Initially, ER targeting of the marker protein fusion was analyzed by CLSM. HEK 293 cells were grown on glass coverslips in 35 mm diameter dishes.

Cells were transiently transfected with the constructs and the GFP fluorescence signals were visualized at room temperature on a Zeiss LSM510-META invert confocal laser scanning microscope. The plasma membranes of the cells were stained using Trypan blue as a fluorescent dye.

In the case of CRF1.NT, reticular GFP signals were detected, indicating endoplasmic reticulum (ER) localization. The nucleus of the cells remained free from

marker proteins (Figure 14). These results demonstrate that this fusion is targeted to the ER and to the secretory pathway as already described (Rutz et al., 2006).

In contrast, in the case of construct CRF2a.NT, diffuse cytoplasmic and nuclear signals of the marker protein GFP were detected, demonstrating that this fusion does not enter the ER and is consequently unable to mediate the insertion of a heterologous protein into the ER as described (Rutz et al., 2006). Nuclear signals were detected because soluble GFP also contains a nuclear targeting signal (Tsien, 1998).

In the case of construct PAR1.NT, a similar pattern of GFP signals was detected as for CRF1.NT demonstrating that this fusion is also able to mediate targeting of GFP into the ER. These results suggest that the PAR1 possesses a functional signal peptide.

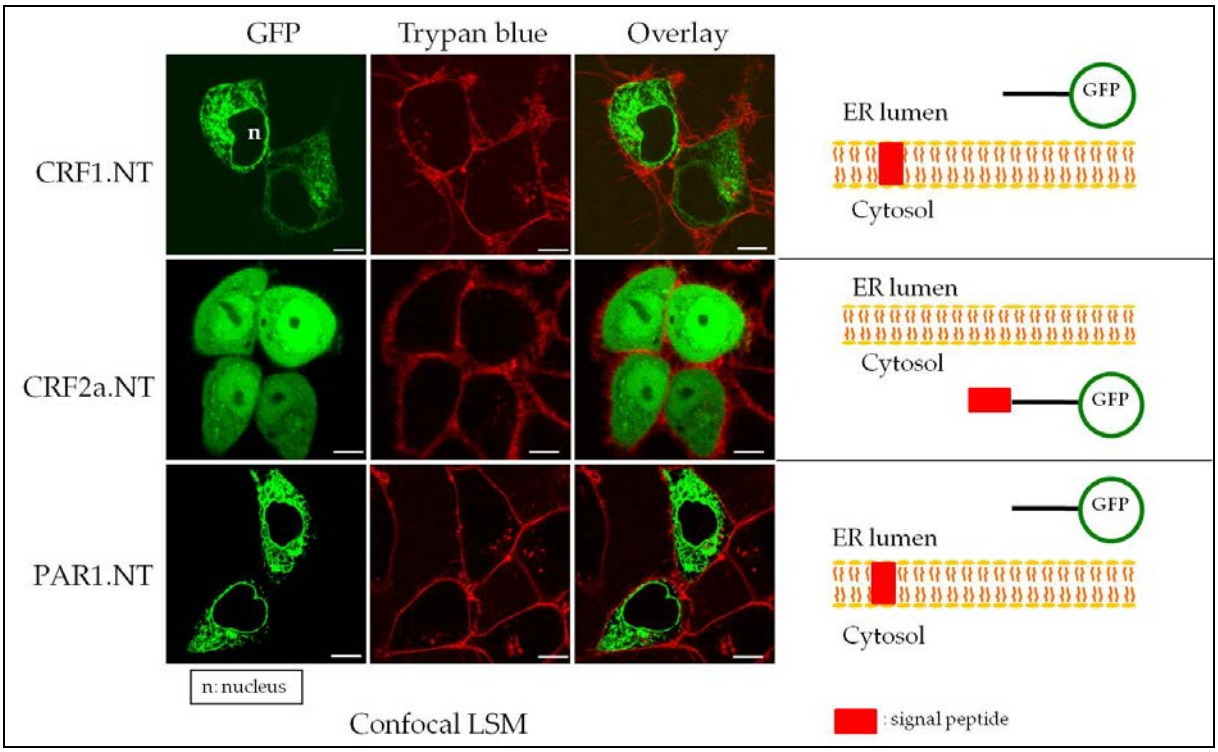

Figure 14. Analysis of the PAR1 signal peptide cleavage using marker protein fusions.

Subcellular localization of the constructs CRF1.NT, CRF2a.NT and PAR1.NT in transiently transfected HEK 293 cells using confocal LSM. The GFP signals of the constructs (green, left panels) and the plasma membrane signals following staining with trypan blue (red, central panels) are shown. GFP and Trypan blue fluorescence signals were computer-overlayed (right panels). GFP fluorescence is detectable only for transfected cells, whereas all cells show cell surface trypan blue fluorescence. Horizontal xy-scans of representative cells are shown. Scale bar = 10 μm, (n) = nucleus (the reliability of this analysis was described previously by Rutz et al., 2006).

To confirm the results obtained from CLSM, secreted marker protein fusions were isolated and analyzed by SDS-PAGE, immunoblotting and fluorimetric analyses.

To this end, HEK cells 293 were transiently transfected with the N-tail constructs CRF1.NT, CRF2a.NT, and PAR1.NT (Figure 12).

Cells were centrifuged and the supernatant was collected and incubated with metal affinity resin beads. One part of the supernatant was used for fluorimetric detection of the green fluorescent protein (GFP), while the second part was used for immunoprecipitation and western blot analysis.

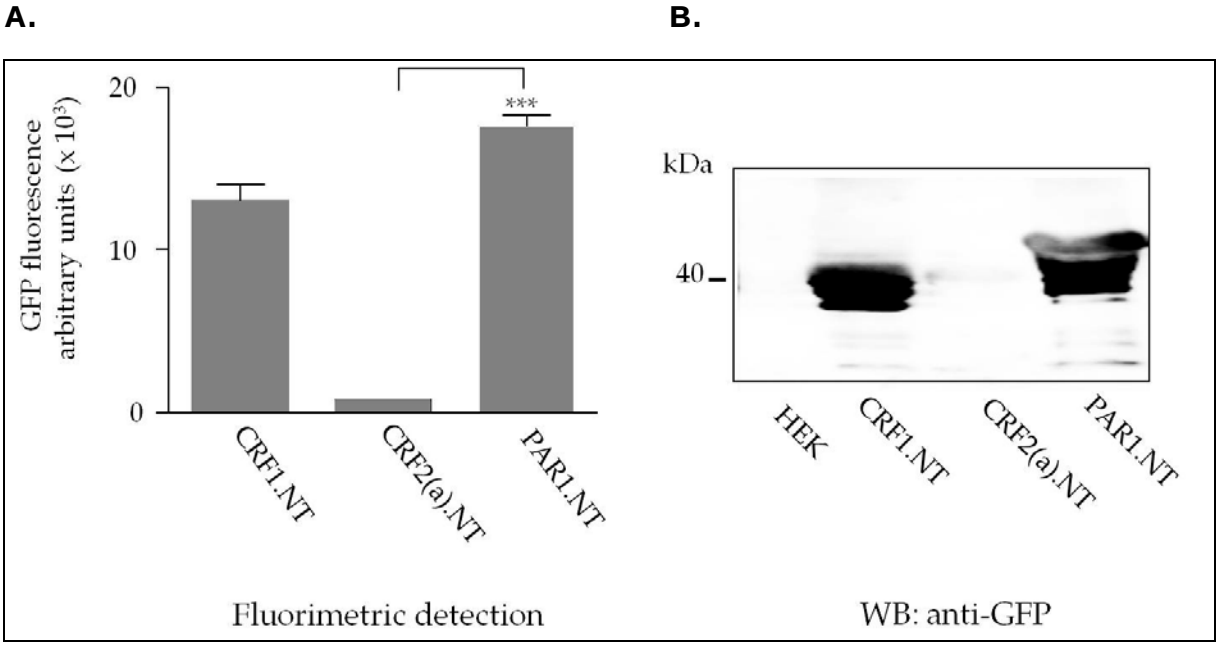

Figure 15. (A) Fluorimetric detection of the constructs in the cell culture supernatant.

Columns represent the fluorescence of the secreted, purified constructs from $4x10^6$ cells, and show mean values of three independent experiments each performed in triplicates (± SD) (***, $p < 0.001$, student's T-test).

(B) Detection of secreted, purified constructs by immunoblotting

Constructs were detected by a monoclonal anti-GFP antibody and a HRP-conjugated anti-mouse IgG. In each lane, the isolated protein of $4x10^6$ cells was loaded. The immunoblotting is representative of three independent experiments

In both fluorimetric analysis and immunoprecipitation experiments, construct PAR1.NT, could be detected in the supernatant indicating secretion of this construct.

Taken together these results demonstrate that the PAR1 contains a functional signal peptide that can mediate the targeting of a heterologous protein to the ER and *via* the secretory pathway finally to the cell culture medium.

3. The Putative Signal Peptide of the PAR1 Receptor is cleavable

The putative signal peptide of the PAR1 is able to mediate ER targeting when it is fused to the GFP protein marker. This raises the question whether the same holds true in the case of the full length receptors.

To address this point, full-length PAR1 and its signal peptide mutant (ΔSP.PAR1) C-terminally tagged with GFP (Figure 16) were constructed.

The GFP tag allows both the immunoprecipitation of the receptors and their subcellular localization by CLSM.

To assess for signal peptide cleavage, all constructs were immunoprecipitated from transiently transfected HEK 293 cells using a polyclonal anti-GFP antiserum, digested with PNGaseF to remove N glycans, and detected by SDS-PAGE/immunoblotting.

If the putative signal peptide is cleaved, the deglycosylated PAR1 and ΔSP.PAR1 constructs should comigrate. If the putative signal peptide is uncleaved, the apparent molecular mass of the PAR1 construct should be about 2 kDa larger than that of ΔSP.PAR1.

The previously described (Alken et al., 2005, Rutz et al., 2006) wild-type GFP-tagged CRF1R and CRF2aR and their respective signal peptide mutants (Figure 16; constructs CRF1 and ΔSP.CRF1; CRF2a and ΔSP.CRF2a) were used as controls for the presence of a cleaved signal peptide and an uncleaved “pseudo” signal peptide.

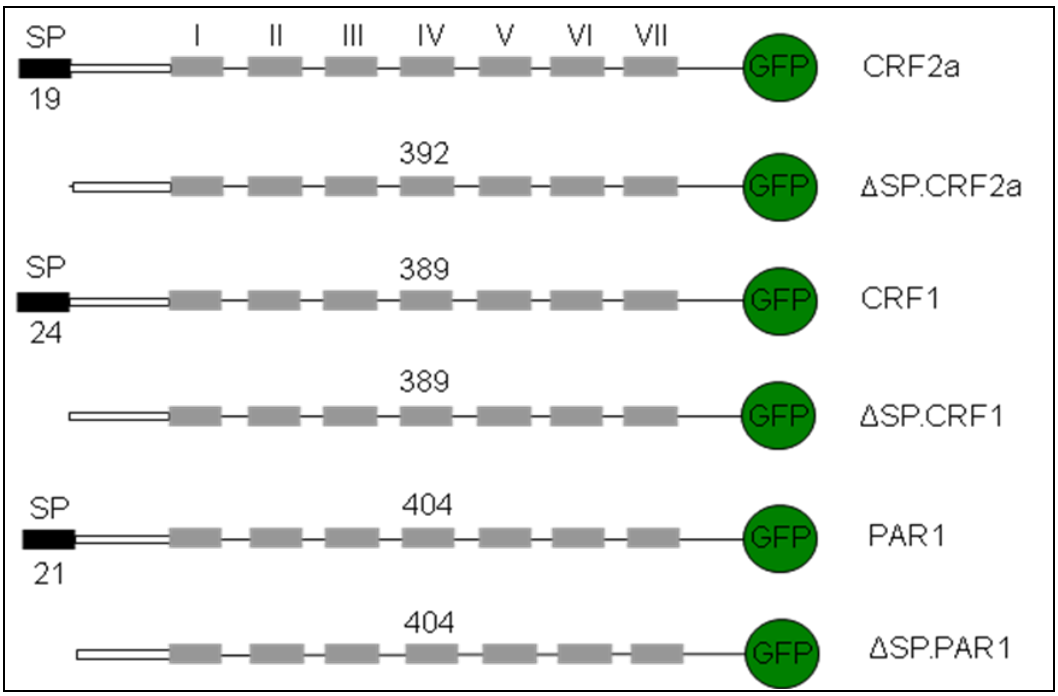

Figure 16. Schematic representation of the full length receptors constructs

The indicated receptors were fused C-terminally with GFP to visualize their expression by confocal LSM and to analyze signal peptide cleavage by immunoblotting. Signal peptides are indicated by black boxes while the N terminus sequences are depicted as open boxes, GFP is indicated by green color. The numbers above each construct indicate the number of N-tail amino acid residues present (without signal peptide); the numerals below the constructs indicate signal peptide length

In the case of the control constructs CRF1 and ΔSP.CRF1, co-migrating protein bands were detected demonstrating signal peptide cleavage as described previously (indicated by * in Figure 17).

For constructs CRF2a and ΔSP.CRF2a, non-co-migrating protein bands were visible demonstrating again that the CRF2aR possesses a "pseudo" signal peptide (indicated by # in Figure 17).

Comigrating protein bands were also detectable in the case of PAR1 with an apparent molecular mass of 72 kDa (indicated by § in Figure 17) indicating that the signal peptide is cleaved in the case of the full length receptor.

However, in the case of the signal peptide mutant, the immunoreactive protein band was only barely visible and in different experiments not constantly detectable.

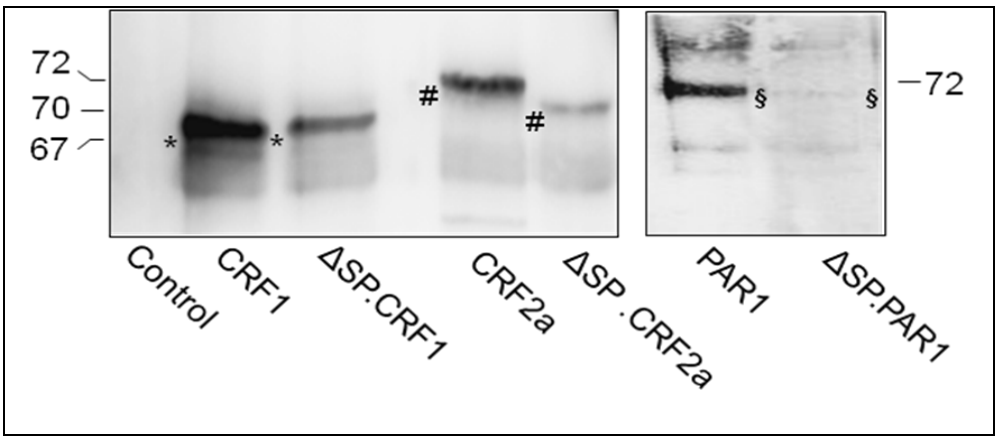

Figure 17. SDS-PAGE/immunoblotting analysis of the full length PAR1 and its signalpeptide mutant

Receptors were immunoprecipitated from transiently transfected HEK 293 cells, digested with PNGaseF to remove all N glycans and detected by SDS-PAGE/immunoblotting using a monoclonal anti-GFP antibody and HRP-conjugated anti-mouse IgG.

Thus, an alternative approach was pursued to confirm signal peptide cleavage in the case of full length PAR1. A FLAG tag was fused N-terminally to the signal peptide of the PAR1 (Figure 18, construct FLAG.PAR1). If the signal peptide is cleaved, the FLAG tag should be removed together with the signal peptide in the early secretory pathway and should no more be detectable at cell surface receptors.

As controls for these experiments, the N-terminally FLAG-tagged CRF1R possessing a cleaved signal peptide (Figure 18; construct FLAG.CRF1) and an N-terminally FLAG-tagged vasopressin V2 receptor (Figure 18; construct FLAG.V2) possessing only a signal anchor sequence were used.

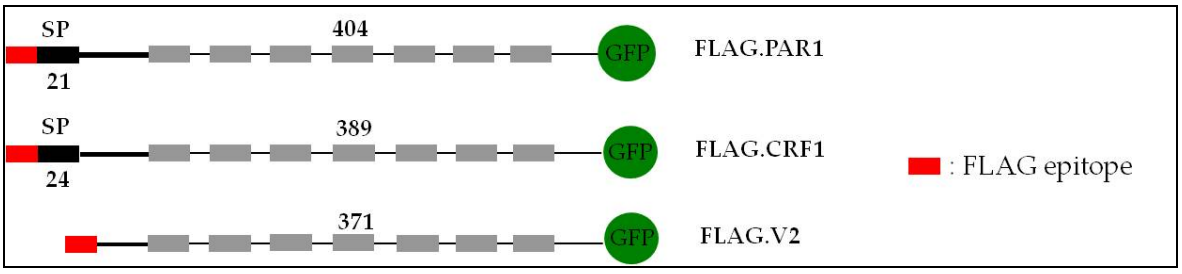

Figure 18. Schematic representation of the constructs used

The signal peptides (SP) and the transmembrane domains (roman numerals) are shown as black and grey boxes respectively. N terminus sequences are depicted as open boxes, FLAG tags are indicated by dark grey boxes. The numbers above each construct indicate the number of receptor amino acid residues present (without signal peptide); the numerals below the constructs indicate signal peptide length.

HEK 293 cells were transiently transfected and receptors were detected on the surface of intact cells using anti-FLAG antibodies and a cell surface ELISA assay (Figure 19). FLAG signals at the cell surface were detectable in the case of the control construct FLAG.V2 but not for constructs FLAG.CRF1 and FLAG.PAR1 confirming that the signal peptide of the PAR1 is cleaved off.

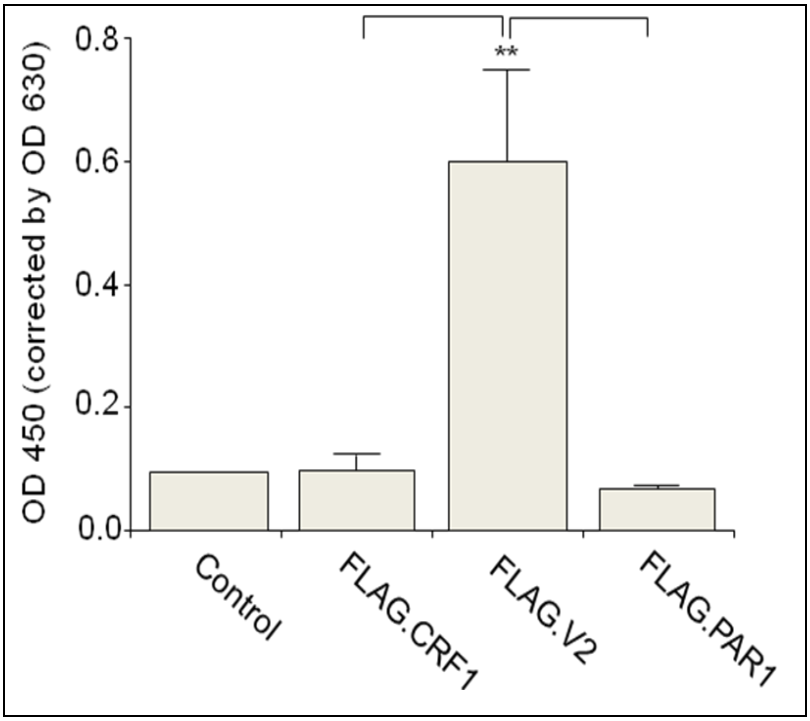

Figure 19. Analysis of the signal peptide cleavage of the full length PAR1 using N-terminal FLAG tags preceding the signal peptide.

In ELISA assay HEK 293 cells were transiently transfected with the constructs FLAG.PAR1, FLAG.CRF1 and FLAG.V2. Cell surface expression on intact cells was quantified using mouse anti-FLAG M2 antibodies and HRP-conjugated goat anti-mouse IgG. Columns represent mean values of three independent experiments (± SD) (***, $p<0.001$, student's T-test)

To confirm the ELISA results immunofluorescence microscopy with intact cells expressing the same constructs. using an anti-FLAG antibody were performed. If the signal peptide is cleaved off, no signals should be detectable in these experiments at the cell surface.

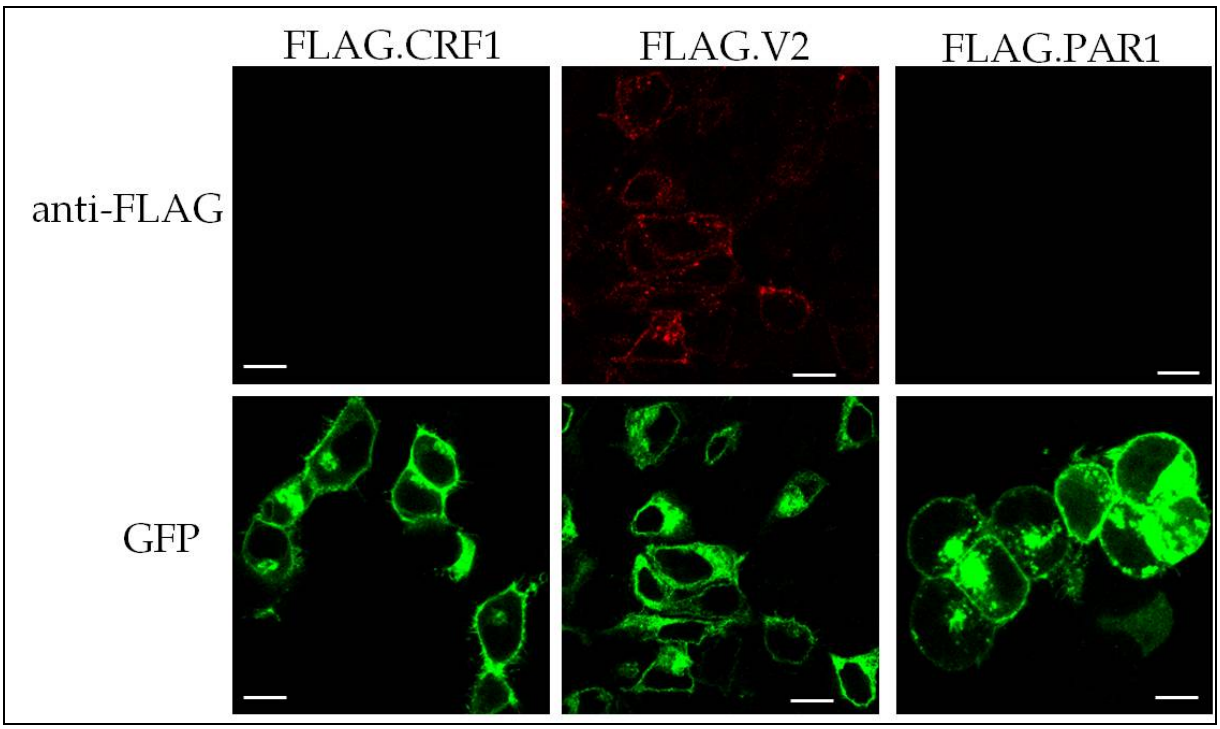

Figure 20. Detection of the constructs in transiently transfected HEK 293 cells by immunofluorescence.

Immunofluorescence microscopy using CLSM. Intact cells were assessed for the presence of an uncleaved FLAG epitope using mouse anti-FLAG M2 antibodies and Cy3-conjugated goat anti-mouse IgG (upper panel). Receptor expression was verified by recording the GFP fluorescence signals (lower panel). Horizontal xy-scans of representative cells are shown. Scale bar = 10 μm.

FLAG signals at the cell surface were detectable in the case of the control construct FLAG.V2 but not for constructs FLAG.CRF1 and FLAG.PAR1, (Figure 20, upper panel), while all three constructs were readily visible when monitoring their GFP signals (Figure 20, lower panel).

These results confirm that the signal peptide of the PAR1 is functional and cleaved off.

Moreover, immunoprecipitation and western blot analysis were performed with the FLAG.CRF1, FLAG V2R and the FLAG.PAR1.

Only the FLAG.V2R was detected on the immunoblot, meaning that the FLAG epitope remains at the N terminus as expected. The failure to detect a band for FLAG.PAR1 is consistent with a signal peptide cleavage (Figure 21).

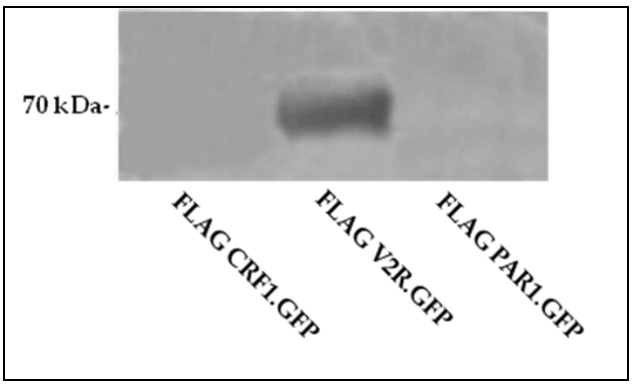

Figure 21. Detection of the precipitated constructs FLAG.CRF1, FLAG.V2R and FLAG.PAR1 by immunoblotting

Receptors were immunoprecipitated from transiently transfected HEK 293 cells using a monoclonal anti-FLAG antibody and a HRP-conjugated anti mouse IgG . In each lane, the isolated protein of $4x10^6$ cells was loaded. The immunoblotting is representative of three independent experiments.

In summary, these results demonstrate that the PAR1 possesses a conventional and cleaved signal peptide and, most importantly, that the signal peptide sequence is consequently not included in the parstatin peptide released by thrombin cleavage at the cell surface.

4. The putative signal peptide of PAR1 is necessary for establishing a functional receptor

The non-constant detection of the ΔSP.PAR1 in the previous SDS-PAGE/immunoblotting experiments suggested that the signal peptide of the PAR1 is necessary for receptor expression.

To address this question in more detail, inositol accumulation assays were performed with crude membrane preparations from transiently transfected HEK 293 cells, expressing the wild type receptor (PAR1) or the signal peptide mutant (ΔSP.PAR1) following thrombin activation (Figure 22).

After 30 min of thrombin exposure (1 IU/ml), a significant increase in inositol phosphates accumulation was detected for cells expressing PAR1 (Figure 22) but not for cells expressing ΔSP.PAR1. These data are consistent with the view that the signal peptide of the PAR1 is necessary for receptor expression.

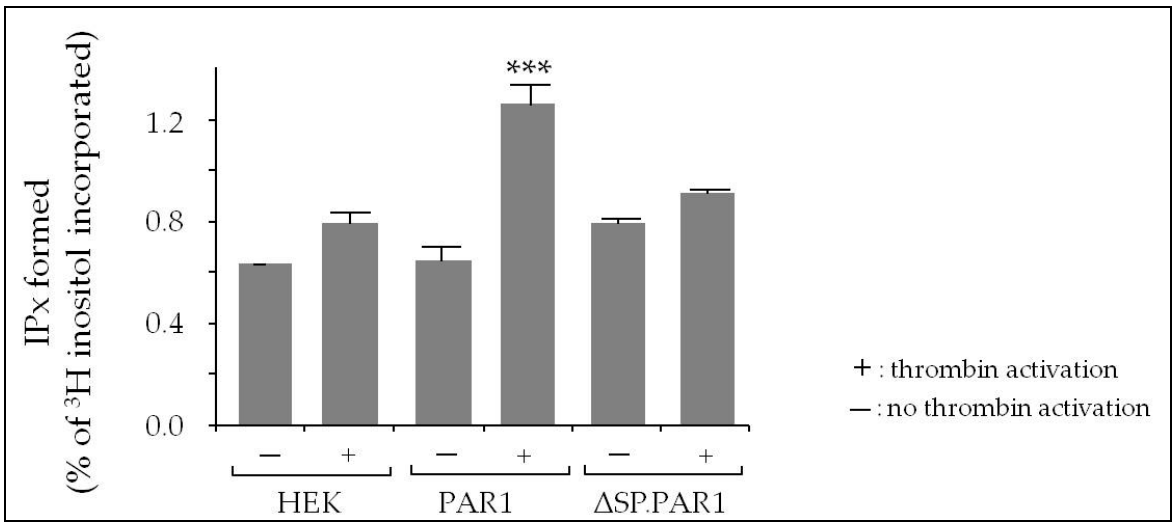

Figure 22. Thrombin-mediated inositol phosphate accumulation in intact HEK 293 cells transiently transfected with the constructs PAR1 and ΔSP.PAR1

Columns represent mean values of three independent experiments each performed in triplicates (± SD) (***, $p<0.005$, two way variance analysis).

Expression of PAR1 in transiently transfected HEK 293 cells by CLSM was also validated. To this end, the GFP signals of the receptors were recorded (Figure 23, left panels in green).

The cell surface of the same cells was stained with trypan blue (Figure 23, central panels in red) and the receptors at the plasma membrane were identified by computer overlay (Figure 23, right panels, co-localization is indicated in yellow).

Receptors at the plasma membrane were only detected for the wild type PAR1 as indicated by the overlap of the receptor's GFP fluorescence signals and the trypan blue signals.

Additional GFP signals were located intracellularly, presumably representing transport intermediates en route to the cell surface or a population of receptors that are stored in intracellular compartments such as endosomes and/or the Golgi apparatus.

No overlap of GFP and trypan blue signals was observed for the signal peptide mutant ΔSP.PAR1. Instead, weak and diffuse GFP signals filled the cytosol and the nucleus.

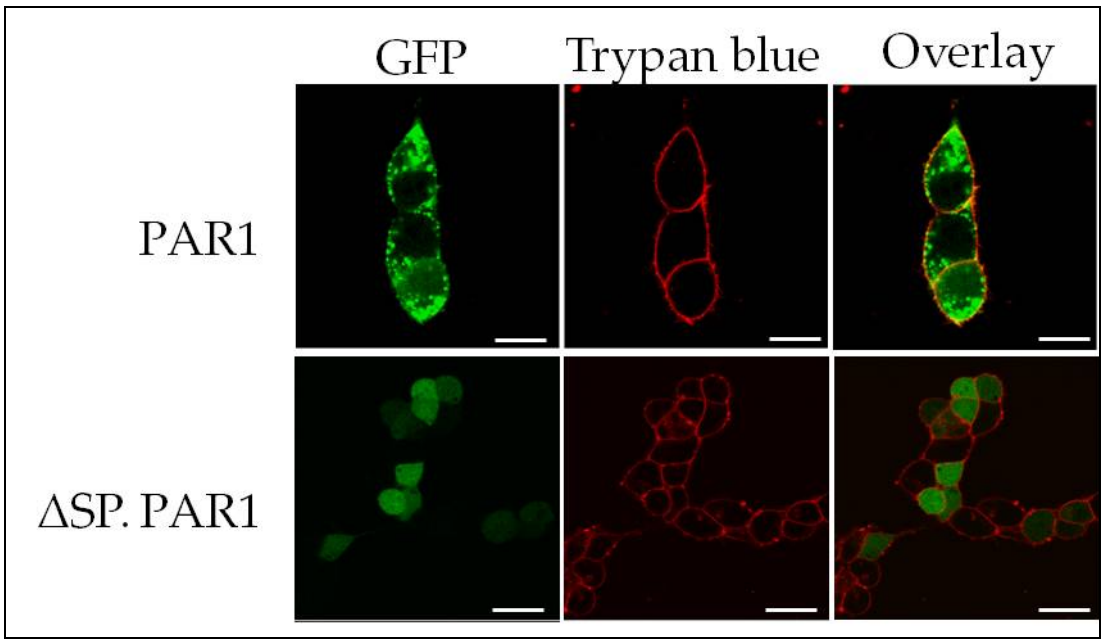

Figure 23. Subcellular localization of the constructs PAR1 and ΔSP.PAR1 in transiently transfected HEK 293 cells using CLSM

The GFP signals of the constructs (green, left panels) and the plasma membrane signals following staining with trypan blue (red, central panels) are shown. GFP and trypan blue fluorescence signals were computer-overlayed (right panels). GFP fluorescence is detectable only for transfected cells, whereas all cells show cell surface trypan blue fluorescence. Horizontal xy-scans of representative cells are shown (Scale bar = 10 μm).

Taking the extremely low expression of the PAR1 detectable after Immunoprecipitation into account, these results strongly suggest that the signal peptide of the PAR1 is necessary for receptor expression.

The overlap of GFP and Trypan blue signals at the cell surface was also quantified using the software version 3.2 SP2 (Figure 24) and no signal could be detected in the case of the signal peptide mutant.

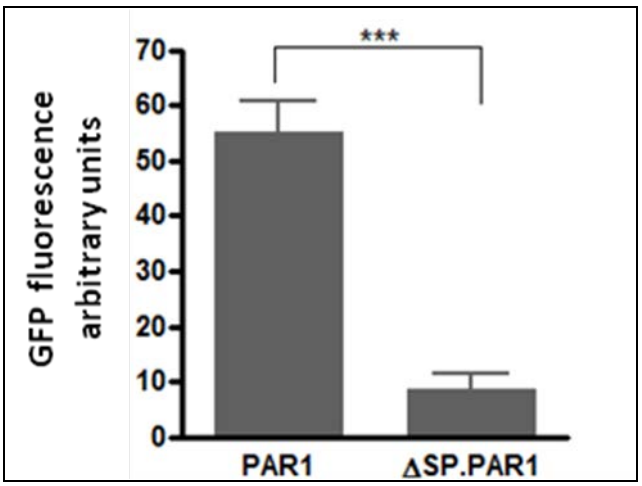

Figure 24. Quantification of the overlap of the receptor's GFP and plasma membrane trypan blue fluorescent signals in transiently transfected HEK 293 cells

Columns represent the mean values ±S.D (n ≥ 45). Statistical analysis was performed using student's t-test.

The insufficient expression of the signal peptide mutant at the cell surface may be the consequence of a folding defect.

Such a folding detect could be recognized by the quality control system of the ER, which ensures the export of only correctly folded proteins (Ellgaard et al., 2003).

Consequently, folding-defective signal peptide mutant could be degraded by the proteasome.

To answer the question whether a rapid protein degradation occurs or not, we used transiently transfected HEK 293 cells expressing PAR1 and ΔSP.PAR1, and inhibited proteasomal degradation by treating the cells with MG132 (30 μM) and performed SDS/PAGE immunoblotting analyses as described above.

Next, CLSM and SDS-PAGE/immunoblotting experiments were performed using the lysosomal degradation inhibitor chloroquine.

The CLSM analysis revealed that chloroquine could enhance the expression of wild type receptor at the plasma membrane of the cells but not that of ΔSP.PAR1 (Figure 25).

SDS/PAGE immunoblotting experiments with the lysosomal degradation inhibitor chloroquine did also not increase the amount of detectable ΔSP.PAR1 (data not shown).

These results indicate that the lysosomal pathway is also not involved in ΔSP.PAR1 degradation

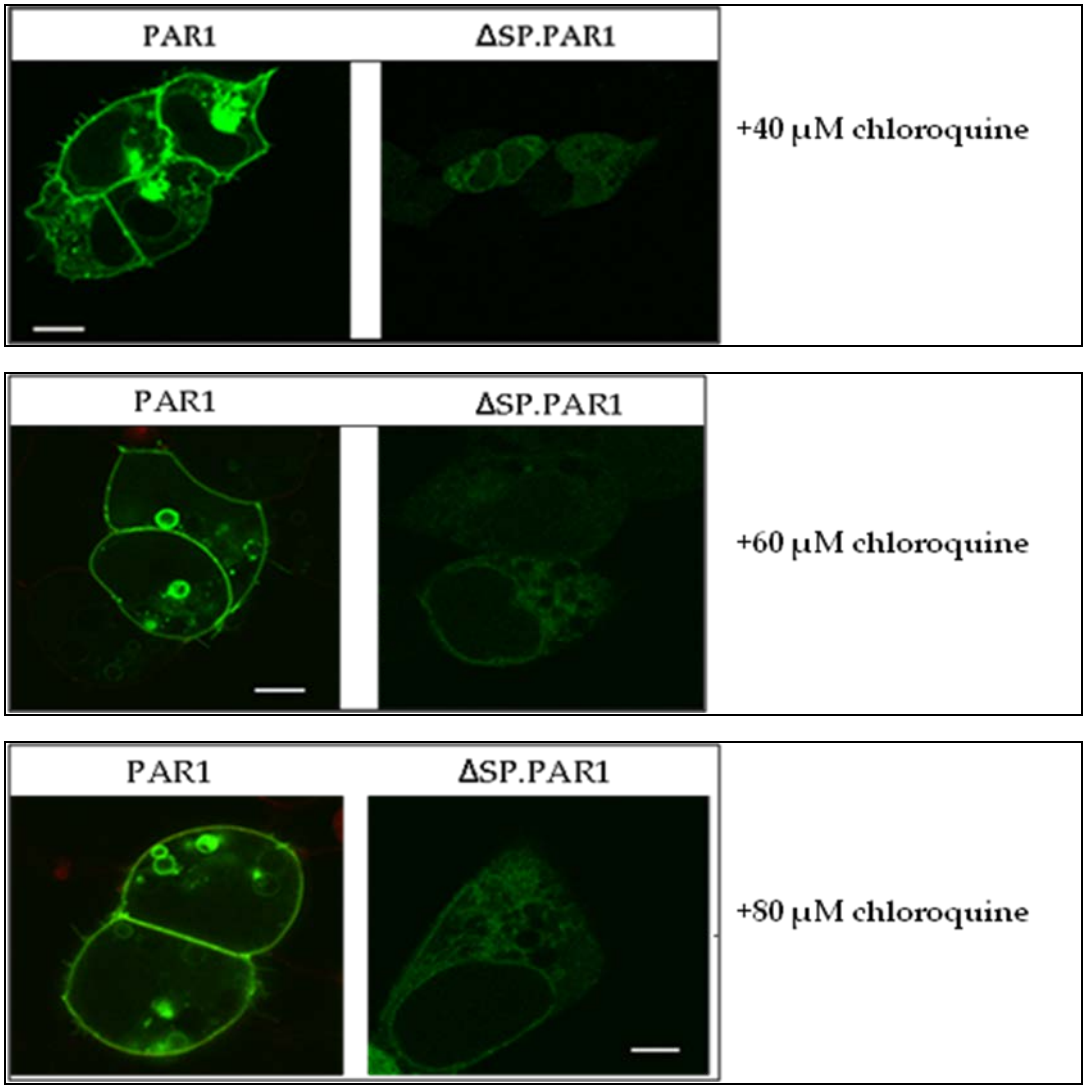

Figure 25. Subcellular localization of PAR1 and ΔSP.PAR1 in transiently transfected HEK 293 cells.

Cells were treated with the lysosomal degradation inhibitor chloroquine. The receptor's GFP signals were detected using CLSM as described above. Representative cells are shown (Scale bar = 10 μm).

The data of the proteasomal and lysosomal degradation assays suggest that ΔSP.PAR1 is not expressed properly.

If a signal peptide of a GPCR is deleted and the protein is not expressed in significant amounts, several possibilities may explain these data, taking into account that the signal peptide is nevertheless able to mediate the ER targeting/insertion process as shown above.

Expression of the signal peptide mutant may be impaired when the transmembrane domain following the signal peptide is unable to function as a signal anchor sequence. On the other hand, the sequence encoding the signal peptide may be necessary for one of the earlier steps of protein biosynthesis such as mRNA synthesis/stability and/or translation. These possibilities were analyzed below. All these possibilities are not mutually exclusive.

5. The TM1 of the PAR1 is insufficient to act as a signal anchor sequence

When a GPCR does not contain a signal peptide, the first hydrophobic sequence of the mature receptor is normally able to target the receptor into the ER membrane and then via the secretory pathway to the plasma membrane (Schöneberg et al., 1999, Edwards et al., 2000).

In the case of opsin, for example, it was shown that five of the six transmembrane segments studied could function as a so called signal anchor sequence (Audigier et al., 1987) helping to express the receptor. In the case of the ETBR (Köchl et al., 2002) and the CRF1R (Alken et al., 2005), the signal peptide mutants were expressed too.

The fact that the ΔSP.PAR1 is obviously only barely expressed raises the question, whether TM1 of the PAR1 could indeed function as signal anchor sequence.

To address the question whether the TM1 of the PAR1 could function as a signal anchor sequence, we used a construct containing the N terminus (including the putative signal peptide sequence), the first transmembrane domain (TM1) and the first intracellular loop (ICL1) fused to a GFP marker protein (construct PAR1.TM1).

A corresponding construct was cloned, lacking the putative signal sequence (construct ΔSP.PAR1.TM1) (Figure 26).

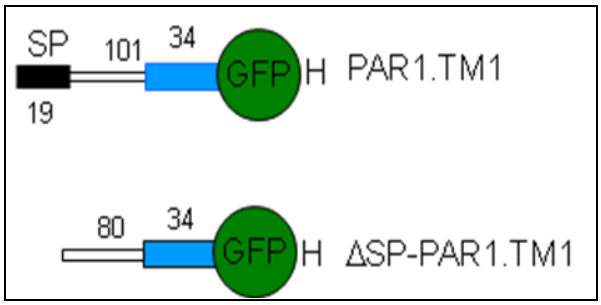

Figure 26. Schematic representation of the PAR1 constructs

The N terminus with or without signal peptide, the first transmembrane domain and the first intracellular loop were fused C-terminally to GFP. Signal peptides are indicated by black boxes while the N terminus sequences are depicted as open boxes. TMs and GFP are indicated by blue and green colors respectively. The numbers above each construct indicate the number of N terminus amino acid residues present (without signal peptide); the numerals below the constructs indicate signal peptide length.

HEK 293 cells were transiently transfected with the constructs and the GFP signals were localized by CLSM.

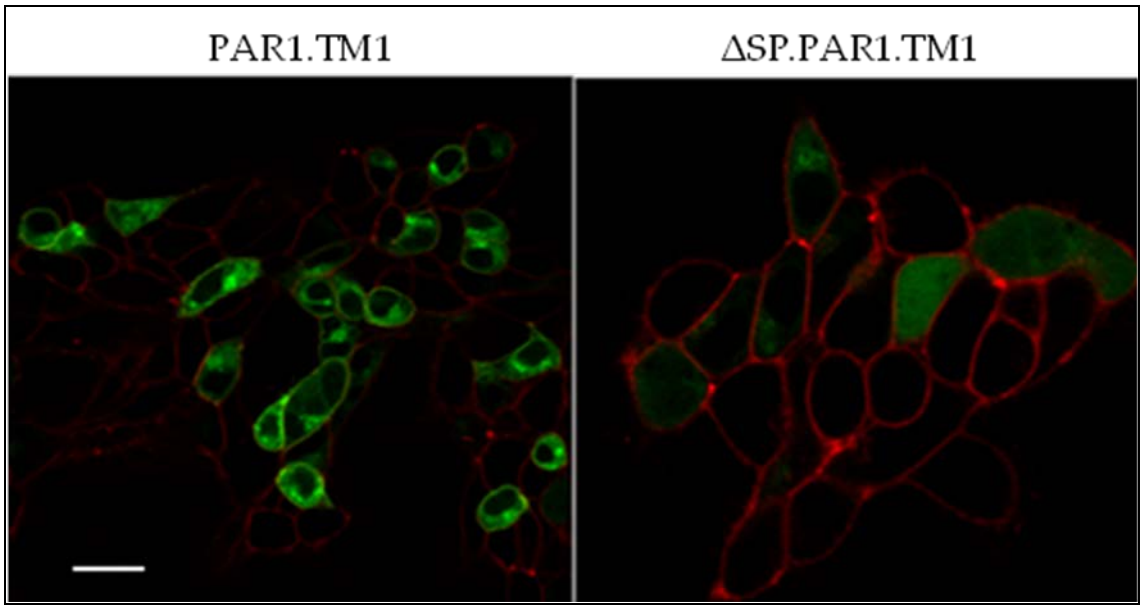

Figure 27. Subcellular localization of the GFP fluorescence signals of PAR1.TM1 and ΔSP.PAR1.TM1

The GFP signals of the constructs were recorded (green) and the plasma membrane was stained with trypan blue (red). The scans show computer overlays of representative cells. (Scale bar = 10 μm).

Reticular GFP signals were detected for the construct PAR1.TM1 indicating ER targeting and insertion of the construct (Figure 27, left panel). In the case of ΔSP.PAR1.TM1, however, only weak and diffused GFP signals were detected (Figure 27, right panel) indicating that TM1 of PAR1 could not function as a signal anchor sequence.

To confirm these results, a SDS-PAGE/immunoblotting analysis was performed using crude membranes of transiently transfected HEK 293 cells expressing PAR1.TM1 and ΔSP.PAR1.TM1. Membranes were treated with PNGase F to remove N-glycosylations prior to immunoblotting.

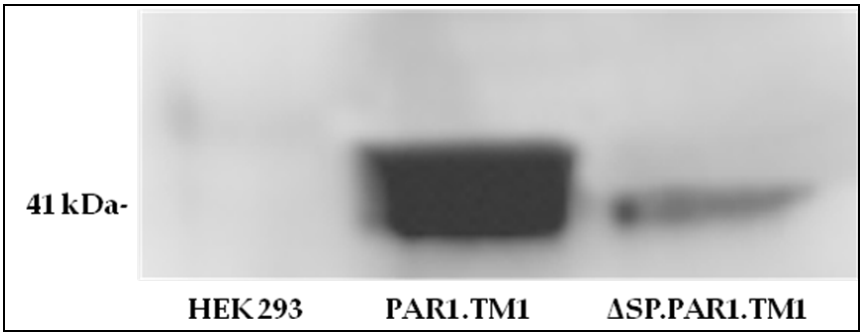

Figure 28. SDS-PAGE/immunoblotting analysis of the constructs PAR1.TM1 and ΔSP.PAR1.TM1

Receptors were immunoprecipitated from transiently transfected HEK 293 cells, digested with PNGaseF to remove all N glycans and detected by SDS-PAGE/immunoblotting using a monoclonal anti-GFP antibody and HRP-conjugated anti-mouse IgG

A broad 41 kDa immunoreactive band was detected for the receptor fragment PAR1.TM1 containing the signal peptide. In the case of the signal peptide mutant ΔSP.PAR1.TM1, this 41 kDa immunoreactive band was only barely detectable (Figure 28) indicating that the TM1 of the PAR1 could indeed not function as a signal anchor sequence.

5. The putative signal peptide of the PAR1 is essential for receptor's mRNA expression

Next we analyzed, whether the sequence encoding the signal peptide could influence mRNA transcription and/or mRNA stability.

To address this question, we first performed an *in vitro* transcription assay using the constructs PAR1 and ΔSP.PAR1 under the control, of the T7 promoter and quantified the mRNA by autoradiography followed by electrophoresis.

Constructs	mRNA (ng)
PAR1	1002 ± 145
ΔSP.PAR1	836 ± 130

Table 2. Amount of mRNA of the constructs PAR1 and ΔSP.PAR1 following in vitro transcription

Constructs were expressed under the control of the T7 promoter and the amount of mRNA was quantified by autoradiography.

Similar amounts of mRNA were obtained for both constructs indicating that transcription itself is not affected when the sequence encoding the signal peptide is deleted.

Next, quantitative real time PCR (qRT PCR) with total RNA isolated from transiently transfected HEK-293 cells expressing PAR1 and ΔSP.PAR1 was performed. As an endogenous control the glycerinaldehyd-3-phosphat-dehydrogenase (GAPDH) was used. The results were normalised to this control and compared to untreated HEK cells (reference control).

The experiment revealed a strong difference between the mRNA levels of PAR1 and the signal peptide mutant ΔSP.PAR1 (Figure 29, left panel).

To address whether this result was specific for the PAR1, we performed a qRT PCR using the CRF1 and ΔSP.CRF1 as a control.

In the case of constructs CRF1 and ΔSP.CRF1, similar amounts of mRNA were detected (Figure 29, right panel) demonstrating that the results were specific for the PAR1.

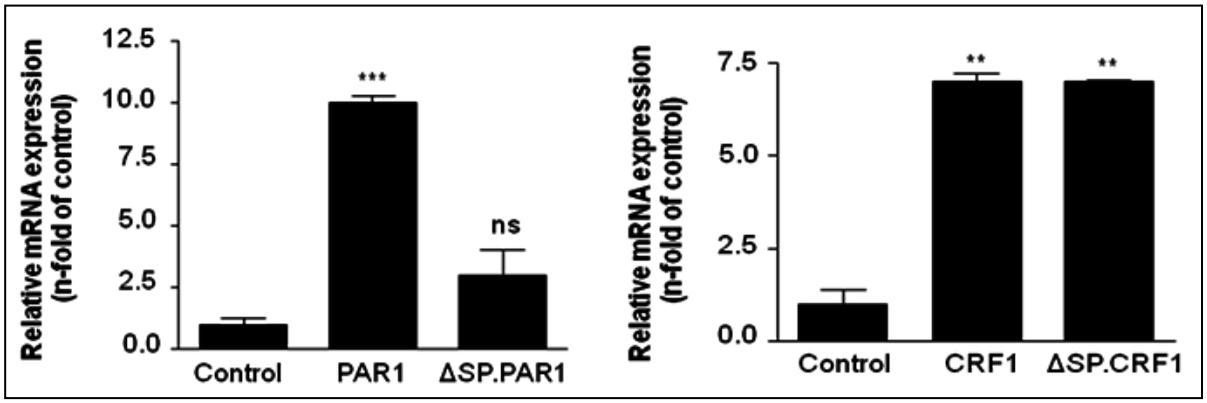

Figure 29. qRT PCR quantification of the mRNA of constructs PAR1 and ΔSP.PAR1 (left panel) and CRF1/ΔSP.CRF1 (right panel)

Columns represent fold difference of mRNA expression after normalization to the GAPDH endogenous control compared to the reference sample (untreated cells) and show mean values of three independent experiments each performed in triplicates (± SD) (***, $p<0.001$; **$p<0.01$, student's t-test).

These results suggest that while transcription itself is not influenced, the sequence encoding the signal peptide of the PAR1 may increase mRNA stability.

To address this question theoretically, we performed bioinformatics analysis of the mRNA secondary structure of PAR1 and ΔSP.PAR1 using the program mFold (Zuker M, 2003). It is known that the secondary structure of the mRNA is very important for the stability of the transcript and consequently for the transcription and translational rates of a protein.

The peptide mRNA secondary structure of PAR1 was compared to those of various other GPCRs possessing putative signal peptides (Table 3).

Receptor	Signal peptide size	GC% content	ΔG
Corticotropin-releasing factor receptor 1	23	68	-24,4
Protease-activated receptor 1	21	71	-32,5
Endothelin B receptor	26	68	-26,8
Glucagon-like peptide 1 receptor	20	77	-27,7
Vasoactive intestinal polypeptide receptor 1	30	53	-19
Somatostatin receptor	24	54	-17,2
Growth hormone-releasing hormone receptor	22	58	-26,7
Metabotropic glutamate receptor 1	18	44	-7,6
Metabotropic glutamate receptor 3	23	52	-9,9
Secretin receptor	22	62	-14,3
Vasoactive intestinal polypeptide receptor 2	23	57	-16,4
Latrophilin-3 receptor	19	44	-2,3
Calcitonin receptor	24	44	-10,3
EGF-like module receptor 4	14	48	-3,8

Table 3. Bioinformatics analysis of the sequences of GPCRs encoding putative signal peptides The GC content of the sequences and their free energy were calculated.

The higher the GC content of a sequence is, the more stable is the secondary structure. Interestingly, the sequence encoding the putative signal peptide of the PAR1 has the second highest GC (71%) content and the highest free energy value (-32.5) and consequently the most stable fold of all sequences.

These results may explain the decreased mRNA expression level of the signal peptide mutant of the PAR1 seen in the qRT PCR experiments. They may, of course also explain the resulting decrease in protein expression.

A plot for the putative secondary structure of the mRNA of PAR1 and ΔSP.PAR1 is shown in figure 30.

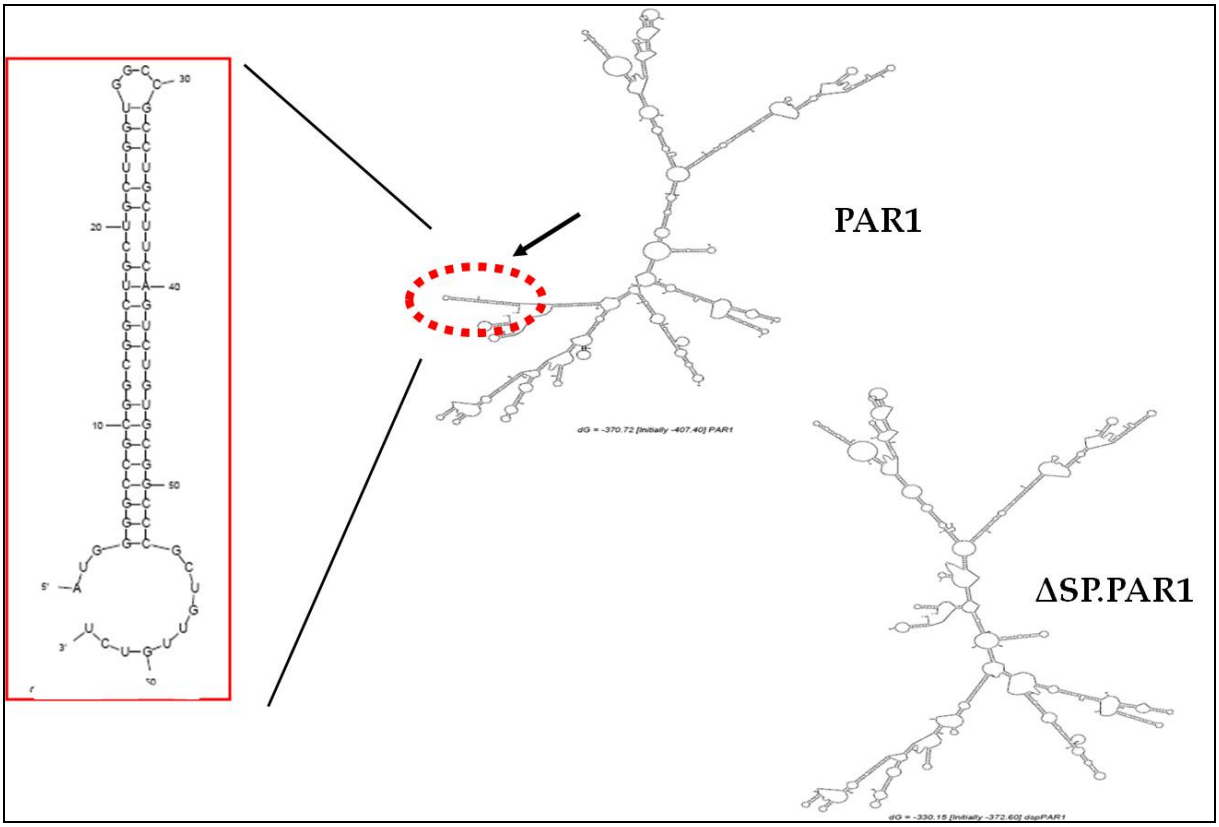

Figure 30. Bioinformatics analysis of the putative mRNA secondary structure of PAR1 (upper panel) and ΔSP.PAR1 (lower panel).

In the case of PAR1, the sequence encoding the signal peptide forms a stem loop structure (arrow and cutaway view) which is missing in the case of ΔSP.PAR1. The analysis was performed using the program mFold.

To assess for the stability of the PAR1 mRNA with or without signal peptide also experimentally, we have performed mRNA degradation assays in transiently HEK293 cells in the presence of the transcription inhibitor Actinomycin D in a time range from 0 to 360 minutes. Total RNA was isolated from the treated cells, and cDNA synthesis and relative quantitative real time PCR (qRT PCR) was performed.

To calculate the mRNA degradation rates, we used the rate of the housekeeping protein GAPDH as a reference control and compared it to the expression rates of the mRNA of PAR1 and ΔSP.PAR1.

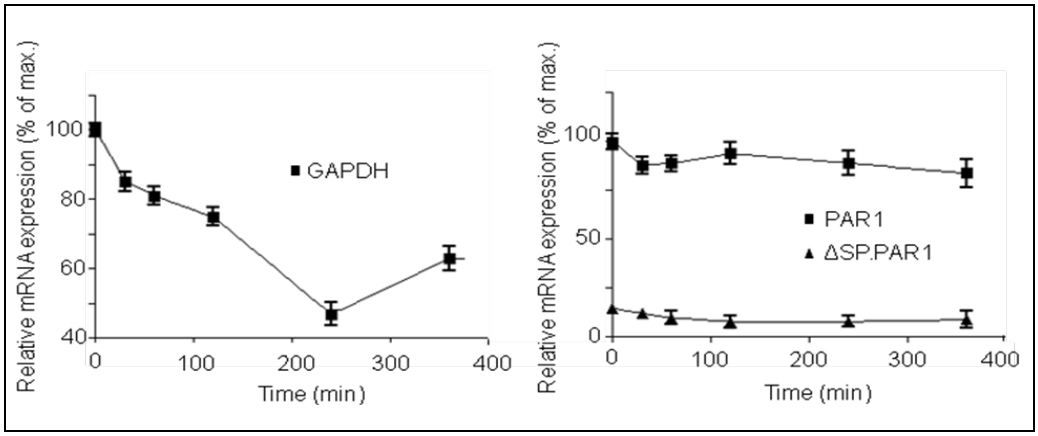

Figure 31. Degradation of the mRNA of PAR1 and ΔSP.PAR1

Transiently transfected HEK 293 cells were treated for different times with the transcription inhibitor Actinomycin D. Data points represent the remaining mRNA of GAPDH (left panel) and that of PAR1 and ΔSP.PAR1 (right panel; normalized to the amount of GAPDH) of one experiment performed in triplicates (± SD). The curves are representative of three independent experiments.

The half time of degradation for the endogenous control GAPDH was approximately 220 min (Figure 31, left panel). In the case of PAR1, quantification of mRNA levels at various time points using qRT-PCR revealed that the PAR1 mRNA levels remained stable for at least 4 hours after actinomycin D treatment (Figure 31, right panel).

In contrast, the very low mRNA levels of construct ΔSP.PAR1 are consistent with a very rapid mRNA degradation.

These results indicate that the sequence encoding the signal peptide of the PAR1 receptor is essential for the regulation and stabilization of the receptor's mRNA.

This result is novel and unique for a signal peptide within the GPCR family.

DISCUSSION

Discussion

PARs constitute a relatively new subfamily of the GPCRs. PARs have a unique activation mechanism different from other GPCRs. Serine proteases recognize a specific region in the extracellular N-terminal domain and mediate its proteolytically cleavage (Coughlin S.R, 2000). In the case of PAR1, thrombin cleaves the peptide bond between Arg^{41}-Ser^{42}.

The thrombin action uncovers a new N tail for PAR1 with the characteristic amino acid sequence SFLLRN, which interacts with the receptor as an internal ligand. It was believed that a peptide representing the first 41 amino acids of the N terminus namely parstatin is released by the proteolytical action of thrombin.

Although parstatin has been shown to exert attractive properties which may be transformed into therapeutic applications in the future, several key questions regarding its sequence and structure were unclear. Most importantly, the precise length of the peptide was still unknown. While it is clear that the peptide is released by cleavage between Arg^{41} and Ser^{42}, its actual length depends on whether the PAR1 possesses an N-terminal cleavable signal peptide.

Cleavable signal peptides of GPCRs and other integral membrane proteins mediate integration of the proteins into the membrane of the endoplasmic reticulum (ER), the initial step of the intracellular transport. Most GPCRs do not possess cleavable signal peptides but instead an uncleaved signal anchor sequence which takes over signaling functions (usually the first transmembrane domain of the mature receptor).

Both types of signal sequences bind to the signal recognition particle (SRP) and mediate targeting of the nascent chains to the translocon complex at the ER membrane (Alken et al., 2009). The signal sequences also facilitate opening of the Sec61 protein-conducting channel of the translocon complex in order to integrate the nascent chain into the bilayer (Schulz et al., 2010).

Whereas signal peptides are cleaved off following ER insertion by the signal peptidases of the ER, signal anchor sequences form part of the mature protein.

Here, we have addressed the question of whether the PAR1 possesses an N-terminal cleavable signal peptide or not. If a cleavable signal peptide is present, the thrombin-released parstatin peptide would only be 19 amino acids long. If not, the actual parstatin length would be 41 amino acid residues.

1. The PAR1 contains a functional and cleaved signal peptide which is necessary for mRNA stability

We found that a construct containing the putative signal peptide of the PAR1 and the N terminus of the receptor (PAR1.NT) was able to mediate ER targeting and consequently secretion of the heterologous protein GFP (Figures 14, 15).

Moreover, we found in the case of the full length receptor's constructs that the signal peptide of the PAR1 is cleaved off during receptor's biosynthesis. The signal sequence of the PAR1 thus represents a functional and cleaved signal peptide.

In the case of the human endothelin B receptor (ETBR) such a signal peptide was also necessary for N-tail translocation. The signal peptide deletion mutant was not functional but it was readily expressed (Köchl et al., 2002).

Similar results were obtained for the human cannabinoid receptor 1 (CB1), (Andersson et al, 2003), the VPAC1 receptor (Couvineau et al., 2004) and the thyrotropin receptor (Akamizu et al., 1990). In all these cases the signal peptides were necessary for N terminus translocation but their deletion did not impair receptor expression.

In the case of the CRF1R, the signal peptide deletion mutant was also expressed although the overall expression was decreased (Alken et al., 2005).

In the case of the PAR1 and in contrast to the signal peptides of the above mentioned other GPCRs, deletion of the signal peptide leads to a very low, only barely detectable expression

The TM1 of the receptor was insufficient to take over signaling functions and act as a signal anchor sequence which could account for the strongly reduced receptor expression.

In the case of the GLP1 receptor, the TM1 was also unable to act as a signal anchor sequence and the signal peptide deletion mutant was consequently not expressed (Huang et al., 2010).

Here we show for the PAR1, however, that the sequence encoding the signal peptide plays an essential role in an earlier step of the receptor's biosynthesis, in the expression of the mRNA.

We found that the sequence encoding the signal peptide dramatically increases the amount of mRNA, most likely by facilitating the formation of a stem loop and in turn preventing rapid RNA degradation. Lack of the signal peptide sequence may consequently cause the observed low mRNA expression of the signal peptide mutant.

In the case of the above-mentioned GLP1 receptor, it was shown that deletion of the sequence encoding the signal peptide did not affect mRNA synthesis and it was speculated that the receptor was degraded proteolytically (Huang et al., 2010).

The results above seem to be substantially different to what was observed here for the sequence encoding the signal peptide of the PAR1.

In comparison to PAR1, a similar function of a sequence encoding a signal peptide has been shown for bacterial proteins such as the PlcH Tat protein in *Pseudomonas aeruginosa.* Here, deletion of the sequence encoding the signal peptide also leads to a substantial decrease of the transcription levels in cells in comparison to those of the wild type. The authors also suggested that the mRNA expression difference was due to decreased stability of the mRNA lacking the signal peptide (Snyder et al., 2006).

Based on these findings, it is likely that the very low amount of mRNA observed for construct ΔSP.PAR1 is a result of a misfolded mRNA secondary structure. The molecular details however, are not clear at the moment.

It is known that mRNA is characterized by different secondary structures such as loops, hairpins and bubbles which possess different stability values. Stem loops or hairpins seem to yield the highest stability values. Moreover, the more GC-rich an mRNA is, the more stable is the whole structure (Serra et al., 1993).

The ability of secondary structures to stabilize transcripts has already been shown for bacterial, plant and viral mRNAs.

In Chlamydomonas disturbing the stem-loop formation of the 5'terminal renders transcripts completely unstable, even if the nucleotide sequence of this element is not altered (Suay et al., 2005).

In *Escherichia coli* it was shown, that the introduction of various secondary structures such as stem loops at the 5'-end of mRNA increases protein expression by increasing the half-lives of the mRNA transcripts (Yoon et al., 2008, Emory et al., 1992).

Moreover, for the HIV viral RNA helix, it was shown that mutation of the stem loop structure at the 3' end leads to its destabilization by altering the free energy value of the hairpin (Berkhout et al., 1997).

Taken these findings into account, it is likely that the sequence encoding the signal peptide of the PAR1 has a function in stabilization of the mRNA.

It must be noted however, that the secondary structure of the mRNA established by a signal peptide sequence may also have implications for gene expression in the other direction i.e. that the presence of a loop may decrease expression.

This was shown in the case of *Neisseria meningitidis* protein GNA33, where the 60 nucleotides encoding the signal peptide were found to exert a negative regulatory effect on the expression of GNA33 (Serruto et al., 2004).

The down-regulation, however, took place at the translational level and not at the level of mRNA stability. A stem–loop secondary structure within the signal peptide encoding sequence seems to inhibit transcription/translation since mutations in this sequence increased the expression of the proteins (Serruto et al., 2004).

Similar results were obtained for the sequences encoding the signal peptide of the protein formate dehydrogenase N (FDH-N) (Punginelli et al., 2004), in the case of the Lc activator protein in Maize (Wang et al., 2001) and in the case of the sxy protein of *Heamophilus influenza* (Cameron et al., 2008). Here, again the stem loops negatively regulated the transcription/translation rates.

Taken together the formation of a stem loop in the mRNA may increase mRNA stability (as it may be the case of PAR1) but also decrease the transcription/translational rates.

To verify whether or not the low amounts of mRNA of the ΔSP.PAR1 mutant were due to transcription failure or rapid degradation, degradation assays were performed, in which the mRNA decay half time was calculated.

From these assays, we were able to determine the degradation rates for the GAPDH, PAR1 and the ΔSP.PAR1 mRNAs. The degradation half time for the endogenous control GAPDH was approximately 220 min. PAR1 mRNA levels remained stable for at least 4 hours after actinomycin D treatment. In contrast, the very low mRNA levels of construct ΔSP.PAR1 are consistent with a very rapid mRNA degradation (Figure 31, right panel)

These results are also consistent with the view that the low amount of the mutant lacking the signal peptide is caused by rapid degradation of the mRNA, presumably due to misfolding.

In summary, in the case of PAR1, the sequence encoding the signal peptide seems to lead to a very stable mRNA. This is important to note since the PAR1 receptor is found abundantly in platelets. These are non-nuclear cells and they do not have any transcriptional machinery. It is thus conceivable that the transcripts of PAR1, which

are made in the megakaryocytes, have to be very stable (Lindemann et al., 2007, Thon et al., 2010, Gnatenko et al., 2009, Kiefer et al., 1997). Thus, the sequence encoding the signal peptide may help to keep the secondary structure of the mRNA stable in order to guarantee sufficient receptor expression in platelets.

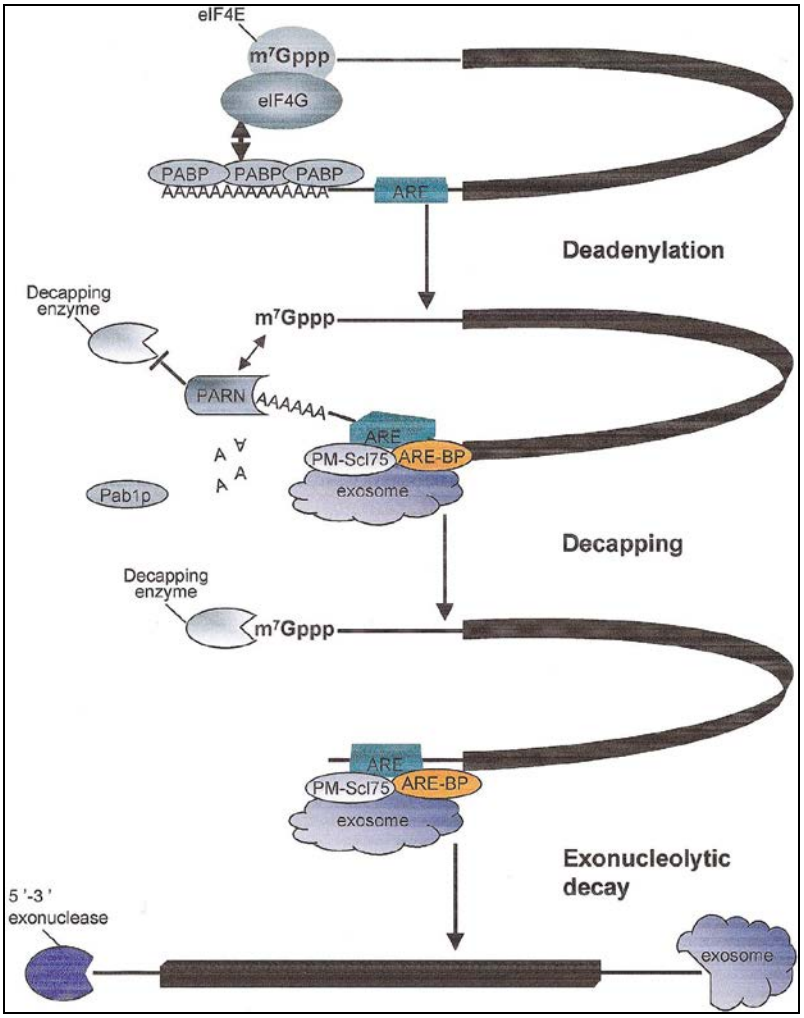

Figure 32. Schematic diagram of the mammalian ARE-dependent mRNA decay pathway

In this model, the ARE sequence, through AU-BP binding, recruits the exosome and destabilizes the translation initiation complex at the cap structure to allow interaction between the cap structure and the deadenylase PARN. After deadenylation, PARN dissociates from the complex, and the decapping enzyme can remove the cap structure. The final step is degradation of the mRNA body by the exosome (Tourrière et al, 2002)

However, how can the stem loop formation mediated by the sequence encoding the signal peptide increase mRNA stability mechanistically?

It is known that in eukaryotic cells, the steady-state levels of mRNAs depend on their combined rates of synthesis and processing, transport from the nucleus to cytoplasm and decay in the cytoplasm. The degradation of mRNA is an essential determinant for the regulation of gene expression and can be modulated in response to developmental, environmental and metabolic signals (Tourrière et al., 2002).

The basic mechanisms by which the cells control mRNA levels through degradation are the, deadenylation of the 3′ end, decapping of the 5′ end and exonucleolytic decay (Figure 32).

Taking these considerations into account, it can be speculated that the putative stem loop formed by the sequence encoding the signal peptide of the PAR1 may influence one of these steps.

2. Parstatin is shorter than previously thought

In this study, we have also analyzed the actual length of the peptide which is cleaved off during PAR1 receptor's activation by thrombin.

This parstatin peptide was believed to consist of 41 amino acids and to have a molecular weight of 4,47 kDa. Its sequence is:

NH_2-M_1GPRRLLLVAACFSLCGPLLSARTRARRPESKATNATLDPR_{41}-OH

Earlier studies indicated that the parstatin peptide promotes platelet aggregation and platelet endothelial cell adhesion in vitro (Furman et al., 1998, Furman et al., 2000, Clayton et al., 2003).

More recent studies showed that parstatin is a potent inhibitor of angiogenesis and protect myocardium from ischemia and reperfusion injury. (Zania et al., 2009, Huang et al., 2010, Strande et al., 2009).

Most importantly, and without knowing that the signal peptide of the PAR1 is cleaved off, it was recently shown that the parstatin fragment consisting only of the

amino acids Met^{1}-Ala^{26} contains the functional domain of the peptide for myocardial protection (Routhu et al., 2010).

In a rat ischemia-reperfusion injury model, the synthetic parstatin (1-26) was more effective and potent in the protection of myocardium, than the "full-sized" parstatin (1-41). In the same model, the synthetic parstatin fragment (24-41) was not functional (Dr. Tsopanoglou, personal communication) indicating that parstatin functions are mediated by its N-terminal part and thus by the signal peptide alone.

Here, we could show that the sequence at the N terminus of the PAR1 represents a functional signal peptide which is removed from the receptor following insertion into the ER membrane. Thus, the peptide which is proteolytically cleaved from the mature receptor at the plasma membrane does only contain residues Ala^{22} to Arg^{41} and not the residues Met^{1} to Ser^{21} forming the signal peptide.

In the original experiments leading to the description of the parstatin effects, the peptide was added to the extracellular side of the cells (Zania et al., 2009, Strande et al., 2009, Routhu et al., 2010). These results do not conflict with the fact that the relevant peptide obviously does not leave the cells. It is conceivable that the hydrophobic signal peptide may easily enter the cells when it is added into the cell culture medium, either by diffusion and/or by endocytosis.

Taking into account that the signal peptide is actually cleaved off in the early secretory pathway, parstatin effects must originate from a peptide which is initially embedded in the ER membrane rather than being released extracellularly.

If so, how can the cleaved signal peptide then fulfill its functions?

It has already been shown in many cases for bacterial, viral and other proteins that the signal peptides or fragments of them are responsible for post targeting functions in cells. The trimmed peptides may be retranslocated into the cytosol by an as yet incomplete understood mechanism and it was proposed that the peptides have physiological functions following their release (Martoglio et al., 1997, Hedge et al., 2006).

For example, the signal peptide of the lymphocytic choriomeningitis virus (LCMV) glycoprotein is synthesized as a precursor protein with an N terminus signal sequence that is cleaved off by the signal peptidase during membrane insertion. The resulting signal peptide ($SP_{GP\text{-}C}$) remains membrane-anchored, associates with the glycoprotein C precursor (GP-C) protein (Froeschke et al., 2003, Schrempf et al., 2007) and plays different roles in both glycoprotein maturation and virus function.

Another possibility for signal peptides to gain function after cleavage, is their release from the ER membrane as soluble peptides. Such a mechanism was shown in the case of the mouse mammary tumor virus protein (MMTV). The cleaved signal peptide, SPRem/Env, initially accumulates in the ER membrane, then appears in the cytosol, and is finally found in the nucleus (Dultz et al., 2008). Here, the SPRem/Env signal peptide acts as a nuclear export factor (Cullen BR., 2003).

A cleaved and released signal peptide may also act as a post-transcriptional regulator of genes. Such a function was described in the case the of signal peptide of the envelope glycoprotein (Env) of the Jaagsiekte sheep retrovirus (JSRV). Here, the signal peptide enhances nuclear export of full length viral RNAs and increases viral particle production by acting at a posttranslational step of the replication cycle (Caporale et al., 2009).

Another example is the signal peptide of prolactin (SPPrl) which is cleaved within its membrane-spanning h-region. An N-terminal fragment initially accumulates in the membrane but is liberated with time from the membrane into the cytosol (Lyko et al., 1995). In the cytosol, the SP_{Prl}-fragment was found to be associated with calmodulin in a Ca^{2+}-dependent manner, suggesting that it has a regulatory function (Martoglio et al., 1995).

Taking the results of this work into account, it is reasonable to speculate that the cleaved signal peptide of the PAR1 is also released from the ER membrane (Figure 33). It may translocate to the cytosol or may enter the nucleus in a similar way as described for the Rem protein to fulfill its functions. Alternatively, the released signal

peptide may have an indirect effect on other proteins in the cytosol or nucleus (Figure 33).

Specific analytical experiments are needed to verify the release of the peptide from the ER membrane and to further investigate its fate and biological and physiological roles.

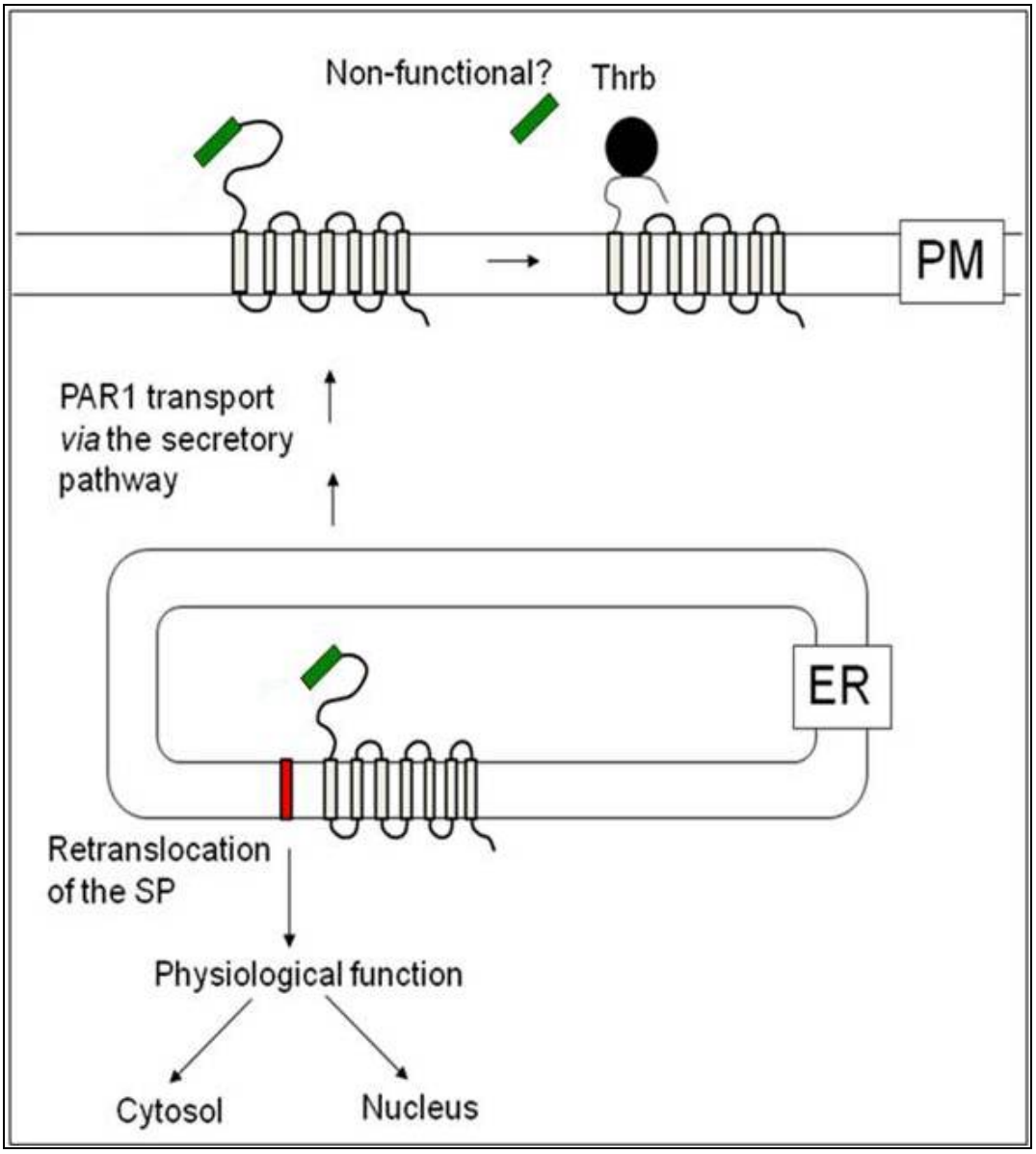

Figure 33. Model of the cellular fate of the N-terminal sequence of PAR1

The signal peptide of PAR1 is cleaved following insertion of the receptor into the ER membrane (Met^{1} to Ala^{26}, red box). From there, it may be retranslocated into the cytosol to fulfill its functions e.g. in the nucleus or in the cytosol. The remaining part of parstatin (green box) is released at the extracellular side by thrombin cleavage. This peptide may be non-functional.

SUMMARY

The heptahelical G protein-coupled receptors (GPCRs) represent a huge protein family and are the most important drug targets in pharmacology. A subfamily of GPCRs forms the protease-activated receptors (PARs) which are activated by N terminus proteolysis. To this subfamily belongs the protease-activated receptor 1 (PAR1) which is activated by cleavage of its N terminus by thrombin. Proteolysis in turn uncovers a tethered ligand transactivating the receptor.

Thrombin cleaves between amino acid residues Arg^{41} and Ser^{42} of PAR1 and releases a peptide into the extracellular space, namely parstatin, which was recently shown to have a regulatory function in angiogenesis and in myocardial ischemia and reperfusion injury. It was previously thought that this extracellular parstatin peptide indeed contains residues Met^{1}-Arg^{41}.

However, the actual length of the parstatin peptide is unclear since the receptor also possesses a putative signal peptide (Met^{1}-Ser^{21}) at its N terminus according to prediction programs. Signal peptides mediate the targeting of nascent chains to the membrane of the endoplasmic reticulum (ER), the first step of the intracellular protein transport. They are normally cleaved off following integration of a protein into the ER membrane (membrane protein) or translocation across it (secretory proteins). Thus, if the putative signal peptide of the PAR1 is functional, parstatin released at the extracellular side by thrombin cleavage would be shorter and only contain residues Ala^{22}-Arg^{41}.

In this work it was studied, whether the signal peptide of PAR1 is indeed functional and cleaved addressing the question of actual parstatin length. By fusing the N terminus of PAR1 to the cytosolic green fluorescent protein (GFP), the GFP moiety was converted to a secretory protein demonstrating that the PAR1 indeed possesses a signal peptide which is able to mediate ER targeting and which is cleaved off in the early secretory pathway. These results were confirmed by experiments using a full length receptor construct possessing a FLAG tag preceding the signal

peptide: in this case, the FLAG tag was removed from the full length receptor together with the signal peptide.

In summary, it was shown that extracellularly-released parstatin contains only residues Ala^{22}-Arg^{41} and not residues Met^{1}-Arg^{41}. Since it is aimed to use parstatin to treat various pathological situations, these results are significant for the future research on this peptide.

During this study, a signal peptide deletion mutant of the PAR1 was constructed. Previous studies with other GPCRs have shown that when a signal peptide is deleted, the first hydrophobic domains of the receptors are able to take over signalling functions for ER targeting/insertion as so called signal anchor sequences.

The signal peptide mutants are thus normally expressed, also sometimes in reduced amounts. In the case of the PAR1, however, expression of the signal peptide mutant was almost completely abolished. The mechanism underlying this phenomenon was addressed and it could be shown that the first transmembrane domain of the PAR1 is unexpectedly unable to function as a signal anchor sequence. However, the impaired expression of the signal peptide mutant of the PAR1 seems to rely on an even earlier step of protein biogenesis.

Experiments addressing PAR1 mRNA degradation indicate that the sequence encoding the signal peptide is necessary for a stable mRNA by forming a stem loop secondary structure, a hypothesis which could be confirmed by bioinformatic analyses. Such a function was never described before for a GPCR.

ZUSAMENFASSUNG

G-Protein-gekoppelte Rezeptoren (GPCR) bilden eine sehr große Proteinfamilie und sind in der Pharmakologie die wichtigsten Zielmoleküle für Wirkstoffe. Die Protease-aktivierten Rezeptoren stellen eine Unterfamilie der GPCR dar, die durch N-terminale Proteolyse aktiviert werden. Zu dieser Gruppe gehört der Protease-aktivierte Rezeptor 1 (PAR1), dessen N-Terminus durch Thrombin gespalten wird.

Dadurch wird ein Ligand freigegeben, der Teil des reifen Rezeptors ist und diesen transaktiviert. Thrombin spaltet den PAR1 zwischen den Aminosäureresten Arg^{41} und Ser^{42} und setzt dadurch auch ein extrazelluläres Peptid frei, das Parstatin genannt wurde. Eine Vielzahl von Arbeiten schreibt diesem Peptid regulatorische Funktionen bei der Angiogenese und bei der Antwort auf einen Myokardinfarkt zu. Da Thrombin zwischen Arg^{41} und Ser^{42} spaltet, wurde bisher davon ausgegangen, dass das extrazellulär freigesetzte Parstatin die Sequenz von Met^{1} bis Arg^{41} umfasst.

Diese Parstatinlänge ist allerdings in Frage zu stellen, da der PAR1 nach bioinformatischen Analysen an seinem N-Terminus auch über ein abspaltbares Signalpeptid verfügen könnte (Met^{1}-Ser^{21}). Signalpeptide vermitteln den Transport neuentstehender Proteine zur Membran des endoplasmatischen Retikulums (ER), d.h. den ersten Schritt des intrazellulären Proteintransports. Nachdem ein Protein in die ER-Membran integriert (Membranproteine) oder über diese transloziert wurde (sekretorische Proteine), werden Signalpeptide normalerweise rasch abgespalten.

Wenn das putative Signalpeptid des PAR1 tatsächlich funktionell wäre, würde dies aber bedeuten, dass extrazellulär freigesetztes Parstatin kürzer wäre als bisher angenommen und nur die Aminosäurereste Ala^{22}-Arg^{41} umfassen würde.

In dieser Arbeit wurde die Frage nach der tatsächlichen Parstatinlänge aufgegriffen und untersucht, ob der PAR1 tatsächlich über ein funktionelles und abspaltbares Signalpeptid verfügt. Hierfür wurde der N-Terminus des Rezeptors mit dem cytosolischen grünfluoreszierenden Protein (GFP) fusioniert. Es konnte mit verschiedenen Experimenten gezeigt werden, dass dadurch das GFP-Protein in ein

sekretorisches Protein umgewandelt wird. Dies beweist, dass der PAR1 tatsächlich ein Signalpeptid enthält, dass das ER-Targeting vermittelt und dann abgespalten wird. Diese Daten wurden durch Experimente mit vollständigen Rezeptoren bestätigt, bei denen ein FLAG-Tag vor dem Signalpeptid fusioniert wurde. In diesem Fall wurde der FLAG-Tag zusammen mit dem Signalpeptid vom Rezeptor abgespalten. Zusammengefasst kann daher festgehalten werden, dass extrazellulär freigesetztes Parstatin nur die Aminosäurereste Ala^{22}-Arg^{41} umfasst.

Da es Planungen gibt, Parstatin auch zu therapeutischen Zwecken einzusetzen, sind diese Ergebnisse für die zukünftige Forschung an diesem Peptid von Bedeutung.

Im Verlauf dieser Arbeit wurde auch eine Signalpeptid-Deletionsmutante des PAR1 konstruiert. Für andere GPCR konnte gezeigt werden, dass die Expression solcher Deletionsmutanten normalerweise nur etwas vermindert ist, da die erste Transmembrandomäne des reifen Proteins die Signalisierung an der ER-Membran als sogenannte Signalankersequenz übernehmen kann. Im Falle des PAR1 wurde die Rezeptorexpression durch diese Deletion dagegen fast vollständig unterbunden.

Es wurde daher noch untersucht, welcher Mechanismus diesem Phänomen zugrunde liegt. Es konnte gezeigt werden, dass die erste Transmembrandomäne des Proteins nicht als Signalankersequenz funktionieren kann. Der tatsächliche Grund für die Verhinderung der PAR1-Expression scheint aber auf einer noch früheren Stufe der Biogenese des Rezeptors zu liegen:

Es konnte gezeigt werden, dass die Sequenz, die das Signalpeptid des PAR1 kodiert, für die Ausbildung einer stabilen mRNA notwendig ist, indem sie die Ausbildung eines Stem-Loops ermöglicht. Eine solche Funktion wurde bisher für einen GPCR nicht beschrieben.

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COOPERATIONS, PUBLICATIONS AND PRESENTATIONS

1. Cooperations

1. **Associate Professor Nikolaos Tsopanoglou, PhD**

Department of Pharmacology, University of Patras

University Campus Rio

26500, Rio-Patras, Greece

2. **Dr. Stefan Kubick**

Group Manager "Cell-free Protein Synthesis"

Fraunhofer Institut für Biomedizinische Technik IBMT

Potsdam-Golm, Germany

2. Publications

Zampatis D.E, Rutz C, Schmidt A, Furkert J, Tsopanoglou N and Schülein R, submitted for publication, (2012). "The protease-activated receptor 1 possesses a functional and cleaved signal peptide which is essential for receptor's expression". FEBS Letters. Jul 30;**586**(16):2351-9

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Zampatis D.E, Tsopanoglou N. (2011). "Parstatin, a novel PAR1-derived peptide, with biological functions". Cell News Journal, Volume **37**, 1/2011:39-41

3. Abstracts, Oral and Poster Presentations

''Biological role and significance of the PAR1 signal peptide''

51th annual meeting of the American Society of Cell Biology,

December 2011 Denver, USA

"Biological function and role of the PAR1 signal peptide"

12th Congress "Medicinal Chemistry: Drug development and design"

April 2011, Patras, Greece

"**The protease activated receptor 1 possesses a functional and cleaved signal peptide essential for receptor's biosynthesis**''

34th Annual meeting of the German Society for Cell Biology

April 2011, Bonn, Germany

"**The PAR1 receptor possesses a functional signal peptide essential for receptor biosynthesis''**

50th Annual meeting of the American Society of Cell Biology

December 2010, Philadelphia, USA

"Biochemical analysis of the cleaved N terminus of the PAR1 receptor"

16th World Congress of Basic and Clinical Pharmacology (WorldPharma 2010)

July 2010, Copenhagen, Denmark

"Functional analysis of the putative cleaved peptide of the PAR1 receptor"

35th Congress of the Federation of the European Biochemical Societies (FEBS)

June 2010, Gothenburg, Sweden

"Functional analysis of the hydrophobic N-terminal extracellular domain of the PAR1 receptor''

60th Congress of the Greek Society of Molecular Biology and Biochemistry November 2009, Athens, Greece

"**Biochemical analysis of the cleaved-off peptide of the N-terminal of the active PAR1 receptor''**

10th Congress "Medicinal chemistry: Drug development and design"

March 2009, Patras, Greece

CURRICULUM VITAE

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