Production of recombinant human endothelin B receptor in different hosts and its subsequent solubilization and purification

Dissertation
zur Erlangung des Doktorgrades der Naturwissenschaften

Vorgelegt beim Fachbereich
Chemische und Pharmazeutische Wissenschaften
der Johann Wolfgang Goethe – Universität
In Frankfurt am Main

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Frankfurt am Main, 2003.
(DF1)

Vom Fachbereich Chemische und Pharmazeutische Wissenschaften der Johann Wolfgang Goethe – Universität als Dissertation angenommen.

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Datum der Disputation: 1. März 2004.

For my parents

The lure of the distant
and the difficult is
deceptive.
The great opportunity is
where you are.

J. B. 1837 -1921

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Summary

The endothelin B receptor belongs to the rhodopsin-like G-protein coupled receptors family. It plays an important role in vasodilatation and is found in the membranes of the endothelial cells enveloping blood vessels.

During the course of this work, the production of recombinant human ET_B receptor in yeast, insect and mammalian cells was evaluated.

A number of different receptor constructs for production in the yeast *P. pastoris* was prepared. Various affinity tags were appended to the receptor N-and C-termini to enable receptor detection and purification. The clone pPIC9KFlagHisET_BBio, with an expression level of 60 pmol/mg, yielded the highest amount of active receptor (1.2 mg of receptor per liter of shaking culture). The expression level of the same clone in fermentor culture was 17 pmol/mg, and from a 10L fermentor it was possible to obtain 3 kg of cells that contained 20-39 mg of the receptor.

For receptor production in insect cells, Sf9 (*S. frugiperda*) suspension cells were infected with the recombinant baculovirus pVlMelFlagHisET_BBio. The peak of receptor production was reached at 66 h post infection, and radioligand binding assays on insect cell membranes showed 30 pmoL of active receptor /mg of membrane protein. Subsequently, the efficiency of different detergents in solubilizing the active receptor was evaluated. N-dodecyl-β-D-maltoside (LM), lauryl-sucrose and digitonine/cholate performed best, and LM was chosen for further work.

The ET_B receptor was produced in mammalian cells using the Semliki Forest Virus expression system. Radioligand binding assays on membranes from CHO cells infected with the recombinant virus pSFV3CAPET_BHis showed 7 pmol of active receptor /mg of membrane protein. Since the receptor yield from mammalian cells was much lower than in yeast and insect cells, this system was not used for further large-scale receptor production.

After production in yeast and insect cells, the ET_B receptor was saturated with its ligand, endothelin-1, in order to stabilize its native form. The receptor was subsequently solubilized with n-dodecyl-β-D-maltoside and subjected to purification on various affinity matrices. Two-step affinity purification via Ni^{2+}-NTA and monomeric avidin proved the most efficient way to purify milligram amounts of the receptor. The purity of the receptor preparation after this procedure was over 95%, as judged from silver stained gels. However, the tendency of the ET_B receptor produced in yeast to form aggregates was a constant problem. Attempts were

made to stabilize the active, monomeric form of the receptor by testing a variety of different buffer conditions, but further efforts in this direction will be necessary in order to solve the aggregation problem.

In contrast to preparations from yeast, the purification of the ET_B receptor produced in insect cells yielded homogeneous receptor preparations, as shown by gel filtration analysis.

This work has demonstrated that the amounts of receptor expressed in yeast and insect cells and the final yield of receptor, isolated by purification, represent a good basis for beginning 3D and continuing 2D crystallization trials.

1 Introduction

1.1 Membrane receptors

The cell is the smallest functional unit of the organism, a closed microsystem capable of maintaining its own homeostasis. It can also function as a part of a larger system – the multicellular organism - and must do so perfectly in accord with the neighbouring cells. A crucial factor making that possible is the communication between the cell and its surroundings. Cells in higher animals communicate by means of hundreds of kinds of signalling molecules, ranging from small peptides and proteins to amino acids, nucleotides, steroids, fatty acid derivatives, even dissolved gasses like nitric oxide. Some of those molecules, being small and hydrophobic, enter the target cell directly by diffusing through the lipid bilayer of the plasma membrane. They then activate intracellular receptor proteins, which directly regulate the transcription of specific genes. But the majority of extracellular signalling molecules are hydrophilic and therefore not able to enter the cell by diffusion. Instead, these molecules initiate the signalling cascade via binding to and activating plasma membrane receptors. Plasma membrane receptors are integral membrane proteins capable of binding a messenger molecule on their extracellular side and transducing the signal to the cell interior.

There are three distinctive types of membrane receptors, classified according to their structure and the type of transducing mechanism:

- transmitter-gated ion channels
- enzyme-linked receptors
- G protein-coupled receptors (GPCRs)

Transmitter-gated ion channels are ionotropic receptors involved in rapid synaptic signalling between electrically excitable cells. This type of signalling is mediated by certain neurotransmitters which transiently open or close the ion channel formed by the protein they bind to, thus briefly changing the permeability of the plasma membrane. Nicotinic acetylcholine receptors, ATP P_2X receptors and NMDA receptors are some examples of this receptor type.

Enzyme-linked receptors represent the class of single transmembrane helix (TMH) receptors, which upon activation function either directly as enzymes or activate the enzyme to which they bind. Their ligand-binding site is situated on the extracellular side of the plasma membrane, and their catalytic or enzyme-binding site, inside the cell. The majority are protein

kinases, *i.e.* when activated, they cause the phosphorylation of target proteins. Receptors belonging to this class mostly form dimers in their active state. Characteristic examples are some cytokine and the growth factor hormone receptors.

GPCRs are a highly diverse group of 7TMH receptors, accounting for 80% of the total membrane receptor gene population. They indirectly, via intracellular G-proteins, regulate the activity of a specific set of target proteins (ion channels and enzymes). Activated ion channels change membrane permeability for ions. If the activated protein is an enzyme, it changes the concentration of one or more intracellular mediators, continuing the signalling cascade and enabling the cell to respond to the external signal.

1.2 Portrait of a GPCR

The largest and pharmacologically most interesting group of membrane receptors is the G protein-coupled receptor (GPCR) superfamily. Genes encoding GPCRs comprise 1-5% of the vertebrate genome (~1% of the human genome). As to their pharmacological importance – GPCRs have emerged as the most prominent class of drug targets for the pharmaceutical industry. It is estimated that around 50% of currently marketed drugs represent modulators of GPCR function (Drews, 2000).

The GPCR superfamily members share two common characteristics:

- a structural pattern consisting of seven membrane-spanning helices
- the ability to couple to the intracellular G proteins upon activation (hence the name)

GPCR superfamily comprises five classes of receptors, classified according to their structural homology (Kolakowski, 1994):

Receptor Family Prototype	Code	Group Members
β2-adrenergic receptor	A	Olfactory and adenosine receptors Biogenic amine receptors Peptide receptors Vertebrate photopigments Invertebrate photopigments Paracrine/autocrine receptors

Receptor Family Prototype	Code	Group Members
Calcitonin receptor	B	Calcitonin-like receptors PTH/PTHrP receptors Secretin-like receptors
Metabotropic glutamate receptor	C	Other metabotropic glutamate receptors
Fungal pheromone receptors	D	STE2 STE3
D. discoideum CAMP receptors	E	Other cAMP receptors

TABLE 1 ***The GPCR receptor superfamily*** (*modified after Kolakowski, 1994*)

Because of their overall importance, research efforts to elucidate GPCR three-dimensional (3D) structures have a long history. The structural analysis of bacteriorhodopsin 2D crystals by electron microscopy and 3D crystals by X-ray crystallography yielded the first reliable insights into the 3D structure of a protein with 7 transmembrane helices (Henderson and Unwin, 1975; Henderson and Baldwin, 1990). Until relatively recently, when the 3D structure of the phototransducer rhodopsin was solved (Palczewski *et al.*, 2000), GPCR structural models were based on the structure of bacteriorhodopsin (which is not a GPCR itself, but shares the seven transmembrane helical motif) and the 2D structure of bovine rhodopsin (Schertler *et al.,* 1993). Recently published 3D structure of bovine rhodopsin based on electron cryo-microscopy (Krebs *et al.,* 2003) provides complementary information to the rhodopsin atomic structure by Palczewski *et al*. and yet more data for reliable GPCR structure modelling.

Rhodopsin is unique among GPCRs for having its ligand, 11-cis-retinal, covalently attached and activated by photons. The X-ray structure of bovine rhodopsin by Palczewski *et al*. was the first, and remains the only, high-resolution structure of any GPCR to date. Owing to the

relatively high resolution of 2.8 Å, it represents a valid basis for structure prediction of the other GPCR superfamily members and for elucidating the mechanism of receptor function.

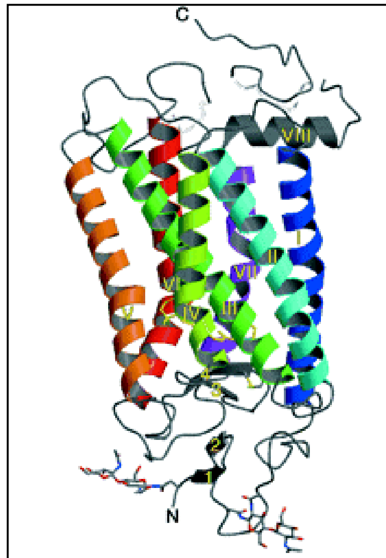

Fig 1.1 ***3D structure of rhodopsin at 2.8 Å resolution.*** *(From Palczewski et al., 2000)*

1.2.1 GPCR mechanism of action

From a functional point of view, all the GPCR superfamily members represent (function as) transmembrane transducer systems, transferring an extracellular signal across the cell membrane and enabling the cell to respond to a broad range of stimuli. Upon ligand binding on the extracellular side, the receptor undergoes a conformational change.

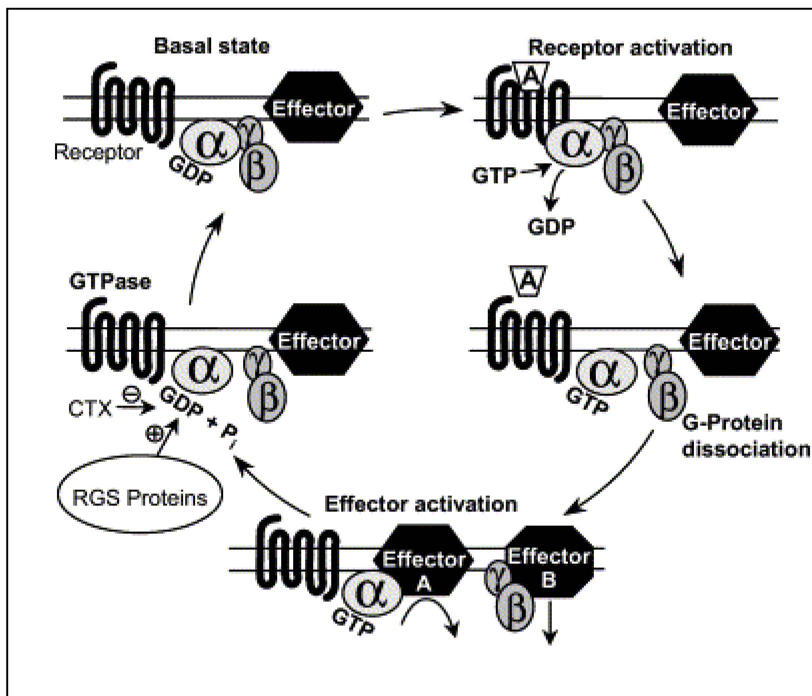

Fig 1.2
G-protein cycle
(*From Offermans, 2003*)

RGS protein = regulator of G-protein signalling, which accelerates G_α GTPase activity. CTX = cholera toxin, which blocks GTPase activity of G_α subunit. Further explanations in the text.

If the ligand is an agonist, this conformational change is followed by coupling with heterotrimeric GDP-bound G-protein on the cytoplasmic side of the receptor, causing the dissociation of the G-protein complex into α and βγ subunits. These subunits further activate networks of various effectors (Fig 1.2).
It should be noted that certain GPCRs exhibit constitutive activity under physiological conditions, *i.e.* can activate G proteins in the absence of ligand.

G-proteins are trimeric GTP-binding proteins, consisting of an α, β and γ subunit. The α subunit is a GTPase and functions as a molecular switch that can flip between two states: active, when GTP is bound, and inactive, when GDP is bound. Activated receptors switch on G-proteins by associating with them and causing them to eject the GDP bound on the G_α, replace it with GTP and dissociate to Gα and βγ subunits. The switch is turned off when the G_α subunit hydrolyses bound GTP, converting it back to GDP, and reunites with the βγ subunit, reconstituting an inactive G-protein trimer (Fig 1.2). Before that occurs, the active subunits have had enough time to diffuse away from the receptor and deliver the message to the downstream targets.

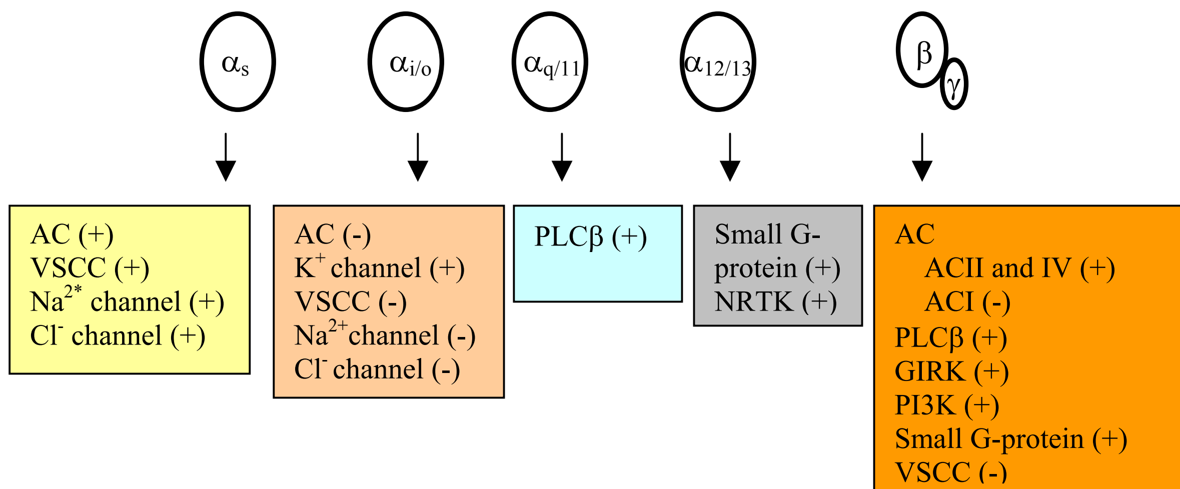

Fig 1.3 ***Effector molecules regulated by G protein family.***
AC = adenylyl-cyclase, VSCC = voltage-sensitive calcium channel, PLC = phospholipase, NRTK = non-receptor tyrosine kinase, GIRK = inward rectifier G-protein gated potassium channels, PI3K = phospatidylinositol-3-kinase. (+) indicates activation, (-) indicates inhibition

In humans, there are 21 different Gα subunits, 5 β subunits and 11 different γ subunits (rev. in Hepler and Gilman, 1992; Hamm and Gilchrist, 1996). Depending on the molecular nature of the α subunit, G proteins are generally classified into four different families: $G_{\alpha s}$ (olf) (Itoh, 1986; Harris, 1988), $G_{\alpha i}$ (Itoh, 1986), $G_{\alpha q}$ (Exton, 1996) and $G_{\alpha 12(13)}$ (Strathmann and Simon, 1990). They activate different sets of effectors, as shown in Fig 1.3. "Stimulatory" G_{α} proteins (*i.e.* G_s) activate the enzyme adenylate cyclase (AC), resulting in an increased level of cyclic AMP (cAMP) in the cell, and "inhibitory" G_{α} proteins (*i.e.* G_i) act as AC inhibitors and lower intracellular cAMP levels. Another class of G_{α} proteins, G_q , activates the enzyme phospholipase C-β, which triggers the events leading to an increase in cytosolic Ca^{2+} concentration. Alteration of cAMP and Ca^{2+} intracellular concentrations leads to activation of different sets of kinases, which further phosphorylate specific target proteins, altering their activity and enabling the cell to respond to a myriad of external stimuli.

Most GPCRs are able to couple to more than one kind of G protein, which provides diversity of signalling even with stimulation of only one particular receptor type.

In addition to G-proteins, GPCRs interact with a number of different proteins that either couple GPCRs to G-protein independent signal transduction pathways or alter G protein specificity and agonist selectivity. These proteins include arrestins, G protein-coupled receptor kinases (GRKs), calmodulin, the Homer protein, SH3 domain-containing molecules and small G-proteins (reviewed in Ferguson, 2001).

It should also be noted that, in contrast to the large number of different GPCRs, the number of identified effectors they indirectly regulate is considerably smaller. This convergence leads to cross-regulation in signalling pathways, yielding yet more diverse physiological responses.

Termination of GPCR signalling initiated by ligand binding is called receptor desensitization, and it represents an important physiological mechanism that protects the cell from both acute and chronic receptor overstimulation. In the course of the desensitization process, uncoupling of the GPCR from G-proteins occurs as a consequence of receptor phosphorylation by intracellular kinases. These G-protein receptor kinases (GRKs) selectively phosphorylate agonist-activated receptors, promoting the binding of β-arrestins to the receptor, which sterically uncouple the receptor from the G protein and target the receptor for endocytosis and subsequent degradation or recycling (Lohse, 1993; Ferguson *et al*., 1996).

1.2.2 Compartmentalization & dimerization of GPCRs

The classical view of the GPCR signalling process is based on random collisions between the proteins that freely diffuse in the plasma membrane, where the specificity of interaction is entirely dependent on the 3D structure of the respective molecules and their matching recognition surfaces. However, recent evidence suggests that the whole system is less mobile than previously predicted, and is organized in distinct microdomains in the plasma membrane, namely calveolae and lipid rafts (Neubig *et al.*, 1994; Ostrom *et al.*, 2000). It has been suggested that caveolae localization represents a mechanism for amplifying extracellular signals by increasing the proximity, and therefore coupling, of receptors and effectors (Lisanti *et al.*, 1994).

Another newly emerging functional aspect of GPCR signalling is dimerization. Although this concept is well known and represents a necessary prerequisite for the activation of enzyme-linked receptors, the conventional assumption in the case of the GPCR superfamily was that monomeric receptors act allosterically with a single heterotrimeric G-protein. But recent evidence for the homodimerization of opioid receptors (Jordan and Devi, 1999), β2 adrenergic receptors (Hebert *et al.,* 1998), vasopressin receptors (Zhu and Wess, 1998) and bradykinin receptors (AbdAlla *et al.,* 2000) suggests that the classical model of GPCR modes of action requires alteration. Additionally, there is ample evidence of heterodimerization of GPCRs, both between different subtypes of the same receptor, as in the case of GABA receptors (Marshal *et al*., 1999; White *et al*., 1998), opioid receptors (Gomes *et al*., 2002) and muscarinic receptors (Zeng and Wess, 2000), and also between different receptor types, as in the case of dopamine and somatostatin receptors (Rocheville *et al*., 2000) and adrenergic and opioid receptors (Jordan *et al.*, 2001), suggesting that this process may be important for GPCR biogenesis, activation and function (reviewed in *e.g.* Rios *et al*., 2001; Bouvier, 2002). In the case of receptor heterodimerization, the generation of sites with novel ligand-binding properties has been reported (Maggio *et al.,* 1999; Jordan and Devi, 1999).

1.3 Endothelin signalling system

In 1988, Yanagisawa *et al*. discovered an active substance in the endothelial cell homogenate, which showed a strong vasoconstrictive effect. The active substance was a 21 amino acid long

peptide, which they named "endothelin". Yanagisawa *et al.* had actually isolated the most abundant peptide isoform of endothelin in humans, ET-1. Since then, two other isoforms of the peptide, termed ET-2 and ET-3, have been found in humans, each coded by a different gene (Inoue *et al*, 1989). Each isoform is first expressed as a precursor protein, the 200 amino acid prepro-endothelin, which is then cleaved by a peptidase to yield "big", 39 amino acid long ET (Fig 1.4). "Big" ET is converted to the mature peptide by the metalloproteinase endothelin-converting enzyme (ECE). Mature peptides are 21 amino acids long, have two intrachain disulfide bonds (Cys1-Cys15 and Cys3-Cys11) and a conserved C-terminal sequence needed for activity (Inoue *et al.*, 1989). The 3D crystal structure of endothelin ET-1 at 2.18 A is available (Janes *et al.*, 1994).

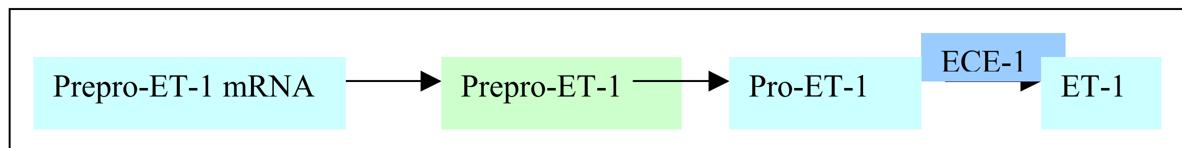

Fig 1.4 ***The synthesis of ET-1***

Endothelins are found in various mammalian tissues, with different distribution patterns. By far the most abundantly expressed is ET-1. This isoform is the only one found in vascular endothelial cells, but it is also found in non-vascular tissues, like brain, kidney, adrenal gland, intestine and myocardium (Sakurai *et al.*, 1991). ET-2 and ET-3 are found in different non-vascular tissues, with ET-3 being relatively abundant in neural tissues (Bloch *et al.*, 1989).

The biological activity of endothelins is regulated by a complex process of synthesis and breakdown. Mature endothelin is packed and transported in secretory vesicles through a cyclic AMP-independent pathway. ET-1 is also stored in Weibel-Palade bodies (with other vasoactive compounds) from which it is released on the cell surface after appropriate stimuli (*i.e.* fluid shear stress, interleukin 1, angiotensin II, TGF β - Russel *et al.*, 1998).

ET-1 is secreted from vascular endothelial cells to the immediate surrounding, provoking directly the contraction of vascular smooth muscles, so its plasma concentration is usually very low – in the low picomolar range.

Endothelin is mainly metabolized by a neutral endopeptidase, a membrane bound enzyme in the proximal tubules of kidney (Abassi *et al.*, 1993), which is widely involved in the degradation of peptide hormones. Endothelin could be also cleared by binding to the ET_B receptor, which then internalizes and is targeted to lysozomes for degradation.

ET-1	Cys	Ser	Cys	Ser	Ser	Leu	Met	Asp	Lys	Glu	Cys	Val	Tyr	Phe	Cys	His	Leu	Asp	Ile	Ile	Trp
ET-2	Cys	Ser	Cys	Ser	Ser	Trp	Leu	Asp	Lys	Glu	Cys	Val	Tyr	Phe	Cys	His	Leu	Asp	Ile	Ile	Trp
ET-3	Cys	Thr	Cys	Phe	Thr	Tyr	Lys	Asp	Lys	Glu	Cys	Val	Tyr	Tyr	Cys	His	Leu	Asp	Ile	Ile	Trp
SRT-6a1	Cys	Ser	Cys	Lys	Asp	Met	Thr	Asp	Lys	Glu	Cys	Leu	Asn	Phe	Cys	His	Gln	Asp	Val	Ile	Trp
SRT-6b	Cys	Ser	Cys	Lys	Asp	Met	Thr	Asp	Lys	Glu	Cys	Leu	Tyr	Phe	Cys	His	Gln	Asp	Val	Ile	Trp

Fig 1.5 ***Endothelins and sarafotoxins - amino acid sequence comparison***

Endothelins show a striking structural similarity (Fig 1.5) with the group of extremely poisonous cardiotoxic snake venom peptides, named sarafotoxins (Kloog *et al.*, 1988). Sarafotoxins are highly lethal peptides: in mice, the LD_{50} is 15µg/kg body weight and the LD_{50} for endothelin is the same (Bdolah *et al.*, 1989), which is quite surprising for a peptide naturally occurring in the plasma of healthy humans. It seems that efficient control mechanisms exist in the human organism, which prevent the build-up of ET concentration above a certain threshold. However, elevated levels of ET-immunoreactivity have been detected in several disease states (*e.g.* hypertension, renal failure, myocardial arrhythmias and ischemia, asthma, arteriosclerosis) (Sokolovsky, 1995 and references therein).

Despite their potent and extremely long-lasting vasoconstrictor activities, endothelins can provoke vasodilatation under physiological conditions as well. Their most prominent role is in regulating blood pressure. They also elicit a wide spectrum of both vascular and non-vascular actions in a variety of tissues, including airways (bronchoconstriction), kidneys (various glomerular and tubular effects), myocardium (positive chronotropic actions), and central and peripheral nervous system (modulation of neurotransmission and release of neuroendocrine hormones) (Yanagisawa and Masaki, 1989). Such a diversity of action of endothelins can be explained in terms of the existence of more than one receptor subtype they bind to, which then activate different sets of effectors.

1.3.1 Endothelin receptors

Two subtypes of endothelin receptors, ET_A and ET_B, have been identified in humans (Arai *et al.*, 1990; Nakamuta et *al.*, 1991). A putative third type of endothelin receptor, ET_C, has been identified in *Xenopus laevis* (Kumar *et al.*, 1994), but no known homologue has been found in the human genome.

Endothelin receptors are members of the class A (rhodopsin-like) family of GPCRs, and share common structural features. They have 63% homology in primary structure, and differ in affinity towards the peptide isoforms of endothelin. ET_A has a higher binding affinity for ET-1 and ET-2 than for ET-3 (Arai *et al*., 1990), whereas the ET_B receptor binds the three isoforms with comparable affinity, *i.e.* is non-selective. The complex formed upon binding of endothelin to the receptor is known to be extremely stable and release or exchange of the ligand from the receptor is very slow under physiological conditions (Martin *et al*, 1989, Clozel *et al*, 1989). Furthermore, the ET-1 complex with ET_B survives in the presence of 1% SDS, at reduced temperature (Fischli *et al*., 1989).

TABLE 2 ***Different tissue distribution of ET receptors and pharmacological effects of endotheline*** *(Modified after Remuzzi et al., 2002)*

Tissue/organ	**Pharmacological effect of ET**	**ET receptor**
Vasculature	-transient vasodilation	ET_B
	-sustained vasoconstriction	ET_A
Heart	-increased coronary vascular resistance	ET_A
	-decreased cardiac output	ET_A
Kidney	-renal vasoconstriction	ET_A
	-increased glomerular afferent and efferent arteriolar tone	ET_A
	-natriuresis, diuresis	ET_A&ET_B
	-increased proton secretion in proximal tubule	ET_B
Lung	-pulmonary-artery constriction	ET_A
	-contraction/proliferation of airway smooth muscle	ET_B
	-mucous gland hypersecretion	ET_A&ET_B
	-potentiation of cholinergic neuronal function	ET_B
Brain	-constriction of cerebral arteries	ET_A
	-activation of Na/K/Cl transporter function on brain capillary endothelial cells	ET_A&ET_B
	-inhibition of generation of second messengers by neurotransmitter catecholamines	ET_B
Parathyroid gland	-increased proliferation of parathyroid cells	ET_A&ET_B

Both subtypes of endothelin receptor possess unusually long N-terminal domains compared to other rhodopsin-like GPCRs. It has been shown that the initial N-terminal 26 residues function as an internal signal sequence necessary for membrane insertion, which is cleaved during translocation (Saito *et al*., 1991). ET_A receptors reside in vascular smooth muscle cells and generally mediate vasoconstriction and cell proliferation (Davenport *et al*. 2002), whereas ET_B receptors are found on endothelial cells and are mainly vasodilatory, inducing NO release upon stimulation (Verhaar *et al*., 1998). ET_B receptors are also clearance receptors for ET from circulation (Fukuroda *et al*., 1994).
The distribution of ET receptors in target organs for the pharmacological effects of endothelin is summarized in Table 2.

1.3.2 ET_A and ET_B mode of action

ET receptor subtypes exhibit different modes of action as a result of the activation of separate effector networks. As depicted in Fig 1.6, the ET_A and ET_B receptors both activate the G_q pathway, which stimulates phosphatidylinositol phosphate (PIP) turnover and increases intracellular Ca^{2+} concentrations. The receptors differ though in their effects on the adenylate-cyclase-dependent pathway: ET_A activates G_s to increase cAMP formation, whereas ET_B attenuates forskolin-stimulated cAMP synthesis via G_i (Aramori and Nakanishi, 1992). Both ET_A and ET_B stimulate arachnidonic acid release from PIP, probably via phospholipase A_2 activation, and potently enhance the mitogenic activity of many cell types (Janakidevi *et al*., 1992; Takuwa *et al*., 1989). Whether these effects are indirectly produced by the increase in Ca^{2+} concentration and/or by modulation of cAMP formation, or whether they are caused by direct activation of effector enzymes is presently unknown (Cramer *et al*., 2001).

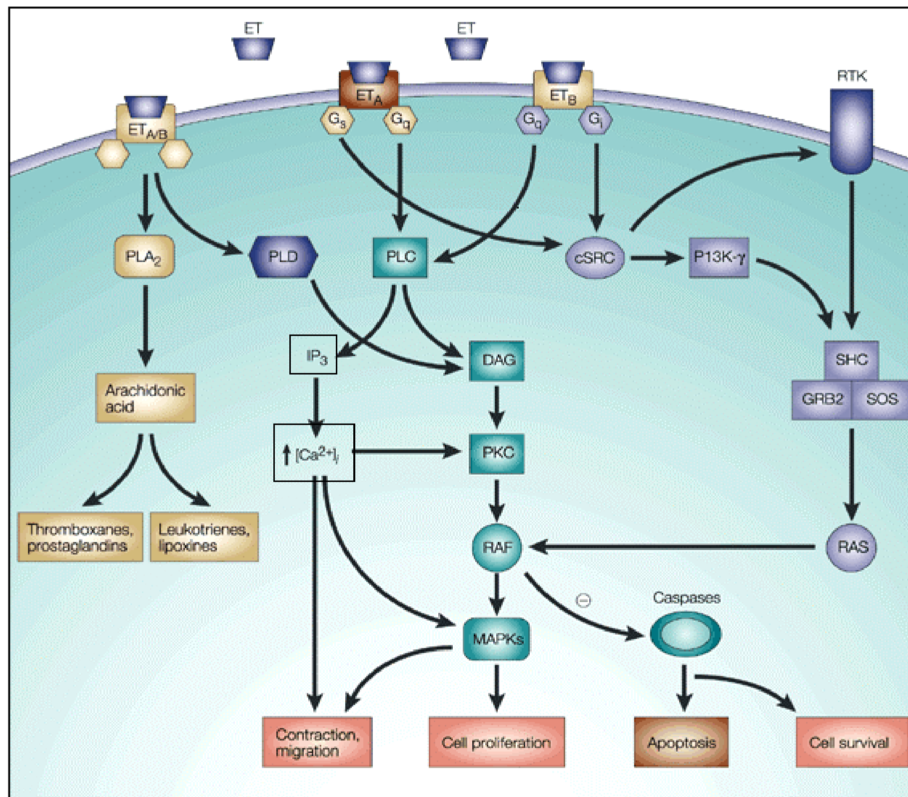

Fig 1.6 ***ET_A and ET_B mode of action.*** IP_3 = inositol triphosphate, PLC = phospholipase C, DAG = diacylglycerol, PKC = protein kinase C, MAPK = mitogen-activated protein kinase, RTK = receptor tyrosine kinase, PLA2 = phospholipase A2, PI3K = phosphatidylinositol 3-kinase, PLD = phospholipase D (after *Remuzzi et al*., 2002)

1.3.3 The ET_B receptor

The protein chain of the human ET_B receptor consists of 442 amino acids (Sakamoto *et al.,* 1991). It has a rather long extracellular N-terminal domain (Fig 1.7) where the first 26 residues represent the cleavable signal sequence (14 of which are hydrophobic). On the extracellular side, one putative N-glycosylation site is found, Asn59. The receptor has seven transmembrane segments, 21-27 amino acids long, three extracellular- and three intracellular loops, and an intracellular C-terminus. The C-terminus has several phosphorylation sites, where either G-protein receptor kinase (GRK) or protein kinase C (PKC) catalyses receptor phosphorylation. Cys402, Cys403 and Cys405 on the C-terminus are potential palmitoylation sites. Between extracellular Cys174 and Cys255 a disulfide bond exists, which is a common characteristic of rhodopsin-like GPCRs (Dohlman *et al*., 1990).

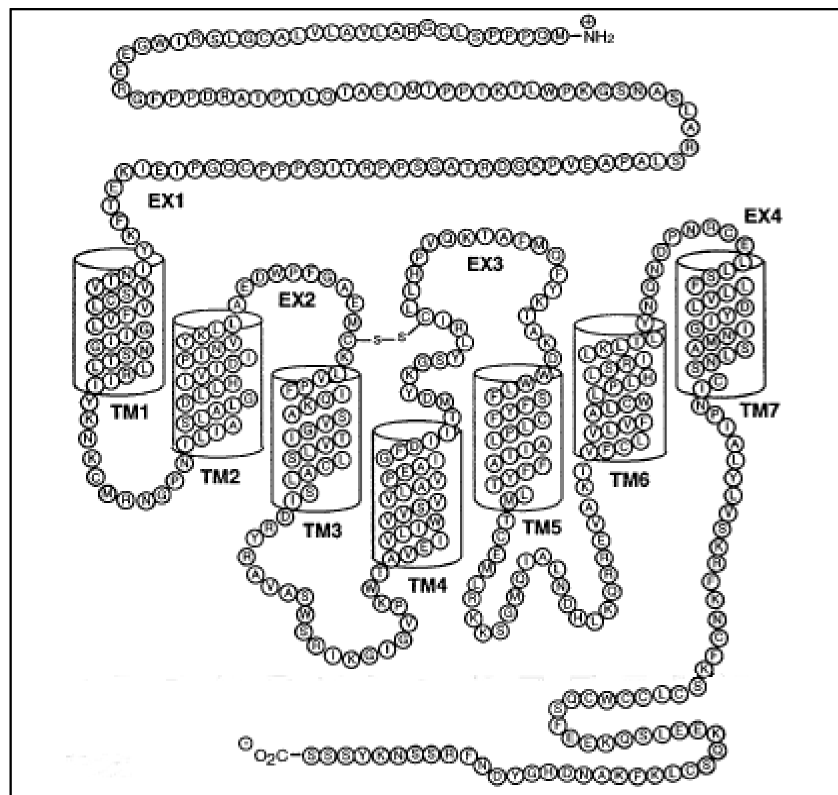

Fig 1.7 ***Schematic representation of the ET$_B$ receptor***

1.3.3.1 Posttranslational modifications of the ET$_B$ receptor

1) Glycosylation – the human ET$_B$ receptor contains a consensus glycosylation site at Asn59, and there is evidence that the receptor is glycosylated when expressed in insect and mammalian cells (Doi *et al.,* 1997). Substitution of Asn59 with alanine causes no change in receptor expression and ligand binding capacity in Sf9 cells (Doi *et al.,* 1997).
2) Phosphorylation – the ET$_B$ receptor is rapidly phosphorylated on the C-terminal serine and threonine residues upon agonist stimulation (Cramer *et al*., 1997). This process is catalyzed by GRK (Bremnes *et al*., 2000). Phosphorylated receptor binds β-arrestin and is thereby internalized and targeted to lysosymes where it is degraded.
3) Palmitoylation – covalent attachment of palmitic acid to the conserved cysteine residues in the C-terminal region of many GPCRs is a modification, which provides a hydrophobic anchor that is inserted to the membrane, thereby creating an extra loop in the cytosolic receptor portion. The ET$_B$ receptor possesses three potential

palmitoylation sites, Cys402, Cys403 and Cys405 (Okamoto et al., 1997). In the case of the ET_B receptor, this covalent modification appears to be constitutive, *i.e.* occurs without agonist stimulation (Okamoto *et al*., 1997), whereas for some other rhodopsin-like GPCRs, β_2 adrenergic receptor for example, it is caused by agonist stimulation (Moffet *et al*., 1993).

Experiments with [^{3}H] palmitic acid incorporation showed that out of three potential palmitoylation sites in ET_B, only two are palmitoylated. An unpalmitoylated triple ET_B receptor mutant, when expressed in CHO cells, appeared on the cell surface, and retained high affinities toward the ligand. However, unlike the wild-type receptor, the mutant one transmitted neither an inhibitory effect on adenylate cyclase nor a stimulatory effect on phospholipase C (Okamoto *et al*., 1997), indicating a critical role of palmitoylation in the coupling with G proteins.

A newly discovered ET_B receptor splice variant (Elshourbagy *et al*., 1996), with a substantially shorter C-terminus and lacking palmitoylation sites, retained ligand binding capacity when expressed, but lacked the ability to activate G protein. This finding is in accordance with the conclusions about the role of palmitoylation in G protein coupling, and also in accordance with the role of the splice variant as a clearance receptor for endothelin, whose further fate is to be internalized upon binding a ligand and targeted to lysosymes for degradation.

1.3.3.2 ET_B receptor ligand binding domain

Because the 3D structure of ET_B is not yet available, various chemical and molecular biological approaches have been employed to study the ligand binding domain (LBD) of the ET_B receptor. Monitoring the ligand binding activity of truncated mutants of ET_B defined the ligand binding domain as the 60 amino acid sequence between Ile138 and Ile197 (Wada *et al*., 1995). Additionally, it was found that part of the N-terminus, in close proximity to the first transmembrane helix, was required for the ligand binding activity as well. Interestingly, it seems that 12 amino acids from the C-terminus of the receptor are necessary to maintain LBD in an active form (Wada *et al*., 1995).

1.4 Different expression systems for GPCR production

Determining the three-dimensional structure of a protein requires milligram quantities of pure material. Historically, the structure determination of a protein was limited to those naturally abundant in certain tissues or cell sources. With the development of molecular cloning and gene expression techniques however, even proteins constitutively present in minute amounts can be produced in heterologous systems, purified and subjected to structural analyses.

GPCRs are proteins that are naturally expressed in very low amounts (there are exceptions to this rule, like rhodopsin, which is highly abundant and very densely packed in retinal membranes). A second problem encountered is the heterogeneity of receptor isolated from native tissue, *i.e.* the presence of different receptor subtypes. In most cases, heterologous expression of the receptor allows the analysis of a single receptor subtype in a defined system and provides the possibility of obtaining it in quantities large enough for structural studies. However, the choice of a suitable host is not an easy task – one has to bear in mind that GPCRs are membrane proteins and that they require the proper surroundings for correct folding and insertion into the membrane lipid bilayer. Furthermore, they are quite different from soluble proteins in that they are largely hydrophobic. In addition, they can bear a number of complex post-translational modifications. All of these factors must be taken into account when choosing the appropriate host for overexpression of GPCRs. Additionally, it is important to decide if the amount of the protein is what is important, in which case the inexpensive and easy-to-handle *E. coli* and yeast expression systems are preferred, or if a protein virtually identical to the one from the natural source, with all the posttranslational modifications, is needed, in which case the mammalian and insect cells are the expression systems of choice.

1.4.1 *E. coli* as a host for protein production

The extensive range of expression vectors available, detailed knowledge about its genetics and ease of manipulation render *E. coli* usually the first expression system of choice for protein overexpression.

However, heterologously produced membrane proteins are often toxic to *E. coli*, which prevents cell growth and limits yield. Misfolding, aggregation, improper transport and insertion into the membrane are further problems researchers are facing when trying to produce a eukaryotic membrane protein in *E. coli*. Milligram quantities of membrane proteins

have been obtained only after different tactics were employed, to produce them in the form of inclusion bodies. Proteins derived from inclusion bodies are mostly misfolded aggregates and have to be refolded to the native state. This has been successful for many soluble, but only for a few membrane proteins (reviewed in Grisshammer and Tate, 1995). In the case of GPCRs, much effort has been put into their production in *E. coli*, with unsatisfactory results: there are only a few reports so far of successful expression in inclusion bodies&proper refolding, yielding a GPCR capable of binding a ligand. One example is an olfactory receptor, expressed as a fusion protein with glutathione-S-transferase (Kiefer *et al.*, 1996). However, there are several reports of functionally active GPCRs expressed in *E. coli* as fusions with maltose-binding protein and inserted in the bacterial membrane: the rat neurotensin (Grisshammer *et al.*, 1993) and NK-2 receptor (Grisshammer *et al.*, 1994), the human ET_B receptor (Haendler *et al.,* 1993), and the human adenosine A receptor (Weiss and Grisshammer, 2002). Generally, the yield of GPCRs in this expression system is low (0.1 mg/L culture) and there is no possibility of *in vivo* post-translational modification.

1.4.2 Mammalian cells

Mammalian cells provide the most authentic background (most natural environment) for the expression of GPCRs. Producing mammalian protein in a mammalian expression system has the distinct advantage that the post-translational modifications are efficiently performed, resulting in a high percentage of functional protein produced in the cases where these modifications are of importance for protein function. This is the main reason that mammalian cells are the preferred expression system in the pharmaceutical industry for high throughput drug screening.

There are three different approaches to protein production in mammalian cells: transient expression, cell infection by recombinant viruses and expression in stable cell lines. However, classical transient expression and cell lines are not very convenient for GPCR expression on a large scale. Transient expression is difficult to scale up, often with the problem that only a subset of the cells is transfected. Conventional stable cell lines are rather time-consuming to establish, and stability problems occur frequently, since the gene expression cassette integrated into the host cell genome can be deleted or otherwise inactivated.

An alternative method for the transient expression of foreign proteins in mammalian cells is provided by recombinant viruses. High infection efficiency in a broad range of host cells is

the main advantage of this system. The replication-deficient alpha viruses Semliki Forest virus and Sindbis virus have been used successfully so far.

1.4.2.1 Semliki Forest virus (SFV)

Alpha viruses such as SFV are small enveloped viruses with a single-stranded RNA genome. Liljestrom&Garoff developed SFV expression vectors specifically for the purpose of producing high levels of recombinant proteins in mammalian cells (Liljestrom and Garoff, 1991). The target DNA is cloned into an SFV plasmid vector, which contains SFV non-structural genes (nsP1-4), the strong SFV 26S promoter and a multi-cloning region for introduction of foreign genes. This vector serves as a template for *in vitro* synthesis of recombinant RNA. SFV structural proteins are provided by the helper vector, containing the capsid and envelope genes needed for virus particle packaging. Therefore, RNA transcripts from both vectors have to be cotransfected into the cell by electroporation in order to obtain new virus particles (Fig 1.8). Due to the presence of an RNA packaging signal only in the recombinant RNA, no helper RNA will be packaged, which renders the generated particles replication deficient. Since this virus has a very broad host range and high infectivity, extremely stringent biosafety standards must be met. To address this issue, a system for production of conditionally infectious, replication deficient recombinant particles has been established (Berglund *et al.,* 1993). The additional safety precaution is that the new virus particles released from the cell have to be activated by a protease (α-chymotrypsin) to be rendered infectious. Activated virus particles are then used as virus stocks for infection of different kinds of mammalian host cells.

Thus far more than 50 GPCRs have been successfully expressed using this system (reviewed in Lundstrom *et al*., 2001, Lundstrom, 2003). Many of them, like the human neurokinin-1 receptor (Lundstrom *et al*., 1994), adrenergic α_1b, α_2b and β_2 (Bjorklof *et al*., 2002) and histamine receptors (Hoffmann *et al*., 2001) have been produced in rather high amounts, ranging between 50 - 150 pmol/mg. The system can potentially be scaled up for production of large quantities of recombinant receptors in mammalian suspension cell cultures, amenable to structural studies.

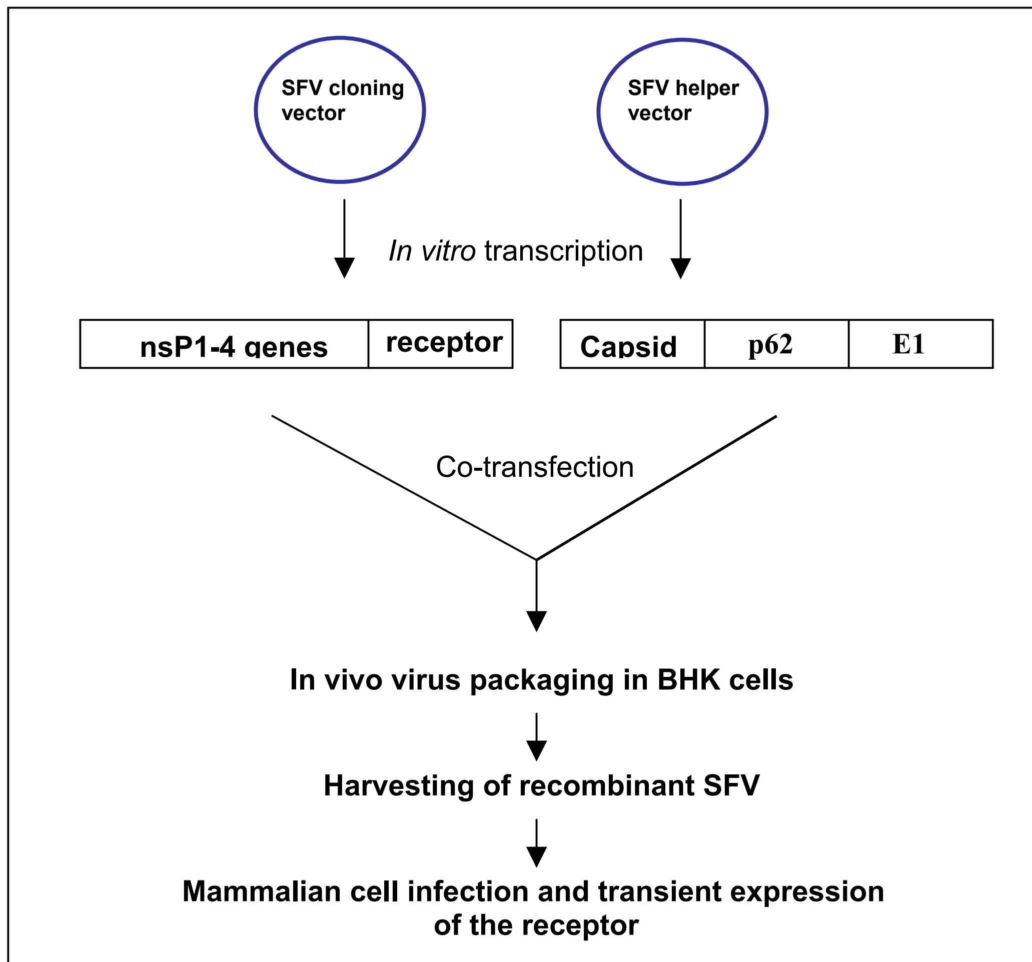

Fig 1.8 ***The SFV expression system***

1.4.3 Insect cells/baculovirus system

The well-established technology of the baculovirus/insect cell system exploits the biological properties of the *Autographica californica* nuclear polyhedrosis virus (baculovirus) that permits the homologous recombination of foreign genes into a specific locus of its genome. In most cases, the target of recombination is the polyhedrin locus, polyhedrin being a non-essential protein produced at extremely high levels in very late stages of viral infection. The very late polyhedrin promotor is the most widely used to drive heterologous protein expression. This yields recombinant viruses that can infect a variety of insect cell lines. The cell line most often used is the Sf9, an ovarian cell derivative from the butterfly *Spodoptera frugiperda.*

The main advantage of the baculovirus expression system is that it expresses GPCRs in a functional form, and sometimes at very high levels. In particular, some GPCRs have been expressed to levels of 10-100 pmol/mg. These include the β-adrenergic, serotonine, muscarinic, dopamine and chemokine receptors (reviewed in Bouvier *et al*., 1998). However, particularly high overexpression leads to the saturation of the biosynthetic pathway of the Sf9 cell, resulting in a portion of the expressed receptor being trapped in the ER or Golgi apparatus in an inactive form. To circumvent this problem, purification strategies should contain a step based on the biological activity of the receptor, such as ligand affinity chromatography, in order to obtain a homogeneous and active protein sample.

The major advantages of insect cell/baculovirus system can be summarised as follows:

- High-level production of mostly functional GPCR
- GPCRs expressed in insect cells are often poorly glycosylated, resulting in a more homogeneous protein sample
- Insect cells are capable of performing other post-translational modifications required for the correct folding and function of expressed mammalian proteins
- Insect cells (Sf9) are deficient in endogenous GPCRs (Butkerait *et al.,* 1995), providing a low-background environment for functional assays on heterologously expressed GPCRs, but they still contain a small number of G proteins

1.4.4 Yeasts

Yeasts are unicellular eukaryotes and they offer the advantage of a eukaryotic host in that they are able to perform the post-translational modifications observed in mammalian cells. In addition, they provide an inexpensive, easily scalable expression system for obtaining large amounts of protein.

However, there are several problems inherent to this specific expression host that must be noted, but are possible to overcome. On of them is that the receptors expressed often remain in internal membrane compartments instead of going to the plasma membrane, which can be circumvented by using N-terminal fusions with a leader peptide (Arkinstall *et al*., 1995; Weiss *et al*., 1995) or with the N terminus of the yeast α factor receptor STE2 (King *et al*., 1990). Proteolytic degradation of GPCRs is another problem, and can be alleviated by using protease-deficient yeast strains for expression (Sander *et al.,* 1994). It should also be noted

that the lipid composition of yeast membranes differs from that of mammalian ones, which can lead to altered ligand-binding properties for some GPCRs (Sander *et al.,* 1994).
However, recently a large number of GPCRs has been expressed successfully and in high yield in *S. pombe*, *H. polymorpha* and especially *P. pastoris* (for an extensive list, see Craig and Cereghino, 2000). This has given legitimacy to the choice of this expression system for producing GPCRs for structural studies.
Since in this work *P. pastoris* was used as a host to overexpress the endothelin B receptor, its features will be presented in more detail in the next section.

1.4.4.1 The *P. pastoris* expression system

The story of *P. pastoris* begins thirty years ago, when Koichi Ogata first described the ability of certain yeasts to utilize methanol as a source of carbon and energy (Ogata *et al*., 1969). These methylotrophs attracted immediate attention as potential sources of single-cell protein (SCP), to be used as high-protein animal feed. In the early seventies, Phillips Petroleum Company developed media and protocols for cultivating *P. pastoris* on methanol at high densities. Soon after, the idea concerning animal feed was abandoned for economic reasons, but the company contracted with the Salk institute of Biotechnology in California and continued developing the *P. pastoris* system for heterologous protein expression. The expression system has been commercially available from the Invitrogen company since 1993.
P. pastoris is methylotrophic yeast, capable of metabolizing methanol as its sole carbon source. The first step in the catabolism of methanol is the oxidation of methanol to formaldehyde. This step is catalysed by the enzyme alcohol oxidase (Douma *et al*., 1983). Since this enzyme has a poor affinity for oxygen (Km 0,1-1,0 mM) (Couderc and Baratti, 1980), the yeast cell compensates this by generating large amounts of the enzyme. Besides formaldehyde, the reaction also generates hydrogen peroxide, which is highly toxic to the cell. For this reason, oxidation takes place in the peroxisome.
Two genes in *P. pastoris* code for alcohol oxidase – *AOX1* and *AOX2*, the former accounting for 95% of the enzyme in the cell. Expression of the *AOX1* gene is tightly regulated and strongly induced by methanol (typically representing 30% or more of the total soluble protein in cells grown on methanol). For this reason, the promoter of the *AOX1* gene is used to drive heterologous protein expression in *Pichia* (Tschopp *et al*., 1987; Koutz *et al*., 1989).

Heterologous protein in *P. pastoris* can be produced either intracellularly or secreted (rev. in Cereghino and Cregg, 2000). Secretion requires the presence of a signal peptide on the protein produced in order to target it to the secretory pathway. The most widely used signal peptide for this purpose is the secretion signal peptide from the *Saccharomyces cerevisiae* α factor prepro-peptide (Cregg *et al.*, 1993).

The major advantages of the *P. pastoris* expression system:

- Using the strong AOX1 promoter, it is possible to achieve high levels of foreign protein expression – 30% of the total cell protein
- *P. pastoris* can be fermented to very high densities (up to 500 g wet weight/L), owing to its preference for respiratory growth and the fact that it is a poor fermenter (won't "pickle" itself in ethanol!)
- The cloned insert with the gene of interest is stably integrated into the yeast genome. Furthermore, it is possible to select clones containing multiple inserts of the gene
- Speed and ease of manipulation – *P. pastoris* expression vectors are designed as *E.coli/P.pastoris* "shuttle" vectors, i.e. they posses the ColE1 origin of replication, which enables their cloning and amplification in *E. coli*
- *P. pastoris* is capable of performing posttranslational modifications (glycosylation, phosphorylation, palmitoylation and disulphide bond formation)
- There is no hyperglycosylation of the expressed protein, as with S. *cerevisiae*

P. pastoris has already proven to be a suitable host for GPCR overexpression – the β_2 adrenergic receptor (Weiss *et al.,* 1998), μ opioid receptor (Talmont *et al.,* 1996) and $5HT_{5A}$ serotonine receptor (Weiss *et al.,* 1995) have already been expressed successfully in this system.

The most common problem with *P. pastoris* is proteolytic degradation of the target protein and the usage of protein deficient strains SMD1163 and SMD1168 is highly recommended. In this work, the strain SMD1163 was used, which has mutations in two protease genes and shows substantial reduction in three protease activities.

1.5 Aim of the work

The goal of this work was to produce large quantities of active endothelin B receptor (ET_B) and to optimize its purification in order to obtain protein sample amenable for crystallization

trials. Despite receptor overproduction in a suitable host, a purification factor of more than 1000 has to be achieved. To minimize the loss of protein, this should be done in as few steps as possible, which can be accomplished only by affinity chromatography. Receptor fusions with different peptides ("affinity tags") were produced and different purification strategies tried out in order to find most suitable one for scaling up.

Three different hosts for protein production were to be tested and compared for receptor overproduction: yeast *Pichia pastoris*, insect cells/baculovirus system and mammalian cells/Semliki Forest virus system.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals

General chemicals used in this work and not listed here were ultra pure chemicals *p.A.* grade from the companies Sigma-Aldrich Chemie GmbH, Deisenhofen, Carl Roth&Co. KG, Karlsruhe or from Merck KgaA, Darmstadt.

Acrylamide ready-made solution for polyacrylamide gels	Carl Roth GmbH&Co. KG, Karlsruhe
Agarose, electrophoresis grade	Bethesda Research Laboratories GmbH, Neu Isenburg
Ammonium-persulfate (APS)	Serva Elektrophoresis GmbH, Heidelberg
Ampicillin, sodium salt	Carl Roth GmbH&Co. KG, Karlsruhe
Bacto Agar	Difco Laboratories, Detroit, Michigan, USA
Bacto Tryptone	Difco Laboratories, Detroit, Michigan, USA
Bacto Yeast Extract	Difco Laboratories, Detroit, Michigan, USA
Biotin	Serva Elektrophoresis GmbH, Heidelberg
Bovine serum albumin (BSA)	Sigma-Aldrich Chemie GmbH, Deisenhofen
5-Bromo-4-chloro-3-indolylphosphate, 4-toluidine salt (BCIP)	Biomol Feinchemikalien GmbH, Hamburg
Bromophenol blue	Sigma-Aldrich Chemie GmbH, Deisenhofen
Coomassie Brilliant Blue R-250	Serva Elektrophoresis GmbH, Heidelberg
Concanavalin A	Sigma-Aldrich Chemie GmbH, Deisenhofen
Deoxynucleoside-5’-triphosphates (dATP, dGTP, dTTP, dCTP)	Pharmacia Biotech, USA
1,4-dithiothreitol (DTT)	Serva Elektrophoresis GmbH, Heidelberg
Ethidium-bromide	Bio-Rad Laboratories GmbH, Munich
Ethylenediaminetetraacetic acid (disodium salt) (EDTA)	GERBU Biotechnik GmbH, Gaiberg
Geniticin sulfate G418	Novabiochem GmbH, Bad Soden/Ts.
Glass beads (0.5 mm)	Biomatik GmbH, Frankfurt
Glutaraldehyde	Sigma-Aldrich Chemie GmbH, Deisenhofen

Glycine	Serva Elektrophoresis GmbH, Heidelberg
Kanamycin	Carl Roth GmbH&Co. KG, Karlsruhe
Non-fat milk powder	Carl Roth GmbH&Co. KG, Karlsruhe
N,N,N'N'-tetramethylethyldiamine (TEMED)	Sigma-Aldrich Chemie GmbH, Deisenhofen
Nitrobluetetrazolium, toluidine salt (NBT)	Biomol Feinchemikalien GmbH, Hamburg
Peptone	Sigma-Aldrich Chemie GmbH, Deisenhofen
Phenol	Carl Roth GmbH&Co. KG, Karlsruhe
Polyethyleneglycol 6000	Serva Elektrophoresis GmbH, Heidelberg
Polyethyleneimine (PEI)	Sigma-Aldrich Chemie GmbH, Deisenhofen
Silver nitrate ($AgNO_3$)	Sigma-Aldrich Chemie GmbH, Deisenhofen
Streptavidine-AP conjugate	Amersham Buchler GmbH&Co, Braunschweig
Tetracycline-hydrochloride	Sigma-Aldrich Chemie GmbH, Deisenhofen
Tris-(hydroxymethyl)-aminomethane	Carl Roth GmbH&Co. KG, Karlsruhe
Tunicamycin	Sigma-Aldrich Chemie GmbH, Deisenhofen
Yeast Nitrogen Base (YNB), w/o amino acids, w/o ammonium-sulfate	Difco Laboratories, Detroit, Michigan, USA

2.1.2 Radioactive labelled chemicals

$[^{125}I]Tyr^{13}$-Endothelin 1 (human, porcine)	NEN™Life Science Products, Inc. Boston, USA

2.1.3 Detergents

Sodium-dodecyl-sulfate (SDS)	Carl Roth GmbH&Co. KG, Karlsruhe
Digitonin	Sigma-Aldrich Chemie GmbH, Deisenhofen
Deoxycholate, sodium salt	Sigma-Aldrich Chemie GmbH, Deisenhofen
n-Dodecyl-β-maltoside (LM)	Glycon Biochemicals, Luckenwalde
n-Decyl-ß-maltoside (DM)	Glycon Biochemicals, Luckenwalde
n-Undecyl-ß-maltoside (UM)	Glycon Biochemicals, Luckenwalde
n-Octyl-ß-D-glucopyranoside (OG)	Glycon Biochemicals, Luckenwalde
N-dodecylphosphocholine, Fos12	Anatrace, Maumee, OH, USA
N-tetradecylphosphocholine, Fos14	Anatrace, Maumee, OH, USA

N-hexadecylphosphocholine, Fos16	Anatrace, Maumee, OH, USA
Tween 20	Koch-Light Ltd., Haverhill, England

2.1.4 Protease inhibitors

Aprotinin	Biomol Feinchemikalien GmbH, Hamburg
Benzamidine	Sigma-Aldrich Chemie GmbH, Deisenhofen
Leupeptin	Calbiochem GmbH, Bad Soden
Pepstatin A	Serva Elektrophoresis GmbH, Heidelberg
Phenylmethylsulfonylfluoride (PMSF)	Carl Roth GmbH&Co. KG, Karlsruhe
Complete®, EDTA free	Roche Diagnostis GmbH, Mannheim
E64	Biomol Feinchemikalien GmbH, Hamburg

2.1.5 Chromatographic matrices and prepacked columns

Anti-Flag™ M2 Affinity Gel	Sigma-Aldrich Chemie GmbH, Deisenhofen
ImmunoPure® Immobilized Monomeric Avidin Gel	Pierce, Rockford, USA
Ni-NTA matrix	Qiagen GmbH, Hilden
Superose 6 PC 3.2/30	Amersham Pharmacia Biotech, Freiburg
UltraLink™ Immobilized Monomeric Avidin Gel	Pierce, Rockford, USA
MicroSpin S-200 HR prepacked columns	Sigma-Aldrich Chemie GmbH, Deisenhofen
Streptavidin-coated magnetic agarose beads	Dynal, Hamburg

2.1.6 Enzymes

Restriction endonucleases	MBI Fermentas GmbH, St. Leon-Rot and New England Biolabs GmbH, Schwalbach
T4 DNA ligase	New England Biolabs GmbH, Schwalbach
T4 DNA polymerase	MBI Fermentas GmbH, St. Leon-Rot
Benzonase	Merck KgaA, Darmstadt
SP6 RNA polymerase	MBI Fermentas GmbH, St. Leon-Rot

2.1.7 Antibodies

Anti-Flag® M2 monoclonal antibody	Sigma-Aldrich Chemie GmbH, Deisenhofen

Anti-Mouse IgG, AP conjugated	Sigma-Aldrich Chemie GmbH, Deisenhofen
Monoclonal anti-polyhistidine antibody	Sigma-Aldrich Chemie GmbH, Deisenhofen

2.1.8 Kits

Qiagen Plasmid miniprep kit	Qiagen GmbH, Hilden
QIAquick Gel Extraction kit	Qiagen GmbH, Hilden
QIAquick PCR Purification kit	Qiagen GmbH, Hilden
HiSpeed Plasmid Maxi kit	Qiagen GmbH, Hilden
BCA Protein Assay Kit	Pierce, Rockford, USA

2.1.9 Marker proteins

SDS-6H High Molecular Weight Markers	Sigma-Aldrich GmbH, Deisenhofen
SeeBlue®Plus2 Pre-Stained Protein standard	Invitrogen GmbH, Karlsruhe

2.1.10 Buffers and Solutions

Agarose gel (1%)	1g agarose
	100 ml 1xTAE buffer
	2.5 µL EtBr (10mg/mL)
Ampicillin stock solution (1000x)	150 mg/mL in H_2O (sterile filtered, stored at -20°C)
AP buffer	100 mM Tris/HCl, pH 9.5
	100 mM NaCl
	5 mM $MgCl_2$
Aprotinin (40x)	20 mg/mL in PBS
APS	10% (w/v) in H_2O
BCIP-solution	50 mg BCIP/mL DMF (stored at –20°C)
Benzamidine (100x)	100 mM in H_2O
Binding buffer	50 mM Na_2HPO_4, pH 7.4
	500 mM NaCl
	5 mM EDTA
	0.1% BSA
Coomassie solution	1.25 g Coomassie Brilliant Blue R250
	200 mL H_2O

	50 mL acetic acid
	250 mL methanol
Conditioner (silver gels)	400 mM sodium-acetate, pH 6.0
	0.5 % (v/v) glutaraldehyde
	0.1% (w/v) $Na_2S_2O_3$
	30% (v/v) ethanol
Destaining solution (Coomassie gels)	1 L methanol
	1 L H_2O
	200 mL acetic acid
Developer (silver gels)	2.5% (w/v) Na_2CO_3
	0.04% (v/v) formaldehyde
Fixier (silver gels)	30% ethanol
	10% acetic acid
Kanamycin stock solution	30 mg/mL in H_2O, stored at –20°C
Leupeptin (500x)	0.25 mg/mL in H_2O
Loading buffer 4x (SDS PAGE)	0.25 mM Tris/HCl, pH 6.8
	40% (w/v) glycerol
	20% (v/v) ß-mercaptoethanol
	8% (w/v) SDS
	0.004% (w/v) bromophenol blue
Loading buffer (DNA)	50 mM Tris/HCl, pH 7.4
	5 mM EDTA
	50% (v/v) glycerol
	0.05% (w/v) bromophenol blue
NBT solution	50 mg NBT/mL in 70% DMF,
	stored at 4°C
Pepstatin A (500x)	0.35 mg/mL in methanol
PMSF (200x)/(500x)	200mM in isopropanol/500 mM in DMSO
Running buffer 10x (SDS PAGE)	500 mM Tris
	1.92 M glycine
	1% (w/v) SDS
rNTP mix	10 mM ATP

	10 mM CTP
	10 mM UTP
	5 mM GTP
Staining solution (silver gels)	0.1% (w/v) $AgNO_3$
	0.025% (v/v) formaldehyde
TAE buffer (50x)	2 M Tris
	1 M acetic acid
	50 mM EDTA, disodium salt
TBS buffer	50 mM Tris/HCl, pH 7.4
	150 mM NaCl
TBST buffer	10 mM Tris/HCl, pH 8.0
	150 mM NaCl
Tetracycline stock solution	12.5 mg/mL in 50% ethanol,
	stored at –20°C
Transfer buffer 1x (Immunoblot)	38 mM glycine
	10 mM Tris
	20% (v/v) methanol
Transfer buffer 5x (Immunoblot)	190 mM glycine
	50 mM Tris

2.1.11 Primers for sequencing

Primers for sequencing for pPIC9K vector:

α*for* : 5' – CTA CTA TTG CCA GCA TTG CTG C – 3'

AOX1*rev* : 5' – GGC AAA TGG CAT TCT GAC ATC C – 3'

Primers for sequencing for pSFV3CAP vector:

CAP*for* : 5' - CTG TCG GTG GTC ACC TGG - 3'

EtBRCT*for* : 5' – CTG AAT TCC TGC ATT AAC CC – 3'

2.1.12 Oligonucleotides

Streptagfor: 5' –AA TTC TCT GCT TGG AGA CAT CCA CAA TTT GGT GGT TAA GC – 3'

Streptagrev: 5' – GGC CGC TTA ACC ACC AAA TTG TGG ATG TCT CCA AGC AGA G – 3'

StreptagIIfor: 5' – AA TTC TCT GCT TGG TCT CAT CCA CAA TTT GAA AAG TAA GC – 3'

StreptagIIrev: 5' – GGC CGC TTA CTT TTC AAA TTG TGG ATG AGA CCA AGC AGA G – 3'

2.1.13 Bacterial and yeast strains

E. coli strain XL1-Blue: *end*A1, *hsd*R17 (rk-, mk+); *sup*E44, *thi*-1, λ–, *rec*A1, *gyr*A96, *rel*A1, Δ(*lac*ZYA-*arg*F)U169 [F': *pro*AB, *lac*I^qZΔM15, Tn10(Tet^r)], Stratagene GmbH, Heidelberg

Pichia pastoris yeast strain:

SMD1163: Mut^+ , His^-, protease-deficient (his4, pep4, prb), Invitrogen Inc., San Diego

2.1.14 Media for bacterial cultures

<u>LB medium</u>

Bacto Tryptone	10 g/L
Bacto Yeast Extract	5 g/L
NaCl	5 g/L
Bacto Agar	15 g/L (for the plates)

<u>SOB medium</u>

Bacto Tryptone	20 g/L
Bacto Yeast Extract	5 g/L
NaCl	10 mM
KCL	2.5 mM

<u>SOC medium</u>

Bacto Tryptone	20 g/L
Bacto Yeast Extract	5 g/L
NaCl	10 mM
KCL	2.5 mM
$MgCl_2$	10 mM
$MgSO_4$	10 mM

Glucose	20 mM

FSB (Frozen storage buffer)

Sodium-acetate	10 mM
Glycerol	10% (w/v)
KCl	100 mM
$MnCl_2x4H_2O$	45 mM
$CaCl_2x2H_2O$	10 mM
$HACoCl_3$	3 mM

2.1.15 Media for yeast cell cultures

Fermentation medium

Basic salt medium	0.93 g $CaSO_4x2H_2O$
(per liter):	18.2 g K_2SO_4
	14.9 g $MgSO_4x7H_2O$
	9 g $(NH_4)_2SO_4$
	40 g glycerol

Fill with H_2O to 900 mL and autoclave in the fermenter vessel. Make separately the solution of Na-hexametaphosphate – dissolve 25 g of the salt in 100 mL H_2O and sterile filtrate. Add this solution to the cold and sterile fermenter medium, as well as 4.35 mL of PTM1 trace element stock solution, via sterile tubing.

Stock solutions

Potassium phosphate buffer (10x)	1M KH_2PO_4 in H_2O, adjust pH to 6.0 with KOH and autoclave
Yeast nitrogen base (YNB) (10x)	34 g YNB w/o amino acids, w/o ammonium-sulfate, 100 g ammonium-sulfate. Dissolve in 1 L H_2O, sterile filter and store at 4°C
Methanol (10x)	5 mL of methanol in 95 mL of sterile H_2O Store at 4°C, max. 2 months
Biotin (500x)	Dissolve 20 mg of biotin in 100 mL H_2O, sterile filter and store at 4°C
Geniticin sulfate G418	50 mg/mL, sterile filtrate, store at –20°C

Glucose (10x) — Dissolve 200 g D-glucose in 1L H_2O, autoclave for 15 min, store at RT

Glycerol (10x) — Mix 100 mL glycerol with 900 mL of H_2O, autoclave and store at RT

PTM1 trace salt solution for the fermentation

0.2 g biotin
0.5 g $CoCl_2$
6 g $CuSO_4 x 5H_2O$
65 g Fe(II)$SO_4 x 7H_2O$
0.02 g H_3BO_3
5 mL H_2SO_4
3 g $MnSO_4 x H_2O$
0.08 g NaI
0.2 g Na_2MoO_4
20 g $ZnCl_2$
Fill till 1L with H_2O, sterile filter and store at RT for max. 6 months

Media:

<u>BMGY medium</u>

Dissolve 20 g peptone and 10 g Bacto Yeast Extract in 700 mL H_2O, 20 min autoclave. When it cools down to 60°C, add:

100 mL potassium-phosphate buffer (10x)
100 mL YNB (10x)
2 mL biotin (500x)
100 mL glycerol (10x)
and store at 4°C max. 2 months.

<u>BMMY medium</u>

Dissolve 20 g peptone and 10 g Bacto Yeast Extract in 700 mL H_2O, 20 min autoclave. When it cools down to 60°C, add:

100 mL potassium-phosphate buffer (10x)
100 mL YNB (10x)
2 mL biotin (500x)
100 mL methanol (10x)

and store at 4°C max. 2 months

YPD medium

Dissolve 20 g peptone and 10 g Bacto Yeast Extract in 900 mL H_2O and autoclave for 20 min. When it cools down to 60°C, add:

100 mL glucose (10x)

Store at 4°C max. 2 months

YPD plates

Dissolve 20 g peptone, 10 g Bacto Yeast Extract and 20 g Bacto agar in 900 mL H_2O and autoclave for 20 min. When it cools down to 60°C, add:

100 mL glucose (10x)

Store at 4°C max. 2 months

YPD-G418 plates

Dissolve 20 g peptone, 10 g Bacto Yeast Extract and 20 g Bacto agar in 900 mL H_2O and autoclave for 20 min. When it cools down to 60°C, add:

100 mL glucose (10x)

Geniticin sulfate G418 in desired concentration

Store at 4°C max. 2 months

2.1.16 Chemicals & media for insect&mammalian cell cultures

Sf9 cells	American Type Tissue Collection, Manassas, USA
TNM-FH medium	ccPro, Neustadt
FKS	GibcoBRL, Life Technologies GmbH, Karlsruhe
L-glutamine	GibcoBRL, Life Technologies GmbH, Karlsruhe
Gentamycin	ccPro, Neustadt
Vitamin B12	Sigma Chemie GmbH, Deisenhofen
Pluronic-68	Sigma Chemie GmbH, Deisenhofen
BHK cells	European Collection of Cell Cultures, UK
CHO cells	Aventis Pharma, Frankfurt am Main
Dulbecco's modified Eagle's medium (DMEM)	Cell Concepts, Umkirch, Germany
Iscove's medium	Cell Concepts, Umkirch, Germany

Trypsin-EDTA	Cell Concepts, Umkirch, Germany
CAP	New England Biolabs GmbH, Schwalbach

2.1.17 Apparatus-general

Cell mill Dynomill KDL A	Willy A. Bachofen, Switzerland
Electroporation device	Biorad, Munich
Microscope	Olympus Inc., Japan
Shakers	Infors AG, Bottmingen, Switzerland
Spectrophotometer	Thermo Spectronic, Cambridge, UK
Vortexer	Bender&Hobein AG, Zürich, Switzerland
Thermomixer 5436	Eppendorf GmbH, Hamburg

2.1.18 Centrifuges

Sorvall RC-5B	Sorvall, Bad Homburg
Avanti J-20 XPI	Beckman-Coulter, Fullerton, CA, USA
Optima LE-80K ultracentrifuge	Beckman-Coulter, Fullerton, CA, USA
Tabletop ultracentrifuge TL100	Beckman Instruments Inc., Palo Alto,USA
Microfuge 5415D	Eppendorf GmbH, Hamburg
Sigma 3K 12 tabletop centrifuge	Sigma-Aldrich Chemie GmbH, Deisenhofen

2.1.19 Filters

GF/C filters for binding assay	Whatman Intl. LtD, Maidstone, England
Prefilters AP25	Millipore GmbH, Eschborn
1,2 µM membranes RA	Millipore GmbH, Eschborn
0,45 µM membranes HA	Millipore GmbH, Eschborn
Net AP32	Millipore GmbH, Eschborn
MediaKap-filters 2L and 5L	INTEGRA Biosciences Germany
Small filters 0,22 µm and 0,45 µm	Schleicher&Schuell GmbH, Dassel

2.1.20 Fermenter and equipment

10 L Bioreactor, magnetic stirrer, pH- and O2-electrode, thermometer, and computer control unit	New Brunswick Scientific, Edison, NJ, USA

Methanol detector and sensor unit	Raven Biotech Inc., Vancouver, Canada
Pump Watson-Marlow 101U/R	Smith and Nephew Watson-Marlow Falmouth, England

2.2 Methods

2.2.1 *E. coli* cultivation

For the overnight culture of *E. coli* XL1Blue, one single colony was picked from an LB agar plate and inoculated into 5 mL of LB medium with tetracycline (12.5 µg/mL final concentration). The culture was incubated overnight at 37°C and 225 rpm.
Large (50-100 mL) *E. coli* cultures were prepared from the small overnight pre-culture. LB medium with the antibiotic was inoculated 1:100 with the overnight pre-culture.

2.2.2 Preparation of competent cells

Competent *E. coli* cells were prepared according to the *TFB based chemical transformation protocol* in DNA cloning 1 – A Practical Approach (Eds. Glover&Hames). Briefly, *E. coli* XL1 Blue cells were grown in 50 mL of SOB medium overnight. The cells were then chilled on ice for 15 min, pelleted for 15 min at 1000xg and resuspended in 1/3 of the culture volume of TBF. Following resuspension, the cells were incubated on ice. Afterwards, the cells were pelleted (1000g, 4°C, 15 min), and resuspended in 1/12.5 of the original culture volume of FSB. DMSO was added (7 µL to 200 µL suspension) and the suspension swirled with a pipette. The cells were incubated on ice for 5 min, a second aliquot of DMSO added, followed by a 15 min incubation on ice. The suspension was aliquoted 200 µL each in pre-cooled 1.5 mL-Eppendorf tubes and flash frozen in liquid nitrogen. The aliquots were stored at –80°C.

2.2.3 Plasmid DNA transformation into competent *E. coli* cells

Competent *E. coli* cells were thawed on ice and 200 µL of cell suspension was mixed with 10 µL of the ligation reaction (s. 2.2.8). After 30 min incubation on ice, the suspension was subjected to heat shock at 42°C for 2 min. Afterwards 800 µL of LB medium was added and the cells were incubated 45-60 min at 37°C with shaking. 100 µL of the suspension was plated on an LB plate containing the appropriate antibiotic and the plate incubated overnight at 37°C.

2.2.4 Fast plasmid DNA transformation after Hanahan (1983)

This method is suitable only for fast transformation of relatively highly concentrated plasmid DNA (>5µg/mL).

A clump of *E. coli* colonies was picked from an LB agar plate and transferred to 200 µL of ice-cold FSB buffer. The cells were resuspended by vortexing and then incubated for 10 min on ice. 10-1000 ng of plasmid DNA (<10µL) was added to the cell sample, mixed with the pipette tip and incubated for further 10 min on ice. After the heat shock at 42°C for 90 s, 800 µl of SOC medium was added. The sample was then incubated for 30 min at 37°C with shaking (this step was optional when more than 100 ng of DNA was used for transformation). Different amounts of cells were then plated on the LB agar plates containing the appropriate antibiotic and the plates incubated overnight at 37°C.

2.2.5 DNA isolation

Isolation of plasmid DNA from *E. coli* and isolation of DNA fragments were carried out following the instructions manual from the relevant QIAGENE kit.

2.2.6 Phenol-chloroform extraction

The procedure developed by Brawerman (1972) is used for removal of proteins from nucleic acid solutions. DNA solution was mixed 1:1 with a phenol/chloroform/isoamylalcohol (25:24:1) mixture and centrifuged at 12000xg for 2 min at RT. Two phases were obtained in the tube. The upper aqueous phase was carefully removed and transferred to another tube. The extraction of the water phase was repeated until no impurities were visible at the phase interface. Traces of phenol were removed by ethanol precipitation.

2.2.7 Ethanol precipitation

Ethanol precipitation is used for removal of phenol traces from a DNA solution after phenol-chloroform extraction.

DNA solution was mixed with 1/10 volume of 3M Na-acetate (pH 5.2) and 2.5 volumes of 100% ethanol (kept at -20°C). The DNA precipitation followed overnight at -20°C or for 30 min at -70°C. The solution was centrifuged at 14000 rpm in an Eppendorf table centrifuge for 10 min at 4°C, supernatant removed and the pellet was washed with 100 µL of ice cold 70%

ethanol. Precipitated DNA was dried in a vacuum centrifuge and afterwards dissolved in water at 65°C. Dissolved DNA was stored at -20°C.

2.2.8 DNA restriction and ligation

In this work only restriction nucleases of Type II were used. These nucleases cut DNA close to the recognition sequence and need no ATP for the reaction.

3 units of enzyme per μg of DNA were used, in the appropriate buffer for the enzyme recommended by the manufacturer. The reaction sample was incubated 1-2 hours at the recommended temperature for every enzyme.

Ligation of the DNA fragments was carried out in the presence of T4 DNA ligase overnight at 16°C or 4 hours at RT.

2.2.9 Yeast cell culture

2.2.9.1 P. pastoris transformation

In this work electroporation was used as a method of choice for *P. pastoris* cell transformation.

One colony of *P. pastoris* strain SMD1163 was picked from the YPD plate, transferred into 5 mL of YPD medium and incubated overnight at 30°C in the shaker with 230 rpm. 500 mL YPD medium was inoculated with the calculated amount of pre-culture in order to get an OD_{600} = 1.4 in a convenient amount of time, taking into account that the doubling time was approx 2 h. The cells were afterwards harvested by centrifugation (1500g, 5 min, 4°C), resuspended in 500 mL of ice cold sterile deionised H_2O and centrifuged again under the same conditions. They were once again washed with 250 mL of H_2O. After the centrifugation, the cells were resuspended in 20 mL of ice-cold 1 M sorbitol and repeatedly centrifuged under the same conditions as previously. Finally, the cells were resuspended in 1 mL of ice-cold 1 M sorbitol, divided into 80 μL-aliquots and placed on ice. One aliquot containing 10-25 μg of linearized DNA (<10 μL) per transformation was mixed with 80 μL of prepared cells, the suspension was transferred into a pre-cooled electroporation cuvette (0.2 cm) and incubated 5 min on ice. Following incubation, the cells were electroporated (1500 V, 25 μF, 400-500 Ω) and immediately after resuspended in 1 mL of cold 1 M sorbitol. The suspension was plated onto three MD plates and the plates incubated for two days at 30°C. After the incubation, clones were picked and used to screen for multiple inserts.

2.2.9.2 Selection of clones with multiple inserts

The selection method is based on the presence of the kanamycin resistance gene in the expression vector. The resulting colonies from one transformation experiment (3 MD plates, see 2.2.9.1) were carefully scraped from the plates and brought together in a 13 mL-tube. The suspension was mixed for 10 s on the vortex, and its OD_{600} measured (with 1:100 dilution in water). Knowing that OD_{600} of 1 means 5×10^7 cells/mL, at least three different dilutions of the cell suspension in H_2O were made, so that 1 mL contained 10^6, 10^7 and 10^8 of cells respectively. From the different dilutions 100 µL each were plated on the YPD-G418 plates with different G418 concentrations, ranging from 0.05 – 1 µg/mL. The plates were incubated 3-4 days at 30 °C and the colonies picked for expression tests. The remaining undiluted suspension was diluted to 30% (w/v) with sterile glycerol, frozen in liquid nitrogen and stored at -80 °C.

2.2.9.3 Storage of **P. pastoris** *clones*

Short-term storage (1-2 months) of *P. pastoris* clones was possible on YPD plates at 4 °C. For long-term storage, glycerol cultures were made. One colony was picked from the plate and transferred to 10 ml of YPD medium. The culture was incubated overnight at 30 °C with shaking at 230 rpm. The following day the cells were harvested after culture OD_{600} measuring (2000 g, 5 min, 4 °C). The pellet was resuspended in sterile 87% glycerol to final 30% glycerol concentration. Aliquots were made, frozen in liquid nitrogen and stored at – 80 °C.

2.2.9.4 Protein production in *P. pastoris*

2.2.9.4.1 Small shaking culture of P. pastoris

One colony of the desired clone from the YPD plate was inoculated into 10 mL of BMGY medium in a sterile 50 mL tube. The culture was incubated overnight at 30 °C in the shaker at 230 rpm. At an OD_{600} of 2-4, the cells were harvested by centrifugation at 2000 g, 5 min, at RT. The cells were resuspended in BMMY medium to an OD_{600} of 1. They were incubated in a 100 mL baffled flask for 24 h at 30 °C and 230 rpm. Care was taken that the culture was sufficiently aerated. After 12-16 h methanol was added to a final concentration of 0.5%. When the incubation was finished, the cells were harvested by centrifugation (2000g, 5 min, 4°C) and either directly used for membrane preparation or frozen in liquid nitrogen and stored at – 80 °C.

2.2.9.4.2 *Large-scale shaking culture of P. pastoris*

30 mL of BMGY medium in a 100 mL baffled flask was inoculated with a single colony of the desired clone and incubated overnight in a shaker (30°C, 230 rpm). With this pre-culture, 2 × 1.5 L of BMGY medium in two 5 L-baffled flasks were inoculated so that the final OD_{600} the following day in each was 4-6. To calculate the amount of pre-culture needed for inoculum, the following formula was used:

$$X\{mL\} = [(OD_{600}\ w / 2^{t/2}) \times V] / OD_{600}\ m$$

OD_{600} w = *wanted OD*

t = *hours of incubation*

V = *culture volume* (mL)

OD600 m = *OD of pre-culture*

Inoculated culture was incubated overnight at 30°C in the shaker at 230 rpm. After the culture reached the desired OD, the cells were harvested in sterile centrifuge tubes (2000g, 10 min, RT). The cells were resuspended in 12 L of BMMY medium, and split into six 5L-sterile baffled flasks. The culture in BMMY medium was incubated 24 hours at 30°C in the shaker with 150 rpm. After 12-16 h, methanol was added to a final concentration of 0.5%. The cells were harvested after incubation (2000g, 10 min, 4°C) and the pellets resuspended in breaking buffer. The suspension was aliquoted in 50 mL tubes, frozen in liquid nitrogen and stored at –80°C.

This was the standard procedure for al 12 L and 24 L shaking cultures.

2.2.9.5 *Fermentor culture of* **P. pastoris**

Fermentation of *P. pastoris* was carried out according to the modified protocols of Invitrogen Inc. in a 10 L-fermentor.

Preparation:

The fermentor vessel was filled with 4 L of basic salt medium (see 2.1.15) and closed. Thermometer, methanol sensor, calibrated pH electrode, O_2-sensor and sampling device were inserted into the vessel. Further, four tubings with closures were attached to the fermentor inlets. These were used during the fermentor run for adding glycerol, methanol, ammonia and anti-foam. The other inlets were closed and the fermentor vessel autoclaved. After cooling down to room temperature, the fermentor vessel was positioned onto the fermentor console and all the necessary connections were made. Temperature was set to 30°C. pH value of the

medium was adjusted with 25% ammoniac solution to 5-6. The agitation speed was set to 600 rpm and the aeration started (set point 10.0). Fermentor was left for 6 hours with these parameters, in order to polarize the O_2-electrode. Afterwards the electrode was calibrated according to the fermentor manufacturer's instructions (BioFlo 3000 Fermentor Handbook, New Brunswick Scientific). Na-hexametaphosphate solution, as well as PTM_1 salts (see 2.1.15), was slowly added to the medium. Fermentor medium was inoculated with 100 mL of BMGY culture so that the starting OD_{600} was 1.

Sample collection:
Samples of the medium were continuously taken during the fermentor run through a special sampling inlet. Wet weight, OD_{600}, ligand binding and cell morphology were monitored. Wet weight was calculated after cells from a 2 mL suspension were pelleted (5000g, 5 min) and the supernatant removed. The pellet was measured on the balance to determine wet weight (g/L). For the ligand binding, small-scale membrane preparations were made, and radioactivity was measured as described in Methods.

Fermentation process

Phase I – glycerol "batch"

During the first fermentation phase, the yeast cells consume glycerol from the medium. The end of this phase was easily recognized, due to the so called "oxygen spike" (the oxygen concentration in the medium rose to 100% in the time window of 10-15 min).

Phase II – glycerol feed

Glycerol solution (50% w/v) containing 12 mL/L PTM_1 salts (see 2.1.15) was added at a rate of 15mL/h/liter fermentor culture. The agitation was increased to 1000 rpm and the oxygen control regime was changed to the "O_2 cascade" (see BioFlo fermentor handbook). The glycerol feed was continued until a wet weight of 250-350 g/L was achieved. It was essential that there was no accumulation of glycerol in the medium. This was to be tested by disconnecting the feed for a short time. If there were no accumulated glycerol in the medium, an oxygen spike would appear in a very short time.

Phase III – methanol induction

Protein production was induced by methanol. Since methanol is rather toxic for yeast cells in higher concentrations, the methanol solution (988 mL of methanol + 12 mL of PTM_1 salts) was fed at a very slow rate. The feeding rates and induction times are described in Results. At the end of the induction phase, the cells were harvested by centrifugation (5000g, 20 min, 4°C), frozen in liquid nitrogen and stored at – 80°C.

2.2.9.6 Cell breaking and membrane preparation – analytical scale

In order to determine the binding activity of the new receptor clones, membranes from 15 mL-*P. pastoris* cultures were prepared. The cells in BMMY medium were pelleted at 3000g, 4°C, for 4 min. The supernatant was removed, the cells were resuspended in 1 mL of Breaking buffer (50 mM Na_2HPO_4, 500 mM NaCl, 100 mM sucrose, 5 mM EDTA, 0.1% BSA) and the suspension transferred to a 2 mL Eppendorf tube. 1mM PMSF was added. Breaking of *P. pastoris* cells was carried out at 4°C in using glass beads. The beads (0.5 mm in diameter) were previously washed with 1M HCl, then with deionized water to neutral pH and dried. 1mL of the beads was added to the tube with cell suspension, mixed and vortexed 10 min in the cold room. Meanwhile, a 5-mL Pierce plastic column with a frit was put into a 13 mL Sarstedt tube and precooled on ice. The suspension was transferred into this column, together with 500 µL of breaking buffer, and centrifuged for 1 min, 2000g, 4°C. In this way the glass beads were separated from the suspension and retained on the column frit. The column was washed with 500 µL of breaking buffer, and centrifuged under the same conditions for a further 4 min. The supernatant in the 13 mL tubes was carefully taken out and transferred to an ultracentrifuge tube. Centrifugation at 100 000g, at 4°C for 30 min followed. The pellet obtained was resuspended in 500 µL of binding buffer (see Materials) and homogenized with three strokes in a hand homogenizer. Two equal aliquots were made, frozen in liquid N_2 and stored at – 80°C until needed.

2.2.9.7. Cell breaking and membrane preparation – preparative scale

Large amounts of yeast cells obtained from the 24 L shaker cultures or from the fermentor were broken mechanically, in a ***Dynomill*** cell mill. The procedure is relatively fast and efficient, since after 3 passages, 80% cells were broken, as determined by visualization under a light microscope.

200-1000 g of the cells were thawed on ice overnight and resuspended in breaking buffer (see the previous chapter), in order to obtain a cell wet weight of 30%. Protease inhibitor cocktail was added (1 mM PMSF, benzamidine, leupeptin, pepstatin A) to the cell suspension. Into the precooled ***Dynomill*** cell mill 500 mL of glass beads (5 mm in diameter, prewashed with 1 M HCl and water) were added. The cell suspension was passed 3x through the mill and the cell breakage rate was checked under the microscope. The glass beads were washed with 100-300 mL of breaking buffer (depending on the starting cell amount). The suspension was subjected to low spin at 5000 rpm (JLA-8 rotor) for 20 min at 4°C, in order to remove cell debris and unbroken cells. The supernatant was, after addition of PMSF to a final concentration of 1mM, centrifuged further at 100 000xg for 45 min at 4°C. The pellets were collected, and 100 mL of wash&storage buffer (50 mM Tris, 1M NaCl, 10% glycerol, pH 7.4) was added to them. The suspension was homogenized 2x10s with the Thorax homogenizer, and then in the hand homogenizer. The mixture was diluted to 360-500 mL with the wash&storage buffer and subjected to ultracentrifugation under above described conditions, in order to wash the pellets. The procedure of pellet homogenization was repeated, homogenized pellets resuspended in 200-400 mL of wash&storage buffer and flash-frozen in liquid nitrogen. Obtained membrane preparations were stored at - 80°C till further use.

2.2.9.8 Precipitation of glycosylated α - factor signal peptide with concanavalin A (ConA)

Half a millilitre of the solubilizate of *P. pastoris* cells expressing FlagHisET$_B$Bio receptor fusion was filtrated through a sterile 0.65 µm microfilter. Concanavalin A solution was added to a final concentration of 1 mg/mL and the sample incubated 1 hour at RT on a rotating wheel. Afterwards the sample was centrifuged for 30 min at 12 000g at 4°C. The supernatant was subjected to SDS electrophoresis, electroblotted and analysed with anti-Flag M2 antibody.

2.2.10 Insect cell culture

2.2.10.1 Virus preparation and titre determination

New virus was prepared from the virus stock (MPI for Biophysics, Frankfurt/M.) when at least 100 mL of the Sf9 cell suspension culture (cell density 5-7,5 x 10^5 cells/mL) was infected with the virus at MOI = 0.1. After 5-7 days of incubation in the shaker at 27°C and

125 rpm, the cells were pelleted by centrifugation at 6000g for 10 min, and the supernatant containing the virus was transferred into sterile flasks and stored at 4°C until use.
The new virus stock was titrated before use. For this purpose, a 96-well plate with $1x10^4$ seeded cells/well was used, and each horizontal row was infected with 10 µL of a different sequential dilution of virus. The plate was incubated for seven days at 27°C and then the number of infected wells at each dilution was determined. Virus titre was calculated according to the End-Point dilution method (Reed and Münch, 1938), and expressed as the number of plaque-forming units of virus (p.f.u.)/mL of the virus stock.

2.2.10.2 Protein expression

Ovary cells from *Spodoptera frugiperda* (Sf9 cells) were used for Baculovirus-mediated expression of the ET_B receptor. The cells were cultivated in mono- and multilayers, in TNM-FH medium supplemented with 5% FCS, 4 mM L-glutamine, 50 µg/mL gentamycin and 10µg/L of vitamin B12. For protein production, the cells were cultivated in suspension. They were transferred from the cell culture flasks into Erlenbach flasks, and grown further in the same medium supplemented with 0.1% Pluronic. The cells were infected with the recombinant baculovirus at a density of at least 2.5×10^6 cells/mL. They were pelleted for 10 min at 1000xg, then resuspended in fresh medium supplemented with 0.1% of Pluronic so that their concentration was at least 2.5×10^6 cells /mL, and infected with the recombinant virus. Multiplicity of infection was 10. Both the cells in culture flasks and in Erlenbach flasks were incubated at 27°C. The cells were harvested 66 h after infection with the virus.

2.2.10.3 Insect cell membrane preparation

66 hours after virus infection, the Sf9 cells were counted, and aliquots containing $6x10^5$ cells were taken for binding assay and electrophoretic analysis. The cells were then harvested by centrifugation at 6000g for 10 min. Supernatant was removed and the cells resuspended in breaking buffer to yield $5x10^6$ cells/mL. The cells were broken in a parr-bomb apparatus for 45 min, under 500 psi N_2. The lysate was spun at 1000g at 4°C for 10 min to remove cell debris and unbroken cells. The supernatant after the low spin was subjected to centrifugation at 100 000g for 1h, at 4°C. This pelleted membrane fraction was resuspended in Tris buffer (50 mM Tris, 1M NaCl, 10% glycerol, pH 7.4), homogenized with a hand homogenizer, frozen in liquid nitrogen and stored at – 80°C until further use.

2.2.11 Discontinuous SDS PAGE

Analytical separation of proteins according to their molecular mass was carried out following the protocol of Fling and Gregerson (Fling and Gregerson, 1986).

The samples were prepared with 4xLoading buffer (see Materials), but were not, as usual, subjected to heat denaturation, since this causes irreversible aggregation of GPCRs molecules. Electrophoresis was carried out in a vertical apparatus (hand-made at MPI for Biophysics), in 10% separation gels, at a constant current of 20 mA per gel. The proteins in the gel were afterwards either stained with Coomassie Brilliant Blue or with silver nitrate, or electrotransferred to PVDF membranes for Western analysis.

2.2.12 Blue native PAGE

The electrophoresis was carried out according to the method from Schägger&von Jagow (Schägger and von Jagow, 1991; Schägger *et al*., 1994), where the proteins are not denatured by detergent. The migration path of the protein through the gel depends therefore not only on the molecular mass, but also on the shape. Polyacrylamide gels with a 6-13% gradient and without stacking gel (self-made) were used for the separation. Electrophoresis was carried out at 4°C, initially at a constant current of 10 mA and later increased to 20 mA. Afterwards, the gels were stained with Coomassie Brilliant blue, according to the standard protocol (see Protein gel staining- Coomassie staining in Methods). 15 µL of 1 mg/mL BSA solution was used as a marker protein.

2.2.13 Electrotransfer of proteins (Western blot)

After electrophoresis, the proteins were transferred to a PVDF membrane by electroblotting. The “sandwich” with 3 Whatman blotting papers (previously soaked in the 1x blotting buffer), protein gel, PVDF membrane (previously treated shortly with methanol and then soaked in blotting buffer) and 3 further Whatman blotting papers on top was made. It was placed to the semi-dry blotting apparatus (made at MPI for Biophysics). Protein transfer was carried out at constant current of 50 mA per gel for 1.5 h. Since the mixture of prestained molecular weight markers was used during the electrophoresis, the efficiency of the electrotransfer could be easily checked.

2.2.13.1 Anti-Flag immunoblot

After the transfer, the PDVF membrane was blocked 1h at RT in TBS buffer with 5% milk powder. The membrane was washed 2x with TBS buffer and incubated in 10 mL of the same buffer containing M2 anti-Flag antibody (10µg/mL) for 30 min. After washing 3x with TBS buffer, the 30 min long incubation with secondary antibody followed. For this purpose, AP-conjugated goat anti-mouse IgG was used, diluted 1:1000 in TBS. The membrane was then washed 3x with the TBS buffer. 10 mL of AP buffer with 66µL of NBT solution and 33µL of BCIP solution was added to the membrane, and incubated till the purple coloured bands appeared. The reaction was stopped by washing the membrane with water.

2.2.13.2 Detection of the ET_B Bio fusion with Streptavidin-AP conjugate

PDVF membrane with transferred proteins was incubated 1h in TBST buffer at RT. Afterwards, the membrane was incubated with streptavidin-AP conjugate (1:5000 dilution in TBST) for 30 min at RT and washed 3x with TBST buffer. Specific band detection was carried out as in the case of anti-Flag immunoblot.

2.2.13.3 Anti-His immunoblot

After electrotransfer, PDVF membrane was blocked in PBS with 5% milk powder for one hour at RT. The membrane was then incubated with anti-His antibody (1:1000) in the same blocking buffer for one hour at RT. After washing 3x with PBS buffer, secondary antibody was added (anti-mouse IgG, alkaline-phosphatase conjugated), in 1:1000 dilution. After incubation with secondary antibody for one hour at RT, the membrane was washed with PBS, and incubated in AP buffer for 3 min. Staining was performed with 66 µL of NBT and 33 µL of BCIP in 10 mL of AP buffer. The reaction was stopped by washing the membrane with water.

2.2.14 Protein gel staining

2.2.14.1 Coomassie staining

After electrophoresis, the gel was incubated 1 h in fresh Coomassie solution (see Materials). Afterwards, the gel was placed into the destaining solution (see Materials), together with Kimwipes® cellulose tissues (which preferentially bind the Coomassie) and destained till the bands without background were clearly visible on the gel.

2.2.14.2 Silver staining

After SDS-electrophoresis, the gel was fixed for 16 min in the fixier (see Materials), and incubated equally long in the conditioner (see Materials). Then, the gel was washed 3x in water. After 20 min of incubation in silver-nitrate solution, the gel was 2x shortly washed with water, and then soaked in the developer until clearly visible bands appeared. The reaction was stopped with 50 mM EDTA solution.

2.2.15 Determination of protein concentration

For the protein concentration measurements the BCA protein assay was used (Smith *et al.*, 1985). This particular method has comparable sensitivity to the Bradford method, without being affected by the presence of detergents and lipids. The BCA assay kit from Pierce (see Materials) was used, according to the instruction manual. For each measurement, a new standard curve with BSA (1-12µg per well) was made. Absorption was measured at 562 nm in the spectrophotometer.

2.2.16 Concentration of proteins

2.2.16.1 Centrifugation

Vivaspin® tubes with 50 kDa-cut-off membranes from Vivascience, Lincoln, UK were used to concentrate the purified protein. The tubes with the protein solution were spun at 3000g at 4°C until the desired concentration was achieved. Concentrated protein was collected from the upper part of the tube.

2.2.16.2 TCA precipitation

A modified protocol after Tornqvist&Belfrage (1976) was used to precipitate proteins with trichloroacetic acid (TCA). To 1 mL sample 25 µL of 1% Na-deoxycholate was added, mixed and incubated 5 min at RT. Afterwards, 1 mL of 12% cold TCA was added and the sample spinned 20 min at 10 000xg, 4°C. Supernatant was removed and the pellet was resuspended in 20-100 µL of 2.5% SDS. The suspension was incubated in thermomixer at 700 upm for 10 min, at 37°C, in order to fully dissolve the pellets. Then the 4x loading buffer was added and the samples loaded to SDS gel.

2.2.17 Receptor solubilization from the membranes

Endothelin B receptor was solubilized from the membranes using 1% n-dodecyl-ß-D-maltoside. Before solubilization, the receptor was saturated with the ligand, ET-1. Membranes were mixed with solubilization buffer (50 mM Tris, 500 mM NaCl, 10 mM imidazole, pH 7.4), to give final concentration of 3 mg/mL. Endothelin-1 solution in 20% DMSO (1mg/mL) was added in equimolar concentration as the calculated receptor concentration. The suspension was incubated for 2 hours with gentle mixing (magnetic mixer) at 4°C. Then, the detergent was added and the mixture was incubated for further 2 hours. During the incubation time, PMSF was added every ½ hour (final concentration 1mM), in order to prevent proteolytic degradation of the receptor. Following solubilization, the suspension was subjected to centrifugation at 100 000g, and the supernatant containing solubilized receptor was taken for further purification.

2.2.18 Affinity chromatography

2.2.18.1 Imobilized metal affinity chromatography (IMAC)

Crude membrane solubilizate (50-500 mL) was filtered through 0.65 µm filter and mixed with previously equilibrated 4-30 mL Ni-NTA matrix (equilibration buffer: 50 mM Tris, 500 mM NaCl, 10 mM imidazole, 0.1% LM, pH 7.4). Protease inhibitor PMSF was added in the final concentration of 1 mM. Batch chromatography was carried out at 4°C for two hours or overnight. The mixture was packed into a column and afterwards washed with Buffer 1 (50 mM Tris, 150 mM NaCl, 10 mM imidazole, 0.1% LM, pH 7.4). The same buffer, but with 30 mM imidazole, was used for further washing step. Receptor protein was eluted with Buffer 3 (50 mM Tris, 150 mM NaCl, 200 mM imidazole, 0.05% LM, pH 7.4).
Ni-NTA matrix was regenerated with 30% CH_3COOH, washed with water and stored in 30% ethanol.

2.2.18.2 Purification on monomeric avidin matrix

Non-specific binding sites on monomeric avidin were blocked according to the matrix producer instructions, and the matrix was equilibrated with Tris buffer (50 mM Tris, 150 mM NaCl, 10 mM EDTA, 0.05% LM, pH 7.4). The elution fraction from the Ni-NTA matrix (5-70 mL) was mixed with 1-20 mL of monomeric avidin matrix, PMSF in DMSO in the final concentration of 1 mM and 50 µM DTT. Endothelin-1 peptide was added (1/5 of ET-1 volume added before receptor solubilization - see 2.2.17). The mixture was rotated on the

wheel at 4°C for two hours or overnight. Following incubation, the mixture was packed into the column and washed with 50 mM Tris, 150 mM NaCl, 0.05% LM, pH 7.4. Protein was eluted from the matrix using competing free biotin added in the washing buffer in the final concentration of 2 mM.

Monomeric avidin was regenerated with 0.1M glycine, pH 2.8, washed with PBS till neutral pH was reached and stored in PBS with 0.02% NaN_3. However, this type of matrix could be used only up to three times, since it rapidly loses its binding capacity and has to be replaced by a fresh one.

2.2.18.3 Purification on M1 anti-Flag antibody –bound matrix

Eluate from Ni-NTA matrix was mixed with 1 mL of M1 antibody matrix prepared according to the instructions manual and equilibrated with TN buffer (50 mM Tris, 150 mM NaCl, pH = 7.4, 0.05% LM). 10 mM $CaCl_2$ was added. The mixture was incubated 2 h at 4ºC. After washing with TN buffer with 1 mM $CaCl_2$, the protein was eluted with 10 mM Flag-peptide in TN buffer.

2.2.18.4 Purification on streptavidin-sepharose

Streptavidin-sepharose was made by Ms. Eva Molsberger by coupling streptavidin produced in *E. coli,* purified and refolded, to sepharose.

10 mL of streptavidine sepharose, equilibrated with TNE buffer (50 mM Tris, 150 mM NaCl, 10 mM EDTA, 0.05% LM, pH = 7.4), was mixed with 40 mL of eluate from Ni-NTA column and with DTT in final concentration of 50 µM and incubated overnight on the rolling wheel. Material was packed into the column and washed with TNE buffer. Receptor protein was eluted with TNE buffer containing 5 mM desthio-biotin.

2.2.19 Analytical gelfiltration

Via gelfiltration (size-exclusion) chromatography, the proteins are separated according to their size.

This method was used to analyse the dispersity of the purified ET_B receptor. Superose 6 PV 3.2/30 column was used, on Smart Chromatographic station from Pharmacia, Upsala, Sweden. After column equilibration with 50 mM Tris –buffer with 150 mM NaCl and 0.05% n-dodecyl-ß-D-maltoside, 50µL of the concentrated protein sample (1-2 mg/mL) was applied. The flow rate of 50µL/min was used, and the run was carried out at 4°C.

2.2.20 Radioligand binding assay

2.2.20.1 Binding assay on membranes

The sample of cell membrane preparation was diluted with the binding buffer (see Materials) to 3μg/mL of protein and incubated with radioactive [I^{125}] ET-1 for 1.5 hours at RT. The amount of radioactivity added per sample was 100 000 cpm. Total binding was always measured in triplicate, and non-specific binding in duplicate. For non-specific binding determination, 10 μM unlabeled ET-1 peptide was added. The reaction was stopped by adding 700 μL of binding buffer and the samples were transferred to prewashed GF/C filters (blocked with 0.3% PEI) on the harvester. The filters were washed 3x with ice cold binding buffer, and then transferred to the plastic vials and put into the γ–counter for measuring.

2.2.20.2 Binding assay for solubilized receptor

Concentration of active receptor in solution was measured with the help of streptavidin-coated magnetic beads (Dynal, Hamburg). 20 μL of beads (10 mg/mL) were washed with ice-cold 50 mM Tris buffer, 150 mM NaCl, 0.1% LM, pH 7.4 and resuspended in 120 μL of the same buffer. 10 μL of solubilized receptor was added, and incubated on ice for 5 min. Radioactive [I^{125}] ET-1 was added to the mixture in the amount of 50000 cpm/sample, and the mixture incubated for 1.5 hours at RT. For non-specific binding was determination, 10 μM unlabeled ET-1 was added. After incubation, the beads were washed carefully in magnetic rack 3 × with ice-cold Tris buffer, resuspended in 200 μL of the same buffer and transferred to the vials for counting.

2.2.21 Mammalian cell culture

For the mammalian cell culture, two types of cells were used: BHK (Baby hamster kidney cells), for the recombinant SF virus production, and CHO (Chinese hamster ovary) K1 cells, for the expression of ET_B receptor. The cells were cultivated in the following medium:
Dulbecco's modified Eagle's medium (DMEM) and Iscove's 1:1, supplemented with 10% FKS and 4 mM L-glutamine. The cells were incubated in the NUNC incubator at 37°C, with 5% CO_2.

2.2.21.1 *In vitro mRNA preparation*

Plasmid DNA (10-20 µg) was linearized with appropriate restriction enzyme (NruI for recombinant DNA with receptor, SpeI for helper DNA) in total volume of 50 µL, for 2 h at 37°C. After running a 2µL aliquot on a 1% agarose gel to check for complete linearization, DNA was purified either over MicroSpin S-200 HR column (according to the instruction manual), or using QIAquick PCR purification kit. Purified DNA was used for the *in vitro* transcription.

Transcription reaction:

5 µL linearized DNA (2.5 µg)

10 µL 5xSP6 buffer

5 µL 10 mM CAP

5 µL 50 mM DTT

5 µL rNTP mix

20 µL H_2O

2 µL Rnasin (40 U)

5 µL SP6 RNA polymerase (100 U)

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50 µL

Reaction mix was incubated at 37°C for 60 min. The result was checked by running a 2 µL aliquot (+ 2µL of DNA loading buffer) on a 0.8% agarose gel.

2.2.21.2 *Electroporation of mRNA into BHK cells*

Growth medium was aspirated from a T175 flask containing confluent BHK cells. Cells were washed twice with PBS, detached with 2.5 mL of trypsine/EDTA and resuspended in 30 mL of growth medium. Cell suspension was spun down in a 50 mL Falcon tube, at 900xg for 5 min. Afterwards, medium was removed and cells resuspended in 30 mL PBS. The centrifugation was repeated as previously, and cells resuspended in the final volume of 2 mL PBS. 400 µL of cell suspension was transferred to 0.2 cm electroporation cuvette. Meanwhile, 25 µL of helper mRNA reaction was added into receptor mRNA reaction, mixed and immediately added to the cuvette. Cells were electroporated with two pulses at RT, whereas time constant was every time 0.7 after each pulse. Electroporation settings:

Capacitance extender 960

Capacitance 25 µF

Resistance &

Voltage 1500 V

The cells were transferred with Pasteur pipette to the T25 flask containing 10 mL of growth medium. Flasks were incubated 24 h at 37°C in the incubator with 5% CO_2, before the virus was harvested.

2.2.21.3 Harvesting the SF virus

Recombinant virus was harvested 24 h after electroporation. Cell medium was transferred to the 10 mL syringe and filtered through a 0.22 µM filter. Solution was aliquoted and kept at –80°C till further use.

2.2.21.4 Recombinant SF virus activation

Virus was activated by adding 500 µg of sterile α-chymotrypsine (20 mg/mL) in PBS/mL virus solution. The mixture was incubated at RT for 30 min. 250 µL of aprotinin in PBS (10 mg/mL) was added to the 10 mL of virus solution in order to inhibit the enzyme. Activated virus was stored in 1mL aliquots at –80°C.

2.2.21.5 Test infection of cells with recombinant SFV

CHO cells were seeded into 6-well plates. When they reached 70-80% confluence, the cells were washed twice with 1.5 mL PBS pro well, and infected with 500 µL of virus solution. After 1h of incubation at 37°C, 1.5 mL of growth medium was added, and the cells were incubated overnight. After washing off the virus with 2x1.5 mL PBS, cells were scraped in 1 mL of PBS, spun down for 10 min at 6000xg, pellets frozen in liquid N_2 and stored at - 20°C till further use.

2.2.21.6 Preparation of the cell lysates for electrophoresis

Prior to electrophoresis, the cells were subjected to SDS lysis and subsequently to benzonase treatment to remove nucleic acids. Cells from the 6-well plate were resuspended in PBS, counted in Neubauer chamber and the aliquot containing 5×10^5 of cells was taken. Cells were spun down at 1000g for 5 min and resuspended in 100 µL of benzonase buffer (50 mM Tris, 1.5 mM $MgCl_2$, pH 8.0) with 1% SDS. Benzonase was added (3 enzyme units/sample) and the samples were incubated on ice for 20 min. Afterwards, 15 µL of 4×loading buffer was added, and the samples loaded to the 10% SDS PA gel.

2.2.21.7 Freezing the cells

In order to obtain frozen stocks of BHK and CHO cells for the experiments, the initial cells obtained from Aventis were grown in usual growth medium till 80-90% confluence. The cells were washed twice with PBS, detached with trypsine/EDTA and spun down for 3 min at 300xg. Afterwards, the cells were resuspended in the growth medium containing 10% DMSO (for the cells from 2xT185 flasks – 5 mL). Aliquots of 0.5 mL were made in cryotubes. The tubes were let 30 min at RT, and then transferred to the polystyrene rack at –80°C for three days. The cells were finally transferred to liquid nitrogen container.

2.2.22 2D crystallization probes

Purified ET_B receptor from *P. pastoris* (see 2.2.18.2) was concentrated to 1 mg/mL and mixed with POPC, DMPC or with *E. coli* polar lipids (each stock 4 mg/mL, in 1% LM) in different ratios, ranging from 1:1.5 to 2:1. The mixtures were incubated for 1 hour at RT with mixing, and then 5 mg of Bio-beads were added. The mixtures were then incubated for additional 2 hours at RT with mixing. 1.3 µL of each sample was transferred to the grids and stained with uranyl-acetate. The samples were examined under electron microscope (CM12, Phillips) for crystalline vesicles.

For freeze-fracture (done by Dr Winfried Haase), droplets of solution were placed on specimen holders of the BAF400T freeze-fracture apparatus (Balzers, Lichtenstein) and frozen in liquid nitrogen cooled ethane. Fractured samples were shadowed with platinum and carbon and the cleaned replicas viewed in an EM208S electron microscope (FEI/Philips company).

3 Results

3.1 *P. pastoris* expression system

3.1.1 Expression vectors for ET_B receptor production in *P. pastoris*

In this work, all the clones used for human ET_B receptor production in *P. pastoris* were cloned into expression vector pPIC9K (Invitrogen Inc., San Diego). The promotor which drives expression of heterologous genes in this case was the promotor for alcohol-oxidase I (AOXI) production.

The receptor constructs FlagHisET$_B$Bio, FlagHisET$_B$Stab and FlagHisET$_B$GFP were obtained from H. Schiller, MPI for Biophysics, and the constructs FlagHisET$_B$StrepI, FlagHisET$_B$StrepII and FlagHisET$_B$TevBio were prepared in the course of this work.

All the receptor constructs have the coding region for α-mating factor prepro-peptide of S. *cerevisiae* (shortly: α - factor) fused to the N-terminal of the receptor coding sequence. The α - factor is yeast secretion signal sequence and therefore should drive the expressed receptor to the cell membrane. The protease-cleavage site is inserted between the α-factor sequence and the receptor, which is recognised by *P. pastoris* Kex-2 protease and should provide efficient α-factor cleavage. The endogenous cleavable signal sequence of the ET_B receptor (see Introduction), comprising the first 26 amino acids of the polypeptide chain, was deleted. A putative N-glycosylation site of the receptor at Asn59 was removed by mutating Asn to Ala. Also, amino acid Arg64 was mutated to alanine, thereby deleting the protease - sensitive cleavage site.

Variety of affinity tags was appended to both receptor termini in different constructs, in order to provide easy detection and purification of expressed receptor protein.

Octapeptidic Flag epitope (with amino acid sequence DYKDDDDK) was introduced at the N-terminus, for subsequent use in immunological detection and receptor purification on Flag-M2-antibody matrix. Other tags used in this work include three short tags – a decahistidine tag and two kinds of nonapeptidic streptavidin-binding tags, StrepI and StrepII. The Bio-tag, 9.7 kDa biotinylation domain of *Propionibacterium shermanii* transcarboxylase (Cronan, 1990) appended to the receptor C-terminus, enabled receptor purification on monomeric avidin and detection with streptavidin. Fusion with the green fluorescent protein coding

sequence from *Aequorea victoria* was used to enable simple determination of the expression and localization of the protein inside the cell via fluorescence.
An overview of the expression vectors described in this work is given in Fig 3.1.

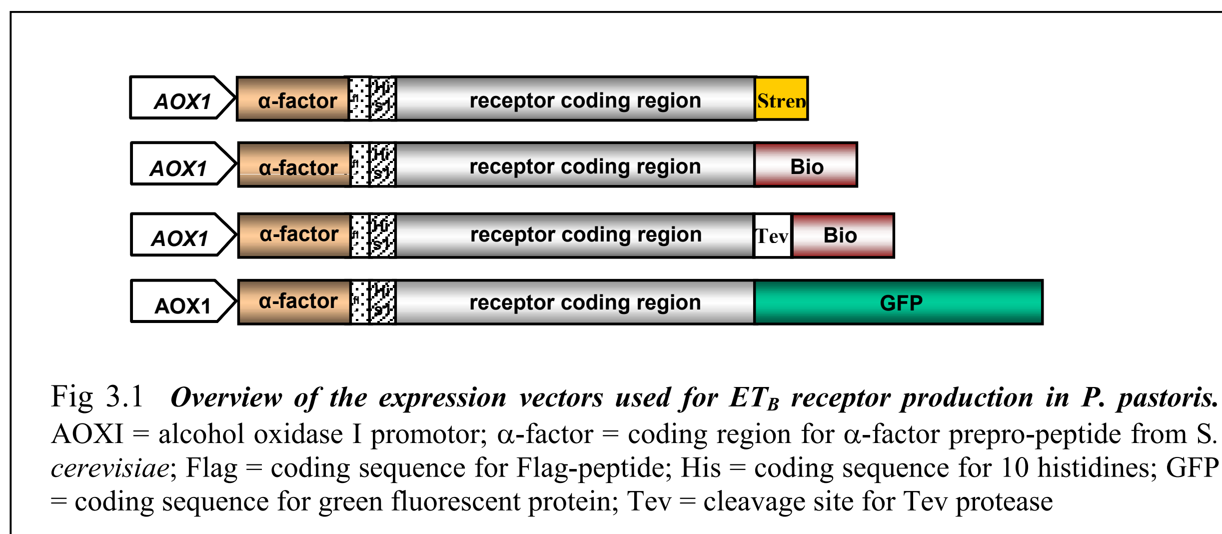


Fig 3.1 ***Overview of the expression vectors used for ET_B receptor production in P. pastoris.*** AOXI = alcohol oxidase I promotor; α-factor = coding region for α-factor prepro-peptide from S. *cerevisiae*; Flag = coding sequence for Flag-peptide; His = coding sequence for 10 histidines; GFP = coding sequence for green fluorescent protein; Tev = cleavage site for Tev protease

3.1.2 Construction of the vectors FlagHisET_BStrepI and FlagHisET_BStrepII

The Strep-tag is a nine amino-acid long tag that was developed as an affinity tool for purification of corresponding fusion proteins on streptavidin. In this work, StrepI (AWRHPQFGG) and StrepII (AWSHPQFEK) tags were used, differing in 3 amino acids and with K_D for streptavidin binding of 37 µM and 72 µM, respectively. From the vector pPIC9KFlagHisET_BBio, the Bio-tag fragment was removed by *EcoR1/Not1* digestion. Hybridised oligonucleotides $StrepI_{for}$+$StrepI_{rev}$ or $StrepII_{for}$+$StrepII_{rev}$ were ligated with the digested vector, yielding a C-terminal protein fusion gene. The sequences of the resulting vector constructs were confirmed by DNA sequencing, and the vectors were linearized with *SacI* for transformation into *P. pastoris*.

3.1.3 Construction of the vector FlagHisET_BTevBio

Because the size of the Bio-tag, 9.7 kDa biotinylation domain from *P. shermanii* transcarboxylase used in this work for purification of the ET_B receptor fusion, might negatively influence receptor crystallization trials, the vector construct FlagHisET_BTevBio

was prepared, with the possibility of Bio-tag removal after receptor purification. The new vector contains a coding region for the Tev protease cleavage site between receptor sequence and Bio-tag sequence. From the vector pPIC9KFlagHisET_BBio, the ET_B fragment was obtained by *BamH1/EcoR1* digestion and ligated into the *BamH1/EcoR1* cut vector pPIC9KFlagHisTevBio (Christoph Reinhart, MPI for Biophysics). The sequence of the resulting construct was verified by DNA sequence analysis, the DNA was linearized with *SacI* and prepared for transformation.

3.1.4 ET_B receptor production in *P. pastoris*

3.1.4.1 Receptor expression in shaking culture

It has been shown that different N-terminal and C-terminal receptor fusions influence the expression of GPCRs in *P. pastoris* (Weiss, 1998). This has been observed in the case of the ET_B receptor as well (H. Schiller, 2000 and this work). Table 3.1 shows different vector constructs used in this work and the respective quantities of active receptor expressed, measured by radioactive [^{125}I]Endothelin-1 ligand binding on the membrane preparations.
The largest amounts of the ET_B receptor per liter of *P. pastoris* culture were obtained in this work with the clone pPIC9KFlagHisET_BBio, which is in accordance with the previous findings about the positive effect of the Bio-tag on active receptor expression. These findings revealed a 6 - fold larger yield of the active receptor in the case of the clone containing the C-terminally fused Bio-tag compared to the one without it (H. Schiller, 2000). The assumption is that relatively long (9.7 kDa) Bio-tag stabilizes the receptor expressed in the yeast cell. Expression levels of *P. pastoris* receptor clones with C-terminally fused nonapeptides StrepI and StrepII were 35 pmoL/mg and 40 pmoL/mg respectively. The receptor fusions with these short affinity tags lack the stabilizing effect of the Bio-tag; therefore, lower active receptor yields were expected. Surprisingly, the introduction of the Tev cleavage site to the pPIC9KFlagHisET_BBio receptor construct lowered the yield of the active receptor to 25 pmol/mg.

Constructs	pmol of receptor/mg membrane protein	μg of receptor per liter of shaking culture
pPIC9KFlagHisET_BBio	60 ± 5	1286
pPIC9KFlagHisET_BStrepI	35 ± 5	786
pPIC9KFlagHisET_BStrepII	40 ± 3	824
pPIC9KFlagHisET_BTevBio	25 ± 4	577

TABLE 3.1 ***ET_B expression levels in P. pastoris shaking culture.*** The values given are for 1L shaking cultures of *P. pastoris*. Radioligand binding assays were carried out on the isolated cell membranes.

3.1.4.2 Production of the ET_B receptor in P. *pastoris* fermentor culture

P. pastoris is an organism extremely convenient for growing in fermentor, since this yeast tolerates cell densities up to 500 g /L of cell suspension. Therefore it is easily possible to grow large amounts of biomass (3 kg of wet weight and more) in a 10L fermentor.

For optimal control of fermentor conditions, yeast was grown in minimal medium (see Materials). Parameters such as pH, O_2 level and methanol concentration were under strict control during the whole fermentation process. Fermentor runs were carried out in a 10L fermentor, and the fermentation process was controlled using the software package BioFlo3000, New Brunswick Scientific.

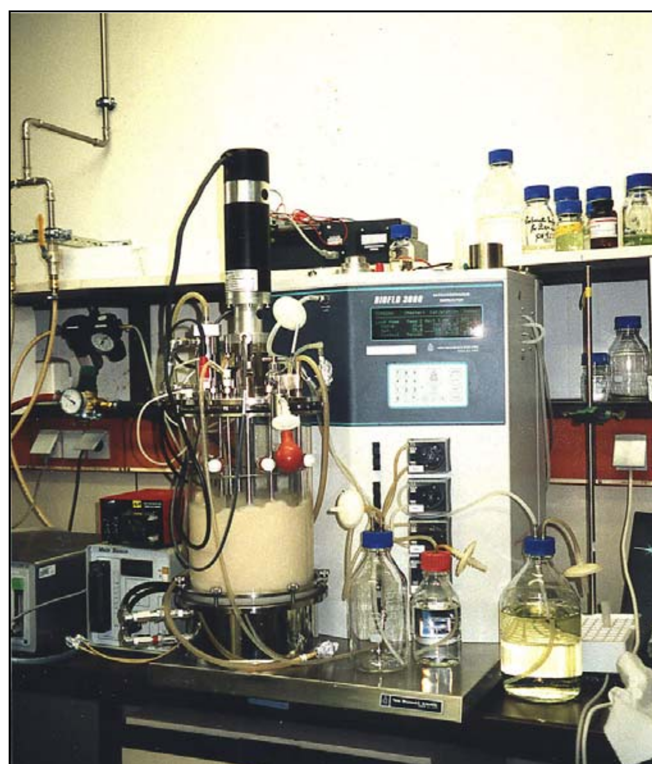


Fig 3.2 ***Fermentor BioFlo3000 during fermentation process.***

P. pastoris cells were grown on glycerol as the carbon source until they reached a wet weight of 350 g/L. Then the glycerol feed was stopped and O_2 consumption closely monitored, until a significant drop in consumption was observed. The so-called "oxygen spike" is the sign that yeast cells in the fermentor are in starvation phase. The methanol feed was started immediately after, and the methanol concentration in the fermentor was kept constant (around 0.5% (v/v)) by methanol sensor controlling. Methanol addition induced production of the receptor construct. Different induction rates of methanol feed were evaluated (2 – 6 mL/L/hour), and different induction times (10-24 h) in order to optimize receptor production in the fermentor. The transition from glycerol as the carbon source to methanol is a rather critical point in *P. pastoris* fermentation process. In this phase, a rise in pH value together with fast fluctuations in O_2 consumption is observed. Therefore oxygen in surplus was provided in order to stabilize the transition.

Fig 3.3 shows the time scale of receptor production and cell wet weight change during fermentation. The observed drop in the cell wet weight after methanol induction is the consequence of methanol toxicity. For this reason, the methanol concentration is kept as low as possible, but still high enough to provide the cells with sufficient carbon source.

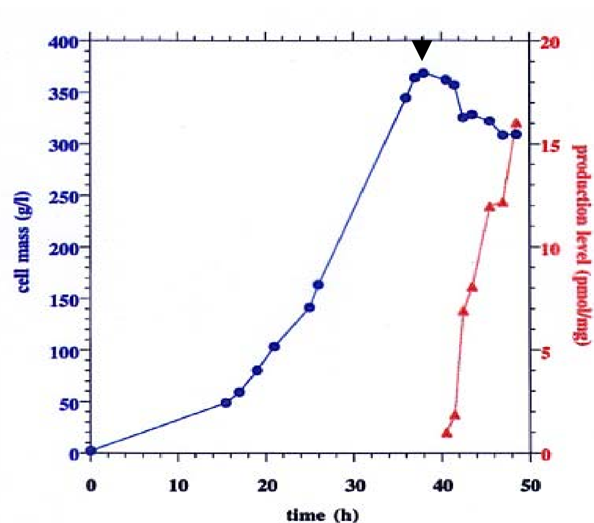


Fig 3.3 ***Receptor production in recombinant P. pastoris strain FlagHisET$_B$Bio in the fermentor.*** Blue line = cell mass changes with time. Red line = receptor production. The arrow shows start of methanol induction.

The cells were harvested 10-24 h after methanol induction. Receptor production was measured on membrane preparations of samples taken at different time points during induction. Maximal production rates of active receptor varied from 8-17 pmoL/mg in different fermentor runs. A 10 L fermentor yielded 3 kg of cells, which contained 20 - 39 mg of the ET_B receptor.

3.1.4.3 Expression of receptor fusion FlagHisET_BGFP in *P. pastoris*

Heterologous expression of receptor – GFP fusions serves as a fast and reliable method to verify receptor expression in the host cell, and to visually check for its localization.
P. pastoris cells transformed with FlagHisET_BGFP were observed under fluorescence 24 h after methanol induction. All cells showed green fluorescence, which verified the production of receptor-GFP fusion protein. However, it was obvious that the fusion protein was not localized in the cell membrane, but rather distributed inside different cell compartments.

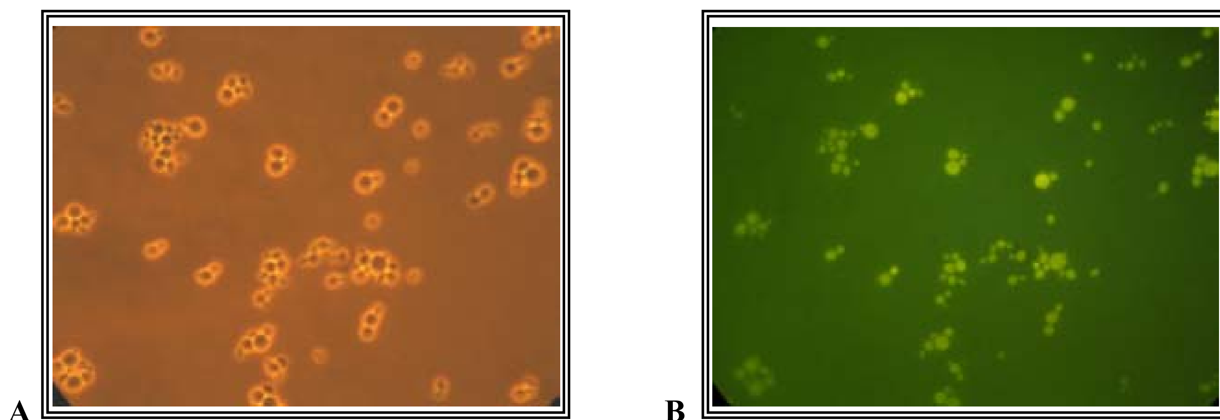


Fig 3.4 ***Expression of FlagHisET_BGFP in P. pastoris.***
A) *P. pastoris* transformed with FlagHisET_BGFP observed under light microscope B) the same field observed under fluorescence. Fluorescence excitation at 488 nm, with a 510 nm filter

3.1.5 Immunological analysis of the FlagHisET_BBio receptor construct produced in *P. pastoris*

In order to analyze the expression profile of the receptor construct FlagHisET_BBio, membranes containing the receptor construct were subjected to Western blot analysis with anti-Flag M2 antibody. As seen in Fig 3.5, two prominent protein bands could be detected.

The lower one has the apparent Mw of 55 kDa, which correlates well with calculated molecular mass of the receptor construct itself. The upper one, with an apparent Mw of 74 kDa, represents the unprocessed form of the receptor construct, where the α-factor signal peptide has not been cleaved by the *P. pastoris* Kex2 - protease. The protein band at approx 40 kDa is a result of receptor degradation.

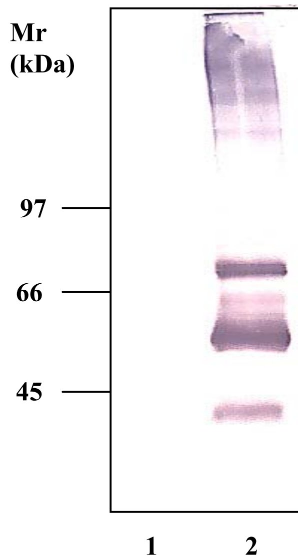


Fig 3.5 ***Immunological analysis of the FlagHisET$_B$Bio receptor construct produced in* P. pastoris**. Yeast cells expressing FlagHisET$_B$Bio were harvested 24h after methanol induction, the membrane fraction was isolated and 20 µg loaded on an SDS gel. After the protein electrotransfer to a PVDF membrane, proteins were probed with anti-Flag M2 antibody. Lane 1: control pPIC9K membranes, lane 2: FlagHisET$_B$Bio membranes.

3.1.6 Biotinylation of the receptor construct FlagHisET$_B$Bio in *P. pastoris*

In the receptor construct FlagHisET$_B$Bio, the 9.7 kDa transcarboxylase domain from *P.shermanii* was fused to the C-terminus of the receptor. As a result, Lys59 of the fusion protein should be biotinylated *in vivo*. This was the necessary prerequisite for subsequent affinity purification of the receptor construct on monomeric avidin.

To check whether FlagHisET$_B$Bio fusion undergoes biotinylation in *P. pastoris*, protein expression was induced with methanol according to the standard procedure (see Methods), membranes were prepared (see Methods) and protein biotinylation was probed with AP-conjugated streptavidin on the blotted PVDF membrane. As a negative control, the receptor construct FlagHisET$_B$Stab was used. The Stab-tag is a derivative of the Bio-tag, with a Lys59 mutated to alanine to prevent biotinylation. As seen in Fig 3.6, the receptor protein band at Mw of 55 kDa is clearly visible in the case of FlagHisET$_B$Bio, which verifies the biotinylation

of the Bio-tag in *P. pastoris*. However, no protein band corresponding to receptor fusion could be detected in the case of FlagHisET$_B$Stab.

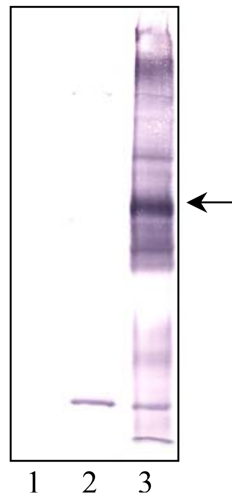


Fig 3.6 ***Western blot analysis of biotinylation of receptor construct FlagHis ET$_B$Bio in*** **P. pastoris.** 10 μg of each membrane preparation was loaded on an SDS gel. Proteins were blotted onto PVDF membrane and analyzed with AP-conjugated streptavidin. Lane 1: control membranes pPIC9K, lane 2: membranes FlagHisET$_B$Stab, lane 3: membranes FlagHisET$_B$Bio. The arrow shows the position of receptor band.

3.1.7 Concanavalin A precipitation of unprocessed receptor fusion protein

The unprocessed form of the receptor fusion protein FlagHisET$_B$Bio, with an apparent Mw of 74 kDa (see Fig 3.5), still contains the α-factor signal sequence. Owing to the fact that this signal peptide is glycosylated, precipitation with concanavalin A (ConA) was attempted. ConA is a lectin and binds sugars with high affinity. It is a dimer at pH 5.7, tetramer at pH 6.8, and at higher pH values builds non-soluble aggregates and precipitates them from the solution. The experiment was carried out as described in Methods, at pH 7.4 and Con A was used at a concentration of 1mg/mL.

Western blot analysis shows the processed form of the receptor at the apparent Mw of 55 kDa, and the four distinctive bands around 74 kDa which represent the receptor fused to differently glycosylated α - factor signal sequence (Fig 3.7, lane 1). After ConA precipitation, the correctly processed receptor band in the solubilizate is the same as in the lane 1, but only one band representing unprocessed form of the receptor could be detected. We assume that this is the receptor fused to the unglycosylated α - factor signal sequence, since it couldn't be precipitated by concanavalin A.

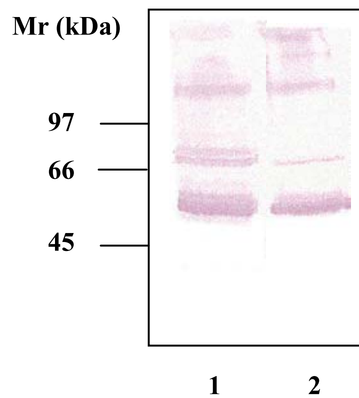


Fig 3.7 ***Concanavalin A precipitation – Western blot analysis with M2 antibody.*** Lane 1: receptor solubilizate without ConA, lane 2: solubilizate after ConA precipitation

3.1.8 Breaking of *P. pastoris* cells and large-scale membrane preparation

Yeast possess exceptionally sturdy cell wall, which is a problem when retrieving heterologously expressed, non-secreted protein from this host system.

In this work the problem of breaking large quantities of yeast cells was solved by using a cell mill-DYNO-mill KDL Typ A, in which the cells were broken mechanically by passing the cell suspension through glass beads (0.5 mm in diameter). Cell suspension in breaking buffer at a wet weight of 30% was determined to be optimal for cell breakage. Under these conditions it was possible to break 1 kg of cells in 30 min by passing a 3L suspension 3-4 times through the cell mill. Breaking efficiency, investigated under light microscope, was between 60-80%.

After the cells were broken, they were subjected to low spin centrifugation (3000g) in order to remove unbroken cells and cell debris. Since the low-spin pellets still showed [I^{125}] ET-1 binding, in order to minimize losses of expressed receptor protein the pellets were passed once more through the cell mill and then subjected to polyethylene glycol 3000 (PEG 3000) precipitation. The membranes precipitated with PEG 3000 yielded 20% of the total active receptor obtained in the whole membrane preparation after ultracentrifugation, as measured via radioligand binding assay.

3.1.9 Purification of the ET_B receptor

Membrane preparations containing active receptor produced in *P. pastoris* were incubated with equimolar quantity of ET-1 in order to stabilize the receptor in its native conformation, and then the receptor was solubilized with ultra pure 1% n-dodecyl-β-D-maltoside. It was

previously shown (H. Schiller *et al.*, 2001) that the solubilization efficiency of this detergent for the ET_B receptor, under the conditions used in this work, is 60-70%.

3.1.9.1 IMAC purification – identification of the chelated metal best suited for purification

Immobilized metal affinity chromatography (IMAC) is routinely used for the purification of His-tagged proteins. In order to test which metal ion is the most suited one for receptor purification, in the terms of highest receptor purity and least aggregation, different divalent metal ions were coupled to Sepharose following the instructions manual. The following ions were tested: Co^{2+}, Ni^{2+}, Fe^{3+} and Cu^{2+}. In parallel, two commercially available metal-coupled matrices were tested for comparison, Ni^{2+} coupled to nitrilotriacetic acid-agarose (Ni-NTA) and Talon (Co^{2+} coupled to Sepharose).

Filtered receptor solubilizate, with the receptor in complex with its ligand, was loaded to the 2 mL of each metal- bound matrix. Columns were washed first with 10 mM imidazole, then with 30 mM imidazole washing buffer (see Methods), and bound receptor was eluted with 200 mM imidazole elution buffer. Washing steps and elution fractions from each column were analyzed on silver stained gels. After the protein concentration determination, identical amounts of each fraction were loaded on the gels. Since purified receptor was in complex with its ligand, no radioactive measurements of active receptor in the elution fraction were possible.

Commercially available Ni-NTA matrix performed the best in the test; as can be seen on the silver gel (Fig 3.8A, lane 9), beside the protein band representing the monomeric form of the receptor at 55 kDa, only two impurity bands could be detected. Receptor eluted from the Talon matrix was also comparatively pure; however, in this eluate aggregated protein was visible in the upper part of the gel. (Fig 3.8B, lane 4). Interestingly enough, the "self-made" matrices loaded with the corresponding ions (Ni^{2+} and Co^{2+}) performed less well than their commercial counterparts, since they yielded less pure receptor with more aggregates (Fig 3.8A and Fig 3.8B, lane 7, respectively). Fe^{3+}-coupled sepharose eluate contained many other proteins beside the receptor (Fig 3.8B, lane 10), and proved the least suitable for receptor purification of all the metal ions tested. The Cu^{2+} matrix eluate (Fig 3.8A, lane 4) yielded a very similar elution profile as the "self-made" Ni^{2+} loaded matrix (Fig 3.8A, lane 7).

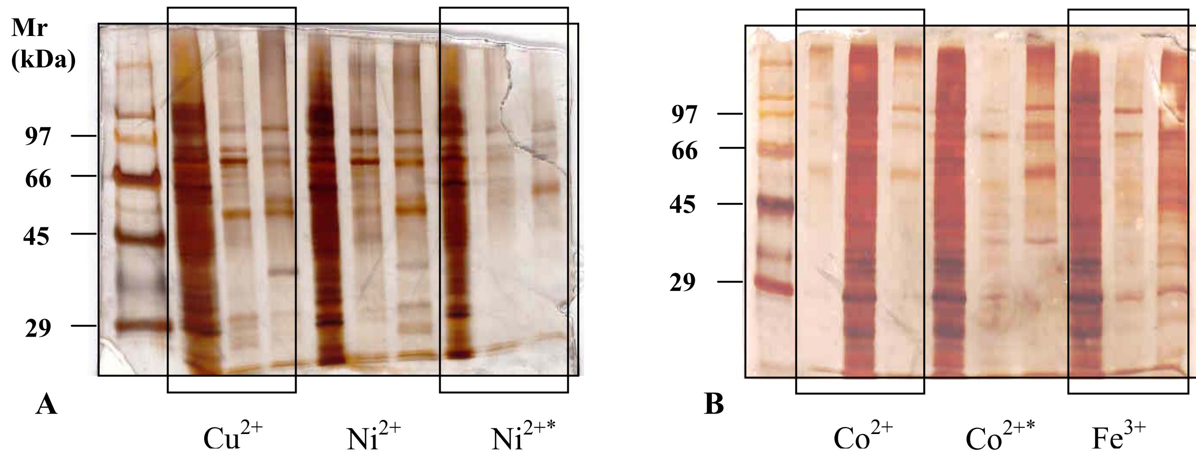


Fig 3.8 ***Different immobilized metal ions on the test for receptor affinity purification via His-tag – silver stained gel.*** For each metal – lane 1: wash 10 mM imidazole, lane 2: wash 30 mM imidazole, lane 3: eluate 200 mM imidazole. (*) – commercially available IMAC matrix

3.1.9.2 Purification on Ni-NTA matrix

The N-terminal decahistidine fusion (His10-tag) enabled the purification of the receptor FlagHisETBBio on Nickel-NTA agarose. Nitrilotriacetic acid (NTA) is a chelating group coupled to the polymerized agarose and charged with Ni^{2+}. Histidine imidazole rings from the His-tag provide interacting partners for immobilized metal ions. Elution of the His-tagged protein from the Ni-NTA matrix is possible under physiological conditions by using high concentrations of competing imidazole.

Crude receptor solubilizate was incubated with Ni-NTA matrix in batch, and subsequently washed with increasing concentrations of imidazole (first 10 mM, then with 40 mM) in order to remove weakly bound proteins. Since a portion of the receptor was eluted by the 40 mM imidazole washing step (data not shown), in subsequent purifications the second wash was performed at 30 mM imidazole concentration instead. The receptor was eluted with 200 mM imidazole.

In Fig 3.9 silver stained gel of the different purification steps is depicted. In addition to the receptor monomer at an apparent Mw of 55 kDa and the Kex2-unprocessed form at 74 kDa, many impurities were detected. The most prominent of these is the protein band at approximately 35 kDa, which represents a *P. pastoris* protein with strong affinity toward the Ni-NTA matrix, since its strong intensity was observed in all cases of receptor purification on Ni-NTA matrix.

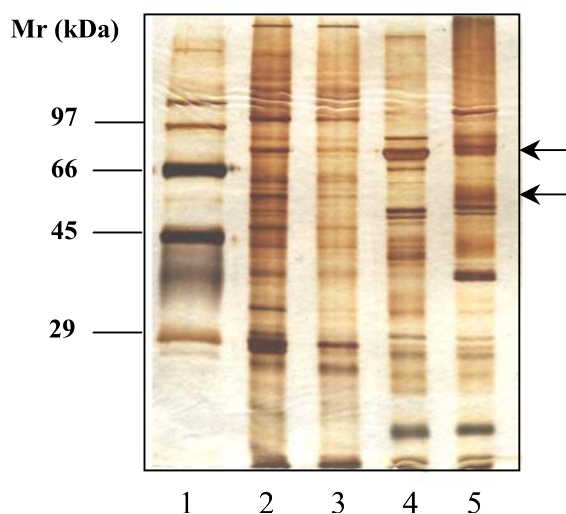


Fig 3.9 ***Silver stained SDS PA gel of receptor purification on Ni-NTA matrix***
Lane 1: molecular weight markers, lane 2: crude receptor solubilizate, lane 3: wash 10 mM imidazole, lane 4: wash 30 mM imidazole, lane 5: elution 200 mM imidazole. Arrows mark the positions of receptor processed (55 kDa) and unprocessed form (74 kDa)

3.1.9.3 Purification on monomeric avidin matrix

Avidin is a tetrameric glycoprotein with a molecular mass of 67 kDa. Its affinity towards biotin is the strongest non-covalent interaction described so far. This interaction can be interrupted only under harsh conditions, which makes avidin unsuitable for affinity purification of native proteins. Monomeric avidin proved a better candidate for this purpose, having much lower K_D for biotin (10^{-7}).

The receptor fusion protein FlagHisET_BBio binds to the monomeric avidin via its biotinylated Bio-tag fused to the receptor C-terminus.

Monomeric avidin affinity matrix was employed as a second step in receptor purification, after the Ni-NTA matrix. It is not suitable for use as a first column for receptor purification since it is expensive and has rather low binding capacity for membrane proteins in detergent.

Purification was carried out in batch, as described in the Methods section. Receptor was eluted with 2mM free biotin.

The elution fraction was concentrated in a concentrator cell with a 50 kDa cut-off to 1-2 mg/mL and its dispersion state analyzed by gel chromatography on Superose 6 resin (Fig 3.10 B). Concentrated protein was also analyzed by silver staining (Fig 3.10A).

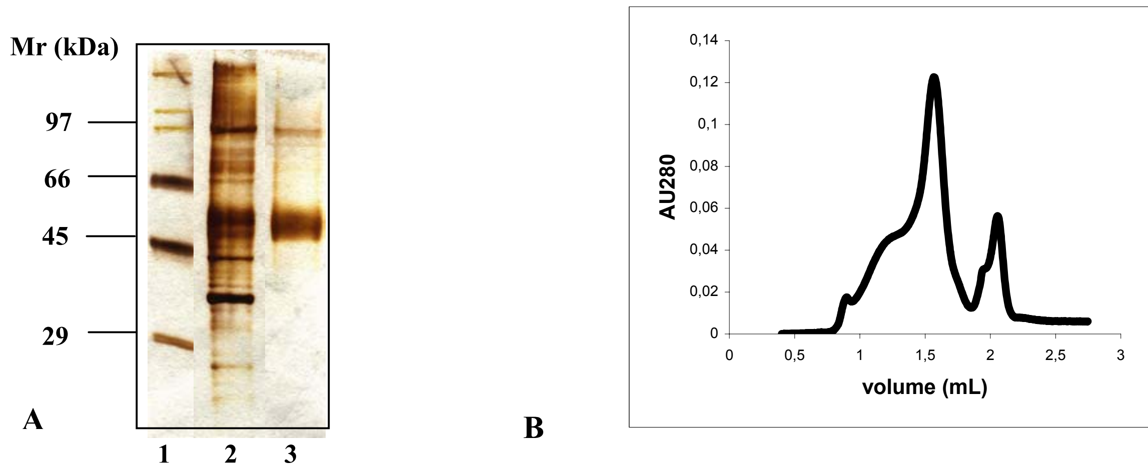


Fig 3.10 ***Purification of FlagHisETBBio on Ni-NTA and monomeric avidin matrix.***
(A) Silver stained gel – lane 1: molecular weight markers, lane 2: Ni-NTA eluate, lane 3: monomeric avidin eluate (B) Superose 6 gel filtration profile of purified receptor

On the silver stained gel, a prominent band of receptor monomer at an apparent Mw of 55 kDa could be seen. Only one more band, a low intensity protein band at approx 100 kDa could be detected. The retention profile from the Superose 6 column shows, in addition to the receptor monomeric peak (retention volume 1.61 mL), aggregated receptor with a smaller retention volume.

Since it was not possible to follow receptor purification by radioligand binding (the receptor was purified in complex with its ligand), estimation of the yield of purified receptor was made based on receptor quantity in the starting membranes and protein concentration determination in the concentrated sample of purified receptor. By this method, it was estimated that the receptor yield after purification was 32% of the starting amount.

3.1.9.4 Purification of the receptor on M1 anti-Flag antibody matrix

Receptor fusion protein FlagHisET$_B$Bio contains an N-terminally fused Flag octapeptide, which permitted receptor purification on an M1 anti-Flag antibody-bound matrix as a second purification step. Every step in the purification procedure was performed in the presence of Ca^{2+} ions, since M1 antibody binds the Flag peptide only when calcium is present in the buffer. Elution under mild conditions with competing free FLAG-peptide was employed, so as not to perturb receptor's native conformation.

The crude solubilizate was first purified on a Ni-NTA column as already described, and then mixed with 1 mL of antibody matrix and 10 mM $CaCl_2$. Chromatography was performed in batch at 4ºC. After a washing step, the receptor was eluted with 100 mM FLAG peptide.

The eluate was concentrated to 1.36 mg/mL and analysed on a Superose 6 gel filtration column and by silver staining.

As depicted in Fig 3.11B, the protein elution profile from Superose 6 shows a dominant elution peak at 0.91 mL, which most probably represents aggregated receptor. The small peak with absorption maximum at 2.04 mL represents the free FLAG peptide present in the elution fraction with the receptor protein.

Eluate analysis on the silver stained gel (Fig 3.11A) shows a monomeric receptor band at 55 kDa, the Kex2-unprocessed form of the receptor at 74 kDa and massive aggregates at higher Mw. The protein band at 35 kDa is most probably a fragment of proteolysed receptor, commonly observed in receptor preparations from yeast.

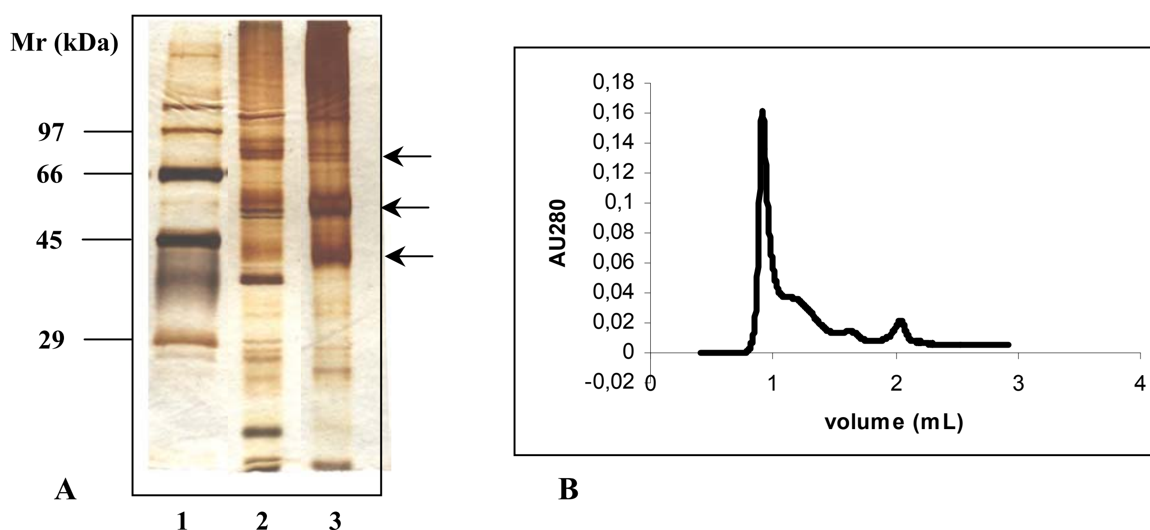


Fig 3.11 ***FlagHisET$_B$Bio purification on Ni-NTA and M1 anti-Flag matrix.***
A) Silver-stained SDS gel Lane 1: molecular weight markers, lane 2: eluate from Ni-NTA matrix, lane 3: eluate from M1 anti-Flag matrix. B) Gel filtration elution profile (Superose 6) of M1 anti-Flag concentrated eluate

3.1.9.5 Purification via Strep-tag

Purification of the receptor on a streptavidin matrix was evaluated as a possible alternative to the more expensive monomeric avidin matrix. For this purpose, the FlagHisET$_B$StrepI clone was used. The StrepI tag nonapeptide binds to streptavidin with an affinity of 34 μM, in the

same binding pocket as biotin. Therefore it is possible to employ a rather gentle elution with a biotin derivative, which competes for the same binding place on streptavidin. The receptor fusion protein was first purified on Ni-NTA matrix as already described, and then incubated with 10 mL of streptavidin-sepharose. After 2 hours of incubation and the washing step, the receptor was eluted with 5 mM desthio-biotin. The eluate was analysed by gel filtration and the silver staining. The gel filtration elution profile of the receptor purified in this way showed massive receptor aggregation (Fig 3.12), very similar in the profile and in the extent with receptor aggregates after purification on the M1 matrix (Fig 3.11). Aggregates are also observed in the eluate analyzed on silver stained gel, where they occur in the form of a broad smear at high molecular weight. The monomeric form of the receptor occurs on the gel with an apparent Mw of 45 kDa (the arrow). This purification technique was abandoned because of the extensive receptor aggregation.

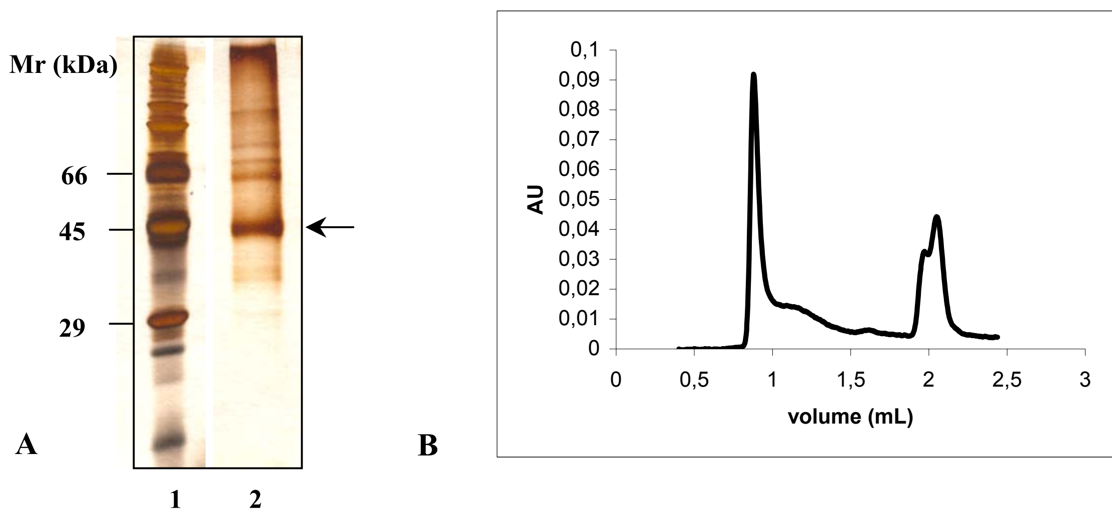


Fig 3.12 **(A)** ***Eluate analysis by silver staining*** **(B)** ***Gel filtration analysis of the receptor purified on streptavidin matrix (Superose 6).***
Lane 1: molecular weight markers, lane 2: streptavidin eluate

3.1.10 Investigating receptor aggregation

3.1.10.1 The influence of the oxidation state and differently processed forms of the receptor on aggregation

In order to investigate whether receptor aggregation is caused by receptor oxidation, non-reductive electrophoresis was run in parallel with reductive. The former contained no β-mercaptoethanol in the loading buffer. As could be seen on the silver stained gel of samples of

purified receptor with or without β-mercaptoethanol in the sample buffer (Fig 3.13), no significant difference in the receptor migration profile was observed. Particularly significant is the absence of a difference in the high molecular weight area on the gel. There, additional receptor bands would be expected to appear under non-reducing conditions due to the intermolecular disulfide bond formation. Since it is not the case, it can be concluded that receptor oxidation is probably not the cause of receptor aggregation.

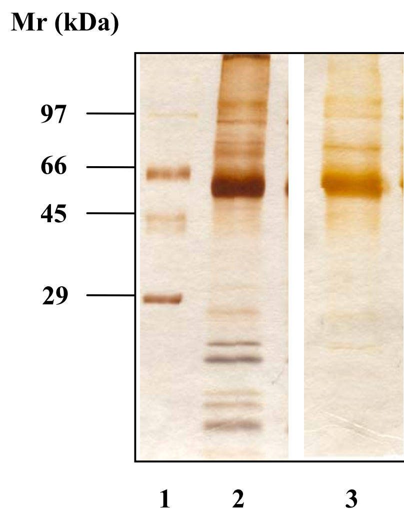


Fig 3.13 ***Silver-stained protein bands after non-reductive and reductive electrophoresis.*** Lane 1: marker proteins, lane 2: protein sample run without β-mercaptoethanol in loading buffer, lane 3: protein sample run with β-mercaptoethanol in loading buffer. 2.5 μg of protein loaded on 10% SDS gel

3.1.10.2 The effect of glycerol on receptor aggregation

Since it is commonly accepted that small amounts of glycerol have a positive influence on native proteins in solution, all buffers in the initial purification trials contained 10% glycerol. Gel filtration analysis of the monomeric avidin eluate from the initial purification trials showed several different absorption peaks at 280 nm, which points to the receptor heterogeneity with aggregation. When the glycerol was omitted from the buffers during receptor purification, subsequent gel filtration analysis of the purified receptor showed the predominant monomeric form of the receptor, although the aggregates were still present (Fig 3.14B).

On the other hand, when receptor purification was carried out with 30% glycerol in the buffers, gel filtration elution profile of the purified protein showed massive aggregation of the receptor (Fig 3.14A).

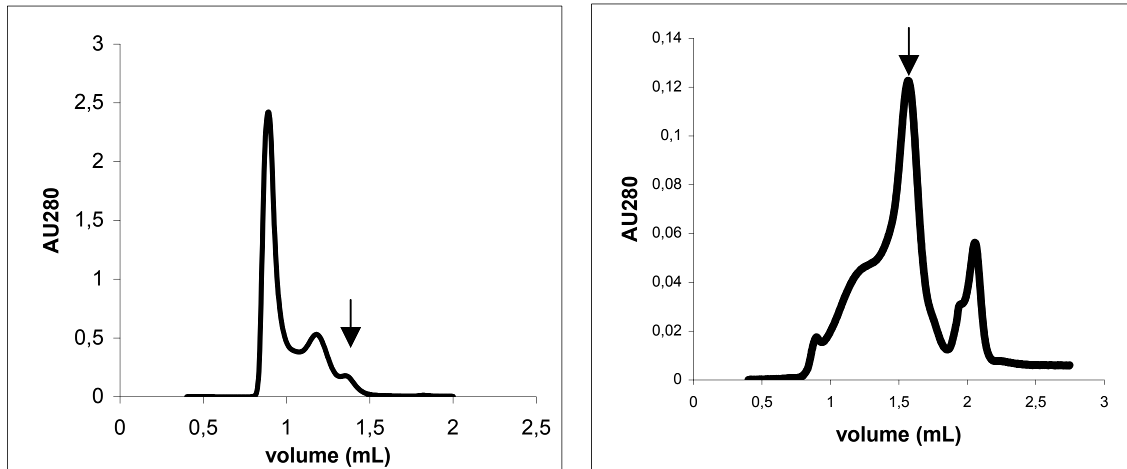


Fig 3.14 ***The effect of glycerol on receptor aggregation***.
Gel filtration analysis (Superose 6) of the purified receptor with (A) 30% glycerol, (B) 0% glycerol. The arrow shows monomeric form of the receptor

3.1.10.3 The effect of buffer additives on receptor aggregation

In order to address the problem of receptor aggregation, it has been tried with addition of many different substances during FlagHisET$_B$Bio receptor fusion purification on Ni-NTA and monomeric avidin affinity matrices.

Short-chain lipids (1,2-dicaproyl-sn-glycero-3-phosphocholine, 0.2%), physical chaperones ectoine and hydroxyectoine (1% each), cholesterol-hemisuccinate (0.5%) and DMSO (2%) were all tested in the hope of reducing the aggregation of solubilized receptor. The additives were present in the buffer in the given concentrations during the entire purification procedure, and were added before receptor solubilization with n-dodecyl-β-D-maltoside. Superimposed Superose 6 gel filtration elution profiles of purified receptor from those trials are depicted in Fig 3.15.

None of the additives helped in obtaining a fully homogeneous purified receptor form.

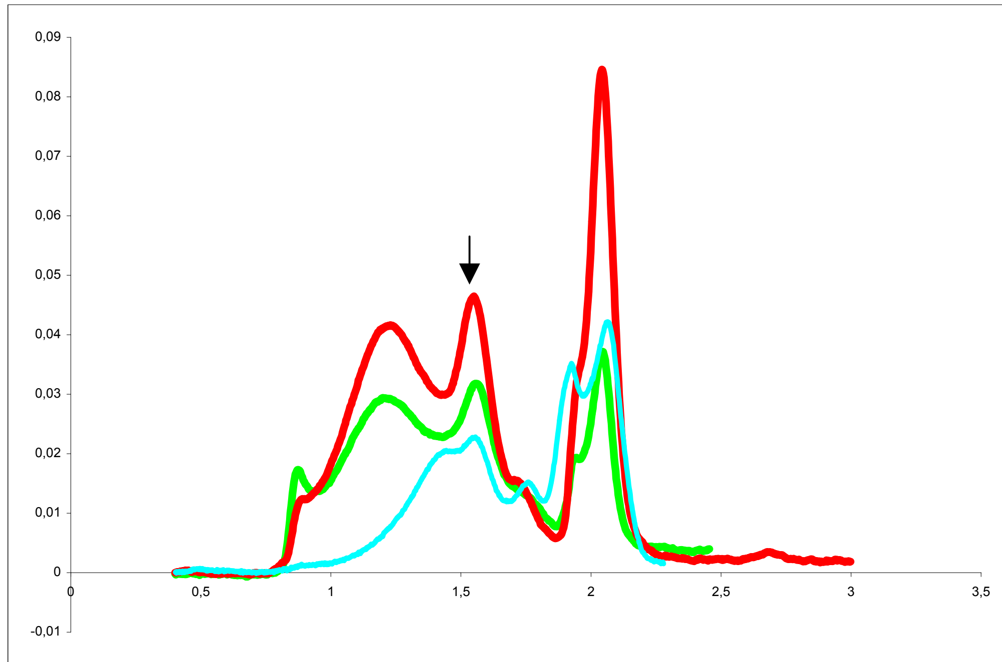


Fig 3.15 ***Superose 6 gel filtration analysis of receptor purified with different buffer additives***. Blue line – Cholesterol-hemisuccinate; green – DMSO; red – ectoines. The arrow shows the position of receptor monomeric form, with the retention volume of 1.61 mL

3.1.10.4 Blue native electrophoresis of the purified receptor

Native electrophoresis was used to further investigate the homogeneity and oligomerization state of the purified receptor sample. The advantage of this method is that the protein retains its native state, since electrophoresis is carried out in the absence of SDS detergent. The blue native electrophoresis technique developed by Schägger&von Jagow (Schägger and von Jagow, 1991) especially for membrane proteins was used. In this system, 6-amino-caproic acid provides the ions – gel current carriers, and Coomassie Brilliant Blue binds to the proteins giving them a uniformly negative charge. A sample of the receptor fusion protein purified by Ni-NTA and monomeric avidin affinity chromatography (see 3.1.9.3) was subjected to electrophoresis. BSA was used as a marker protein, since it exists as monomer (66 kDa), dimer (132 kDa) and trimer (198 kDa) under native conditions. The gel was stained with Coomassie Blue following the standard procedure (see Methods) to visualise the protein bands. The most prominent receptor band is found at Mw ~130 kDa on the gel, indicating the predominant existence of dimerized receptor in the sample. Much weaker band at ~180 kDa could be detected, indicating the trimeric form of the receptor. Since it was possible to visually follow the protein band separation and migration during the run because of the nature

of the running buffer (it contained Coomassie Brilliant Blue), it should be noted that no monomeric form of the receptor was detected during electrophoresis.

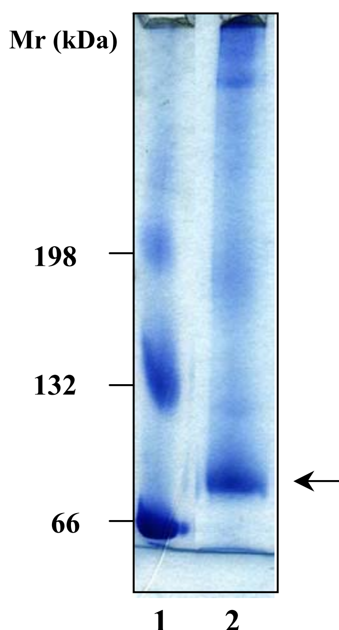


Fig. 3.16 ***Blue native electrophoresis of ET_B receptor fusion protein.*** Lane 1: BSA, 15 µg, lane 2: purified receptor protein, 20 µg. Arrow shows the position of receptor dimer band

3.2 Expression of the ET_B receptor in insect cells

3.2.1 Time scale of receptor production in Baculovirus expression system

A suspension culture of *Spodoptera frugiperda* (Sf9) cells was infected with recombinant virus pVlMelHisET_BBio (titre 1.39 × 10^8 p.f.u/mL) at a MOI = 10. After 0, 24, 36, 42, 48, 54, 60, 66 and 72 h cell aliquots were taken, cells pelleted by centrifugation and the pellets stored at - 80°C. The membrane preparations were subsequently subjected to radioligand binding measurements in order to establish the level of receptor production.

The results show that receptor production in Sf9 cells starts between 24 h and 36 h after virus infection, and reaches its peak approximately 66 h after infection.

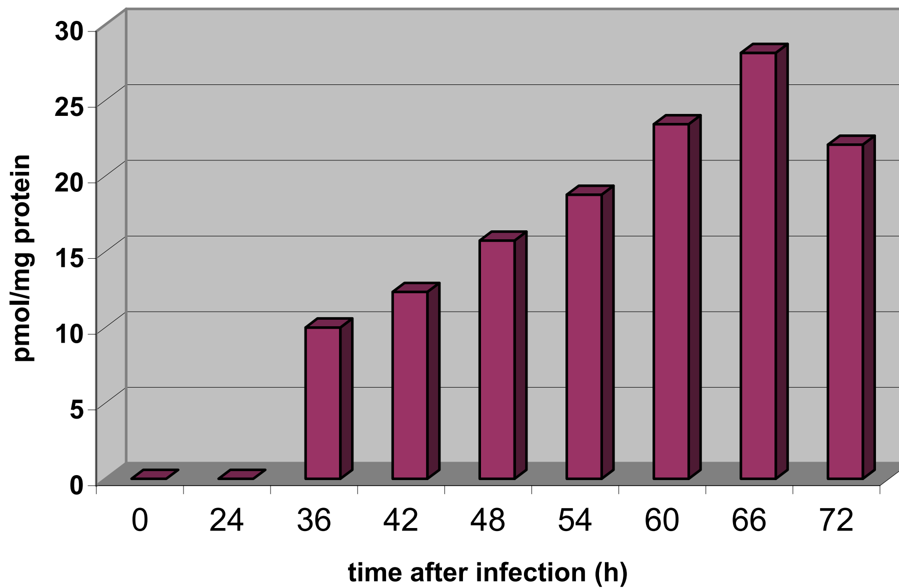


Fig 3.17 ***Dependence of receptor production levels in Sf9 cells on the time after infection***. Mean values with standard deviation are shown, out of three independent radioligand binding measurements with [I^{125}] endothelin-1 on membrane preparations

3.2.2 Expression of ET_BGFP fusion in insect cells

Expression of receptor-GFP fusion represents a fast and easy way to visualize receptor expression in the host system. For this purpose, pVLMelFlagHisET_BGFP recombinant baculovirus (H. Reiländer, MPI for Biophysics) was used. Adherent Sf9 cells were infected with recombinant virus and observed 72 hours post infection by fluorescence microscopy.

As depicted in Fig 3.18, nearly all the cells showed fluorescence. Different cell fluorescence intensities can be explained by different number of recombinant viruses which infected each cell, since the multiplicity of infection was >1. Most of the expressed protein was observed in the cell membrane. Diffuse fluorescence was observed in the cell cytoplasm as well, which means that part of expressed receptor fusion stayed intracellularly.

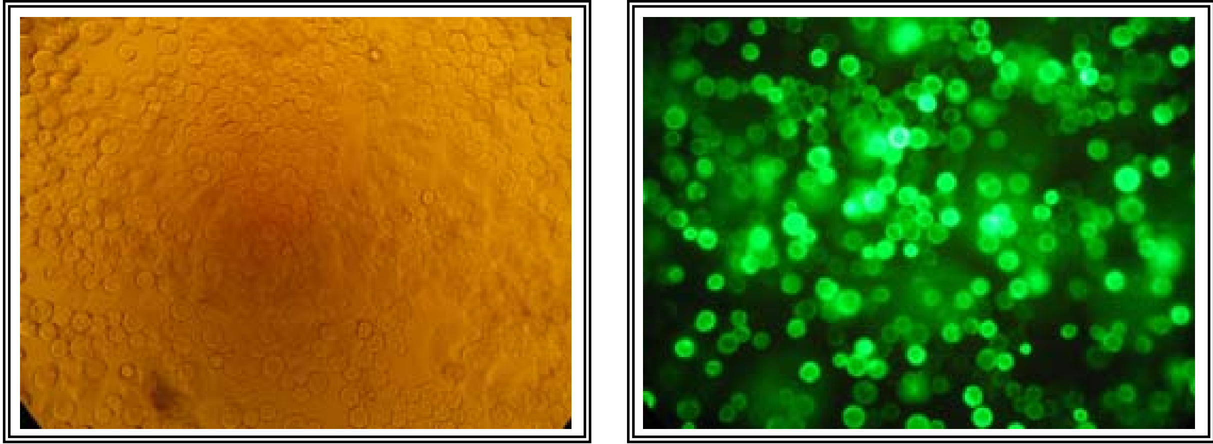


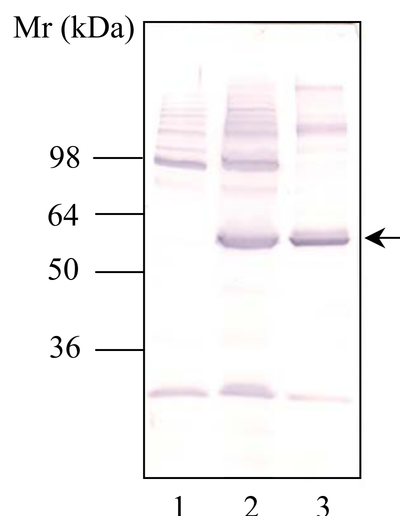
Fig 3.18 ***Sf9 cells expressing ET_B-GFP fusion.***
a) Sf9 cells infected with pVlMelFlagHisET_BGFP observed under white light with phase contrast b) the same cells observed under fluorescent light. Fluorescence excitation at 488 nm, with the filter at 510 nm

3.2.3 Immunological analysis of ET_B receptor produced in insect cells

Sf9 cells infected with pVLMelFlagHisET_BBio recombinant baculovirus were harvested 66 h after infection, an aliquot was taken for Western blot analysis and from the rest membranes were prepared according to the procedure described in Methods. Benzonase-treated cell extract (see Methods) from approx 50 000 Sf9 cells was loaded on a 10% SDS gel, together with 20 µg of membrane preparation, and after electrophoretic separation, transferred to PVDF membrane. Immunological analysis was performed with anti-Flag M2 antibody in order to confirm receptor expression and with streptavidin-AP conjugate to confirm biotinylation of the receptor construct.

A single band at an apparent Mw of 55 kDa representing monomeric receptor was detected in the cell extract of Sf9 cells infected with the pVLMelFlagHisET_BBio recombinant virus (Fig 3.19, lane 2). Control uninfected Sf9 cells show no immunostaining at the same apparent Mw. Western blot analysis with streptavidin shows the protein band in the cell extract of infected Sf9 cells at the same apparent Mw of 55 kDa (Fig. 3.20). Since the streptavidin-AP conjugate recognizes only receptor with a biotinylated Bio-tag, it could be concluded that the receptor fusion protein undergoes biotinylation in Sf9 cells.

Fig 3.19 ***Immunological analysis of FlagHisETBBio produced in Sf9 cells.***
Western blot analysis with anti-Flag M2 antibody. Lane 1: Sf9 cells, lane 2: Sf9 cells infected with pVlMelFlagHisET$_B$Bio, lane 3: membrane preparation from Sf9 infected with pVlMelFlagHisET$_B$Bio. The arrow shows the ET$_B$ receptor band position.



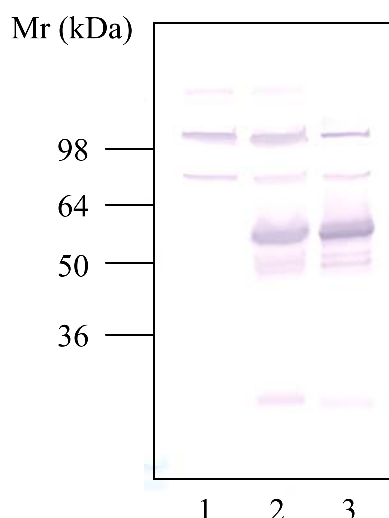


Fig 3.20 ***Biotinylation of FlagHisET$_B$Bio produced in Sf9 cells.*** Western blot analysis with streptavidin – AP conjugate. Lane 1: Sf9 cells, lane 2: Sf9 cells infected with pVlMelFlagHisET$_B$Bio, lane 3: membrane preparation from Sf9 infected with pVlMelFlagHisET$_B$Bio

3.2.4 Solubilization of the ET$_B$ receptor from insect cell membranes

Different detergents were tested in order to find one that yielded the highest amount of active solubilized receptor. Non-ionic, mild detergents – maltosides with different carboxydrate-chain lengths of and Fos-cholines were used at the final concentration of 1% to solubilize membranes of Sf9 cells expressing FlagHisET$_B$Bio (only the detergent Fos-16 was used at the final concentration of 0.1%, because of its low CMC, and the digitonin/cholate mixture was 1.25%/0.3%). Membranes were incubated with detergents for 2 h at 4°C, the suspension centrifuged at 100 000g for 1h and the supernatant containing solubilized receptor was taken for further analysis. The amount of solubilized active receptor was determined based on [I^{125}] endothelin-1 binding to the receptor bound to streptavidin-coated beads. Radioligand binding assay showed that n-dodecyl-β-D-maltoside (LM), lauryl-sucrose (LS) and digitonine/cholate solubilized the comparatively largest amounts of active receptor (Fig 3.21). These detergents

yielded nearly equal amounts of solubilized active receptor, whereas the Fos16 detergent was less efficient. The detergents Fos 12 and Fos 14 solubilized negligible amounts of active receptor, as well as octyl-glycoside. The corresponding solubilized receptor bands for these detergents on the Western blot are less intensive (Fig 3.22, lanes 3, 8 and 9), so it can be concluded that they are generally less efficient in receptor solubilization. Western blot analysis with anti-Flag M2 antibody shows a single band of solubilized receptor at an apparent Mw of 55 kDa in each detergent solubilizate.

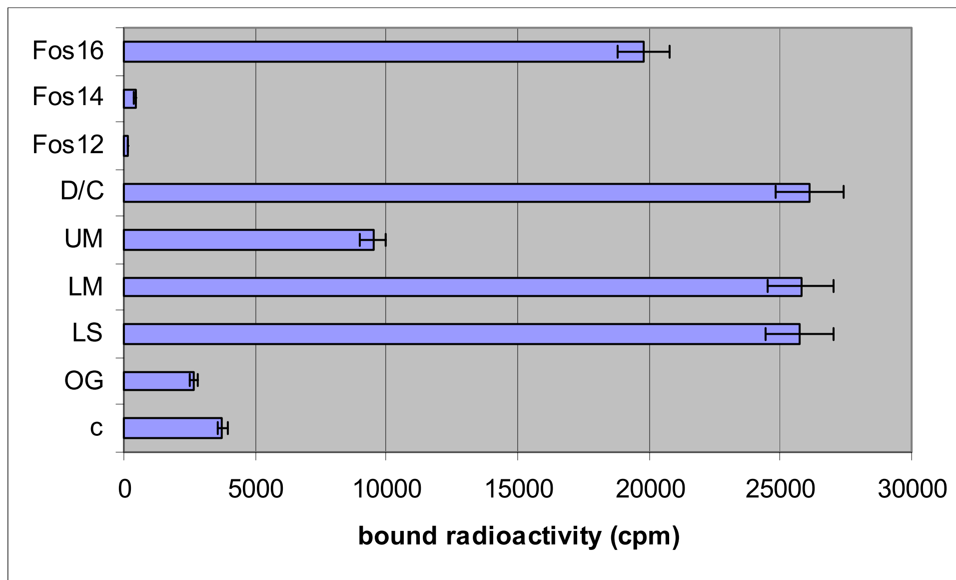
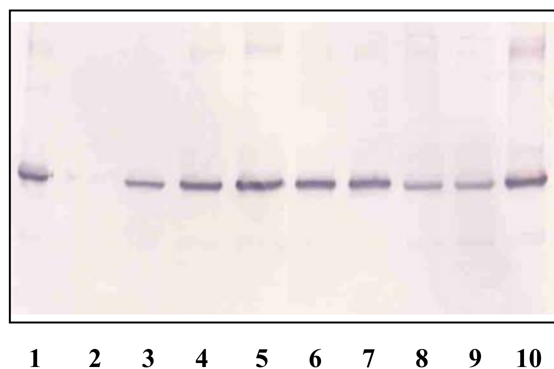


Fig 3.21 **Efficacy of different detergents in the ET_B receptor solubilization from the membranes of Sf9 cells.** The amount of active receptor in the solubilizate was determined by [I^{125}] ET-1 binding on the receptor bound to the streptavidin beads.



1 2 3 4 5 6 7 8 9 10

Fig 3.22 **Western blot analysis of solubilized ET_B in different detergents.**
Lane 1: FlagHisET_BBio membrane preparation, lane 2: empty, lanes 3-10: Octyl-glucoside, LS, LM, UM, Digitonin/Cholate, Fos12, Fos14 and Fos16 solubilizates respectively.

3.2.5 Purification of ET_B receptor produced in insect cells

Membranes of the Sf9 cells producing FlagHisET_BBio were solubilized with 1% n-dodecyl-β-D-maltoside, which was chosen as one of the three best performing detergents tested (see previous chapter) and the solubilized receptor subjected to two-step affinity chromatography. The receptor was again purified in complex with its ligand, ET-1, to render it more stable during the purification process. First, receptor was purified on Ni-NTA matrix. After washing steps with 10 mM and 30 mM imidazole, bound receptor protein was eluted with 200 mM imidazole. Monomeric avidin was used as the second affinity matrix. The Ni-NTA eluate was incubated in batch with this matrix and during the washing step a low molecular impurity copurified on the Ni-NTA matrix could be removed (Fig 3.23A, lane 6, 7). Following this method, receptor was purified to high homogeneity. A single receptor band was obtained when the eluate was analysed on a silver gel (Fig 3.23A, lane 8, 9).
The aggregation state of the receptor in the eluate was analyzed via Superose 6 gel filtration. The elution profile depicted in Fig 3.23B shows a single symmetric peak with an elution volume of 1.61 mL, which we assume is a monomeric form of the ET_B receptor within a detergent micelle.
Since the receptor was purified in complex with its ligand ET-1, the radioligand activity measurements on the purified receptor were not possible.

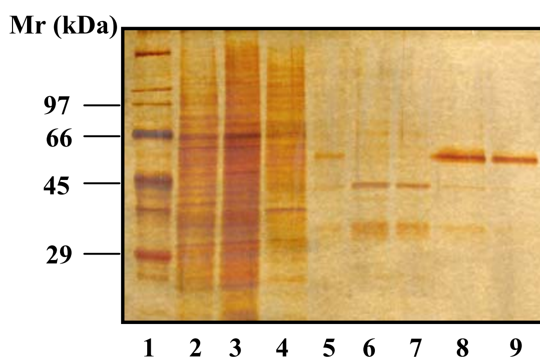
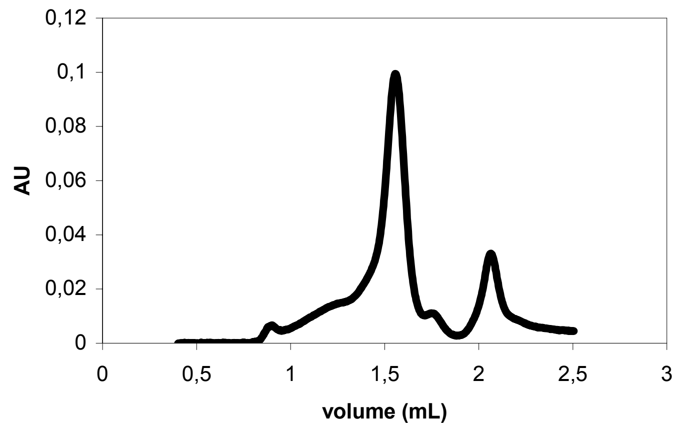


Fig 3.23 ***Purification of FlagHisET_BBio from insect cells.*** A) Different steps during purification analyzed on a silver stained gel – lane 1: molecular weight markers; lane 2: solubilizate; lane 3: wash 10 mM imidazole; lane 4: wash 30 mM imidazole; lane 5: elution 200 mM imidazole; lane 6,7: wash monomeric avidin; lane 8: eluate monomeric avidin; lane 9: eluate from (7) concentrated 40-fold with 50 kDa cut-off membrane.

B) Elution profile of purified receptor on Superose 6.



3.3 Expression of ET_B in mammalian cells

3.3.1 Construction of the expression vectors pSFV3CAPET$_B$His and pSFV3CAPET$_B$GFP

In this work, two different vector constructs for ET_B expression in mammalian cells were prepared: pSFV3CAPET$_B$His and pSFV3CAPET$_B$GFP. The expression vector pSFV3CAP was provided by Dr. Christoph Reinhart, MPI for Biophysics. The ET$_B$His fragment was obtained by *BamH1/EcoR1* digestion from the pPIC9KFlagET$_B$His expression vector, gel purified and ligated into the *BamH1/EcoR1* digested pSFV3CAP vector. The ET$_B$GFP fragment was obtained by *BamH1/Not1* digestion from the vector pPIC9KFlagET$_B$GFP, gel purified and ligated with the pSFV3CAP DNA. The sequence of the resulting expression vector constructs was verified by dideoxy - DNA sequencing.

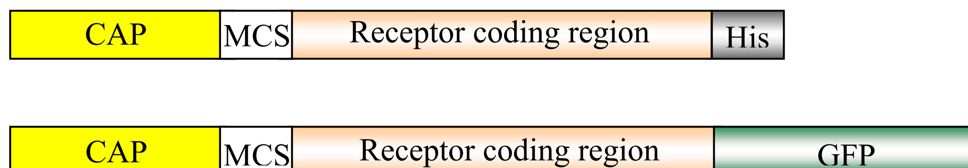


Fig 3.24 ***Vector constructs for ET_B expression in mammalian cells.*** CAP = capsid protein coding sequence; MCS = multiple cloning site; His = decahistidine fusion; GFP = green fluorescent protein from *Aequorea victoria*

3.3.2 Expression of ET_BGFP fusion protein in BHK cells

In order to visually investigate receptor expression in adherent BHK cells, the cells were infected with pSFV3CAPET$_B$GFP recombinant SFV. The cells were infected at 70% confluence and observed under the fluorescence at different time points after infection. The fluorescence in the cells could be observed already 8 hours after virus infection, but the strongest intensity was observed 24 h after infection. Seventy-two hours after infection, no fluorescence could be detected in BHK cells. In the Fig 3.25B, diffuse fluorescence throughout the cell could be seen, indicating that the expressed receptor is found in different cell compartments.

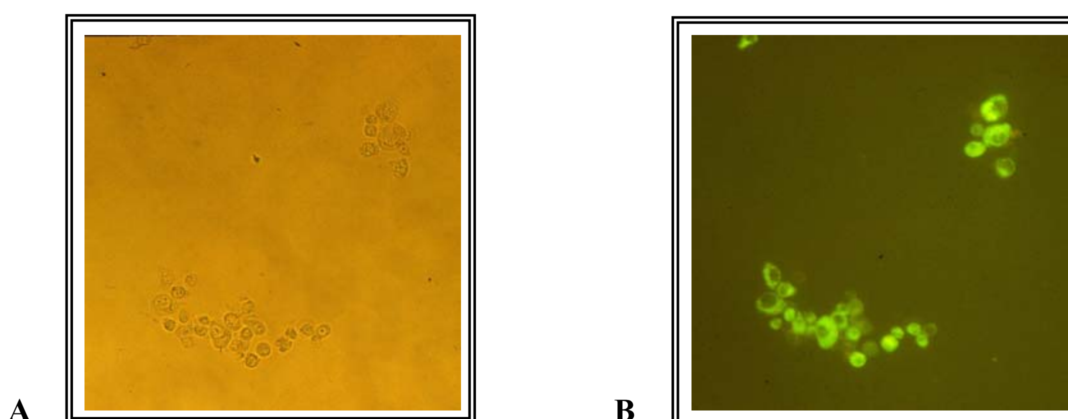


Fig 3.25 ***Expression of ET_BGFP fusion protein in BHK cells.*** a) BHK cells expressing ET_BGFP fusion 24 h after infection, observed under white light with phase contrast b) same section of BHK cells expressing ET_BGFP fusion, observed under fluorescence. Fluorescence excitation at 488 nm with 510 nm filter

3.3.3 Expression of ET_B receptor in CHO cells

Adherent CHO cells were infected with recombinant virus pSFV3CAPET$_B$His and harvested 24 h after infection. Western blot analysis with anti-His monoclonal antibody showed three bands in the CHO cells infected with the virus. The protein band at an apparent Mw of 80 kDa can be explained as the receptor fusion with CAP protein, which has a Mw of 30 kDa. The protein band at 50 kDa corresponds to the full-length ET_B receptor. Somewhat lower band at

approx 40 kDa may correspond to shortened ET_B receptor, which underwent proteolytical cleavage.

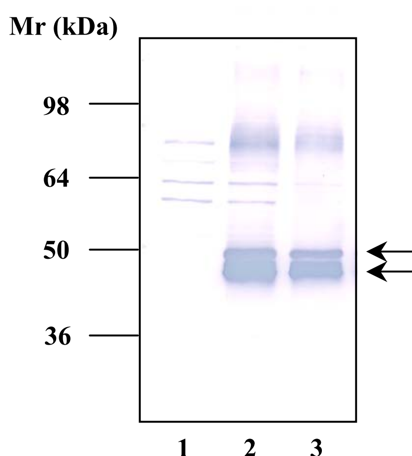


Fig 3.26 ***Western blot analysis of His-tagged ET_B receptor expressed in CHO cells.*** Lane 1: CHO cells; lane 2: CHO cells infected with pSFV3CAPET$_B$His, 8 µL; lane 3: the same as (2), 4 µL.

The maximal expression rate achieved in CHO cells was 7 pmoL/mg protein, as measured by radioligand binding assay with [I^{125}] ET-1.

3.4 2D crystallization trials

The ET_B receptor (construct FlagHisET$_B$Bio) produced in *P. pastoris* was purified on Ni-NTA and monomeric avidin matrix (see 3.1.9.3) and used in 2D crystallization trials in final concentration of 1.0 mg/mL. Crystallization set-up probes contained the protein mixed with different phospholipids (DMPC, POPC, *E. coli* polar lipid mixture) in 1% LM, as described in Methods chapter. Detergent was slowly removed by adsorption with Bio-beads, and negatively stained probes were investigated by electron microscopy.

In all the probes vesicles could be detected, ranging in size from 50-700 nm. The largest vesicles, some of which were multilayered, were found in the probes with DMPC (Fig 3.27) and POPC. In the sample with *E. coli* polar lipids, 4-5 fold smaller vesicles were found compared to the other two lipids. However, none of them looked crystalline. Between the vesicles, protein precipitates were frequently found. The question was whether the protein was incorporated into the vesicles at all, which was hard to judge on negatively stained vesicles.

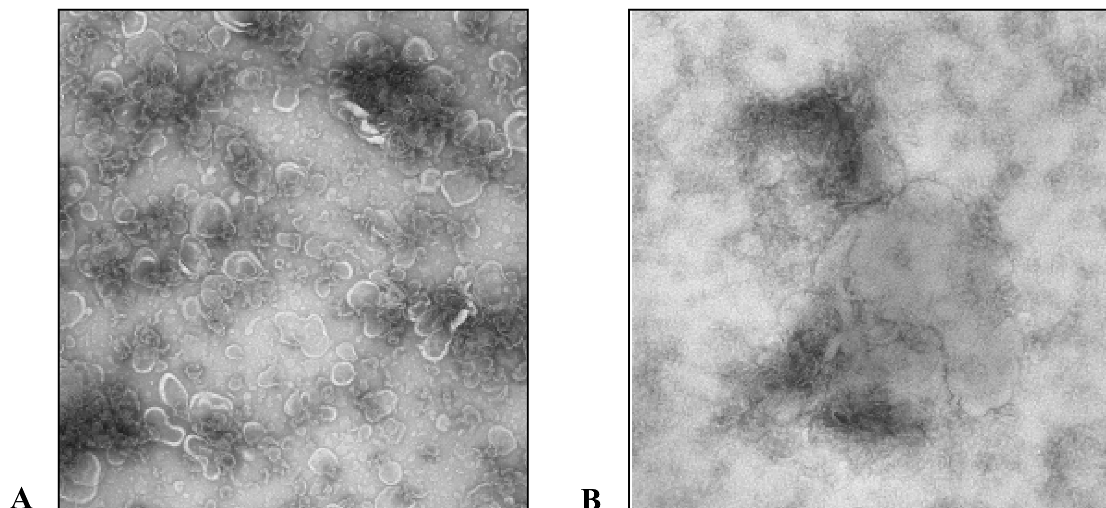


Fig 3.27 ***Electron micrographs of phospholipid vesicles in crystallization set-ups.***
A) vesicles in protein sample with DMPC B) multilayered vesicle in the DMPC sample. The protein concentration was 1 mg/mL, and the protein:lipid ratio 1.3:1

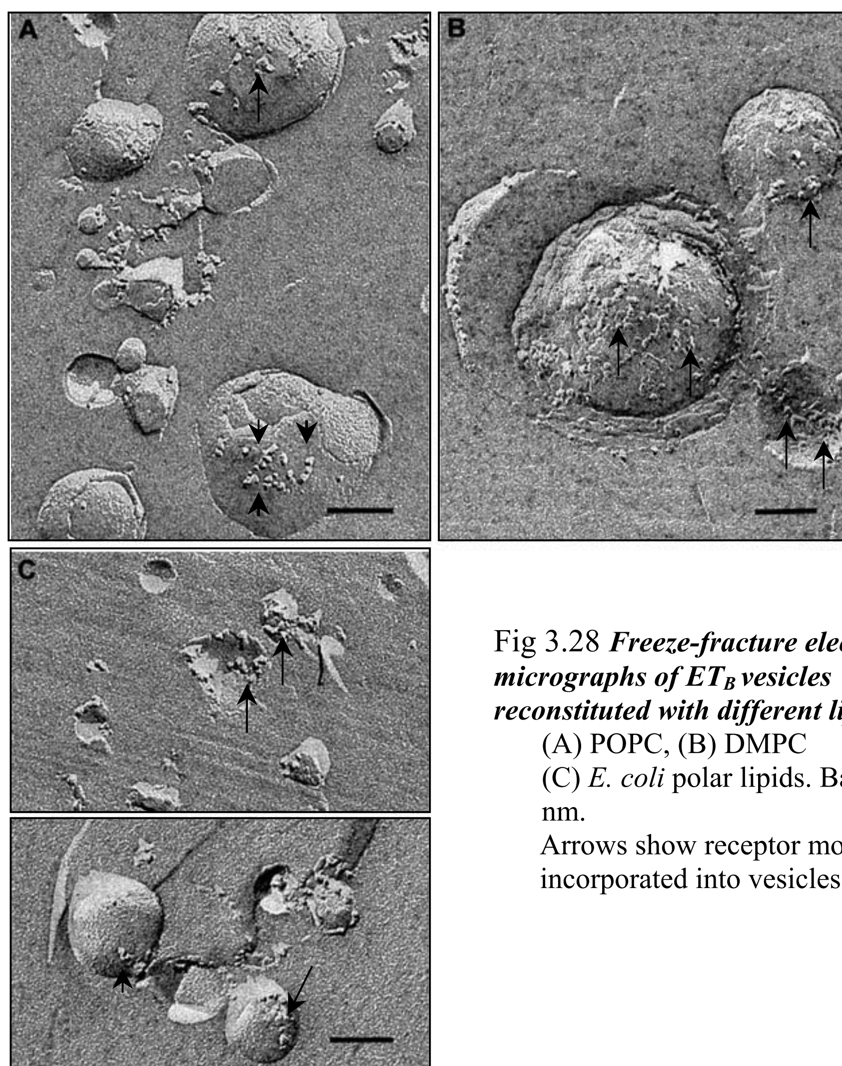


Fig 3.28 ***Freeze-fracture electron micrographs of ET_B vesicles reconstituted with different lipids.***
(A) POPC, (B) DMPC
(C) *E. coli* polar lipids. Bars 100 nm.
Arrows show receptor molecules incorporated into vesicles.

In order to verify the presence of the receptor in lipid vesicles, freeze-fracture experiments were performed. Freeze-fracture of the vesicles (kindly done by Dr. W. Haase) showed protein incorporation in all the samples where vesicles were found (Fig 3.28). Especially large number of multilayered lipid vesicles with a high density of inserted protein was found in the sample with DMPC lipids. A characteristic example of such a vesicle is presented in the Fig 3.28B.

No crystalline surfaces within any of the vesicles were found.

4 Discussion

The first crystal structures of membrane proteins were obtained with proteins isolated from naturally abundant sources, and recombinant proteins still represent minority of the membrane proteins that have been crystallized to date. In the case of soluble proteins, over 90% have been crystallized using recombinant protein, which is in contrast to the 21 recombinant of 50 total structures for membrane proteins (Loll, 2003). Only three mammalian membrane proteins to date have had crystal structures determined using recombinant protein: prostaglandin H_2 synthase-2 (COX-2), produced in insect cells (Barnett *et al.,* 1994; Kurumbail *et al.,* 1996), monoamine-oxidase B, produced in yeast (Binda *et al.,* 2002) and fatty acid amide hydrolase, produced in bacteria (Bracey *et al.,* 2002). All three proteins are monotopic membrane proteins and therefore have little hydrophobic surface. It should also be noted that the only GPCR crystallized to date, bovine rhodopsin, was obtained from the natural source, from bovine retina where it is found in large amounts. These statistics reflect the fact that active recombinant membrane proteins are difficult to obtain.

It is not surprising that heterologous production of membrane proteins poses big challenges. Unlike their cytoplasmic counterparts, membrane proteins have a longer way to the fully functional form. Once their synthesis begins, the complicated secretory machinery is engaged in order to achieve proper targeting and insertion into to the membrane. The major part of protein synthesis yield in many cells are cytoplasmic proteins; such cells have no sufficient capacity to handle large amounts of newly synthesized membrane proteins. Therefore, when produced in a heterologous system, membrane proteins can saturate the secretory pathway of the cell, leading to protein aggregation inside different cell compartments.

Therefore, the choice of expression system is a crucial step towards obtaining large amounts of receptor in its active form. Devising a successful strategy for its subsequent purification is nonetheless important.

4.1 Comparison of receptor production in different hosts

The endothelin B receptor was produced during the course of this work in three different expression hosts: yeast *P. pastoris*, Sf9 insect cells and mammalian CHO cells.

The highest receptor yield was obtained using yeast as a host; the best expressing clone, FlagHisET_BBio, with an expression of 60 pmol/mg of membrane protein, yielded 1.2 mg of receptor/L shaking culture. By fermentation of the same *P. pastoris* clone in the 10L

fermentor, it was possible to obtain 3 kg of yeast cells, which contained 20-39 mg of the receptor.

The quantity of active ET_B receptor produced in Sf9 insect cells with a maximal expression of 30 pmol/mg protein was 0.7 mg receptor/L cell culture. This value is in accordance with previously reported data obtained from insect cells (Doi *et al.*, 1997). In this report, wild-type ET_B receptor was produced at 100 pmol [I^{125}] ET-1 binding activity/mg of membrane protein, whereas the deletion of the first 36 receptor amino acids lowered the expression to 30 pmol/mg. Compared to receptor production in yeast, the receptor yield from the insect cells is lower and the system handling is more time-consuming, but the choice of this host can be rewarding in terms of obtaining homogeneous receptor preparation after purification.

The expression of active ET_B receptor of 7 pmoL/mg in adherent CHO cells is lower than expected**,** taking into account that the vector construct containing the capsid protein sequence (pSFV3CAP) was used for receptor production. The capsid gene contains a translation-enhancement signal on its 5' end, which reportedly resulted in 5-fold higher expression of human neurokinin 1 receptor compared to a construct lacking the capsid protein (Lundstrom *et al.*, 2001).

CHO cells are certainly financially the most challenging host for receptor production, and with an expression level for the ET_B receptor of 7 pmol/mg achieved in this work, can not be recommended for large scale receptor production.

It should be mentioned that the most often used host for protein production in general, *E. coli,* was not evaluated as a host for ET_B production in this work, but earlier trials of receptor production in *E. coli* yielded 41 binding-active receptors/cell (Haendler *et al.*, 1993). This amount of the active receptor obtained is too low for *E. coli* to be seriously considered as a competitive candidate for large-scale heterologous expression of the ET_B receptor.

4.2 Production of the ET_B receptor in *P. pastoris*

The ET_B receptor constructs used for production in yeast have various affinity tags fused to the receptor in order to allow its easy detection and purification.

The amount of active receptor obtained from *P. pastoris* is strongly dependent on the vector construct used for transformation. It was previously reported that the highest yields of the ET_B receptor were obtained with clones containing the Bio-tag fusion (H. Schiller *et al.*, 2000), which was verified during the course of this work. The same report showed that, on the other

hand, the introduction of a His-tag at the receptor C-terminus dramatically lowers the yield of active receptor. Therefore, all the vector constructs used in this work had N-terminally fused decahistidine tag. The highest amount of active receptor in this work - 60 pmoL/mg membrane protein - was obtained for the FlagHisET_BBio receptor fusion. Previously reported expression level for the same clone was 40 pmoL/mg (Schiller *et al.,* 2000). The clones FlagHisET_BStrepI and FlagHisET_BStrepII, prepared in order to enable receptor purification on streptavidin, had expression levels between 35-40 pmol/mg membrane protein. Here the stabilizing effect of the relatively large Bio-tag on the expressed receptor was abolished by having much shorter, nonapeptidic Strep-tags fused to the receptor C-terminus and therefore the yield of active receptor was lower.
Unexpectedly, the introduction of the 8 amino acid-long Tev protease cleavage site to the receptor clone FlagHisET_BBio, yielding the clone FlagHisET_BTevBio, lowered the yield of active receptor to 25 pmol/mg.

4.3 Large scale expression of ET_B in *P. pastoris* yeast cells

After testing several ET_B receptor constructs, the receptor fusion FlagHisET_BBio was chosen for obtaining large amounts of protein for purification. The reasons were the high expression rate in *P. pastoris* of 60 pmol/mg of membrane protein, and the presence of three different affinity tags, Flag- and His10 on the N-terminus and Bio-tag on the C-teminus, which were used for subsequent receptor detection and purification.
The highest expression rates of this receptor clone were achieved in shaking culture, where upon methanol induction up to 1.2 mg of receptor per liter of culture were obtained.
Fermentor culture of the same receptor clone, however, had a lower expression yield. It has been attempted, by applying different methanol induction rates and different induction times, to increase the overall yield of the receptor in the fermentor, but the maximal expression achieved was 17 pmol/mg of membrane protein. This is three-fold lower than expression of the same clone in the shaking culture. Still, in total, the 10L-fermentor yielded 39 mg of receptor protein. Since larger amounts of receptor were obtained by fermentation of a same receptor clone (H. Shiller, personal communication), fermentation of FlagHisET_BBio needs further optimization in order to achieve the receptor production level as in the shaking culture.

4.4 Protein localization after heterologous expression

Expression of the receptor fusion with GFP in *P. pastoris* and BHK cells clearly showed that a large portion of expressed receptor could be found intracelullarly. This is in accordance with previous findings on ET_B and other GPCRs produced in the same host. Although all the vector constructs for ET_B receptor production in *P. pastoris* have the N-terminally fused α-factor signal sequence from *S. cerevisiae*, which naturally leads to protein secretion and drives protein transport to the plasma membrane (Romanos, 1995), only a small fraction of the receptor produced was actually found in the yeast plasma membrane. One reason could be that the overproduced receptor is a burden for the secretion machinery in the yeast cell, and only small amount of produced protein is transported to the membrane.

Immunogold electron microscopy revealed that ET_B receptor produced in *P. pastoris* was mainly located intracelullarly (Schiller *et al*., 2000). Interestingly enough, this report showed that, in the case of expression of the receptor fusion FlagET_BGFP, the receptor was found in bulky vesicular structures consisting of stacked membranes. When the localization of the same receptor fusion lacking a glycosylation site was analysed, these structures were not found. Very similar observations were made for other GPCRs expressed in the same host (Reiländer, 2000).

From the experiments with the ET_B-GFP fusion expressed in BHK cells, it seems that the majority of the receptor expressed is localized in the cell interior and not on the cell plasma membrane. This is also the case with the β_2 adrenergic receptor (Darui Huo, personal communication) and with the human neurokinin 1 receptor (Lundstrom *et al*., 2001), both expressed in the same host. It has been previously reported that during the course of transient expression of human neurokinin 1 receptor in mammalian cells after infection with recombinant Semliki Forest virus, 80% of the expressed receptor was found intracellularly, but was active (Lundstrom *et al*., 2001).

The endothelin B receptor expressed in Sf9 insect cells infected with pVlMelFlagHisET_BGFP recombinant baculovirus shows localization in the plasma membrane of the insect cell, although part of the expressed receptor could still be found in the cell interior. It seems that the signal sequence of the insect protein mellitine, N-terminally fused to the receptor construct, is efficient in driving receptor transport to the plasma membrane of Sf9 cells.

4.5 Biotinylation of FlagHisET$_B$Bio receptor fusion

Biotinylation *in vivo* on the transcarboxylase domain of *P. shermanii* (Cronan, 1990), C-terminally fused to the receptor, represents an artificially introduced receptor post-translational modification. Since the amino acid sequences that lead to *in vivo* biotinylation of the protein are highly conserved in different organisms (Samols *et al*., 1988), it occurs also in *P. pastoris* and in insect cells.

Quantitative measurements of the biotinylation rate in *P. pastoris* for the β_2 adrenergic receptor fusion β_2ARBio showed 95 ± 5% biotinylation efficiency (Reinhart, 2000).

Biotinylation efficiency in insect cells was quantitatively investigated in the case of Bio-"tagged" β_2 adrenergic receptor (50 ± 10%) (Reiländer, 2000) and serotonine 5HT$_{5A}$ receptor, where biotinylation rates between 25-50% were measured (Röhrig, 1997).

Biotinylation of ET$_B$ receptor fusions from both *P. pastoris* and insect cells could be easily confirmed by immunoblot analysis with streptavidin (see Results). This biotinylated "tag" was used in this work for the purification of receptor fusion protein on avidin matrix, its immunological detection during different purification steps and for receptor radioligand binding assays on streptavidin-coated beads.

4.6 Large scale membrane preparation from *P. pastoris* cells

With the introduction of the cell mill Dynomill KDL A, it was possible to mechanically break large amounts of yeast cells in a relatively short time (1 kg wet weight of cells were broken in 30 min with 60-80% efficiency). Additionally, with repeated passage of the low-spin pellets through the cell mill and subsequent membrane precipitation with PEG 3000, it was possible to recover additional membranes, which contained up to 20% of the total active receptor.

However, the bottleneck in membrane preparation lies in the subsequent high spin centrifugation step at 100 000g, which is performed in order to pellet the yeast cell membranes. Large volumes of supernatant obtained after removal of unbroken cells and cell debris had to be centrifuged in a Beckman Ti45 rotor, which has a maximum capacity of 410 mL per run. With 2.5 L of low spin-supernatant to be centrifuged, membranes to be pelleted and then pelleted again after washing, this remains the most time-consuming step in the course of large scale membrane preparation.

4.7 Partial Kex-2 protease processing of the receptor produced in *P. pastoris*

Western blot analysis of the receptor produced in yeast showed the presence of two receptor forms in the membranes: correctly processed receptor with a Mw of 55 kDa, and the receptor form still containing the α-factor signal sequence with a Mw of 74 kDa. This indicates that the α-factor cleavage by the yeast Kex-2 protease was not complete and yielded a heterogeneous receptor preparation. An attempt was made to remove the unprocessed portion of the receptor with concanavalin A, based on the fact that the α-factor signal sequence is glycosylated. However, it was not possible to completely remove the unprocessed receptor from the solubilizate, since a portion of unprocessed receptor fused to the non-glycosylated α-factor could not be precipitated with Con A.

4.8 The exceptional characteristics of ET_B-ligand complex in comparison to other GPCRs

The endothelin B receptor binds its ligand, ET-1, with a K_D 7 pM, which is an exceptionally tight (Takasuka *et al*., 1991) and the complex reportedly survives even in 1% SDS at low temperature. The ligand-receptor complex is stable in the cell for at least 2 hours, although it reaches the lysosome within 30 min after internalization. A full explanation for this phenomenon has not yet been found. It can be attributed in part to the role of ET_B as a clearance vehicle for ET-1 from circulation, taking into account that endothelin's lethal dose is 15 μg/kg body weight and that its levels in human plasma must therefore be strictly controlled.

Since binding of the ligand renders the receptor more stable and less prone to aggregation (Doi *et al*., 1997, H. Schiller *et al*., 2001), receptor in this work was always purified in complex with ET-1. However, the disadvantage of this approach was that radioactive ligand binding measurements of the active receptor quantities during different purification steps were not possible.

4.9 Protein aggregation

The tendency of the ET_B receptor produced in yeast to aggregate represented the most serious obstacle in obtaining the homogeneous protein preparation required for crystallization

attempts, and considerable efforts were made during this work to find a solution for this problem.

There are many possible causes of receptor aggregation, and a number of them were addressed in the course of this work.

GPCRs are aggregation prone proteins since they have large hydrophobic domains. With soluble proteins, it is quite common that aggregation is mediated by non-specific interaction of hydrophobic surface areas between monomers. The presence of detergent and the relatively low ionic strength of the buffers used in this work should minimize this kind of interaction.

Aggregates can also be formed as a result of receptor oxidation, since receptors possess free cysteines (the ET_B receptor has 16 in total; one in extracellular loop, six in the transmembrane domain and nine in intracellular loops) in its polypeptide chain. When this is the case, such aggregates can be easily observed on polyacrylamide gels under non-reducing *vs.* reducing conditions. Since the same protein band pattern was obtained in both cases, it can be concluded that receptor aggregation is not due to the formation of intermolecular disulfide bridges between receptor molecules. During purification, 50 μM DTT was used as a reducing agent. There is a possibility that this reduces the naturally occurring disulfide bridge in the receptor (A. Oksche, personal communication), but no difference in the receptor aggregation pattern was observed when purification was performed in the presence and absence of DTT.

Yet another cause of receptor aggregation could be the Ni^{2+} ions released from the Ni-NTA affinity matrix during purification. These ions could concurrently bind several His-tags fused to receptor molecules and in this way cause receptor aggregation. For this reason, in this work 10 mM EDTA was added to every protein eluate from Ni-NTA matrix.

Because it is commonly accepted that glycerol has a positive effect in stabilizing proteins in solution, initial receptor solubilization and purification were always performed in the presence of 10% glycerol. However, after aggregation problem was observed, many of the receptor purification parameters were changed. Omitting glycerol from the buffers resulted in an increased level of the monomeric receptor form and in fewer receptor aggregates. Conversely, when receptor purification was carried out in the presence of 30% glycerol, the receptor was mainly found in its aggregated form (see Results section). It seems that glycerol promotes aggregation of the ET_B receptor, and it was subsequently omitted from further receptor purification protocols.

Delipidation of the receptor protein could be yet another cause of its aggregation, since lipids have stabilizing effect on membrane proteins. Since it has been shown that during purification

of the ET_B receptor solubilized with n-decyl-ß-D-maltoside, the receptor undergoes total delipidation (Doi *et al.*, 1997), it seemed a reasonable idea to try to provide external lipids. Purification in the presence of short-chain lipids - 0.2% 1,2-dicaproyl-*sn*-glycero-3-phosphocholine was attempted, hoping that it could have protective role against receptor aggregation, since lipids bind to exposed hydrophobic receptor domains and prevent their interaction. Unfortunately, short-chain lipids didn't give any positive results in stabilizing the receptor in its native form. Addition of 1% cholesterol-hemisuccinate (a water-soluble derivative of cholesterol) during receptor purification gave the same result.

Yeast membranes have a different lipid content, qualitatively and quantitatively, than those of mammalian cells. The major difference is that yeast membranes contain ergosterol, which gives them the necessary rigidity. In mammalian cells, cholesterol has this role. It has been tried to change yeast membrane lipid composition-ergosterol was replaced with cholesterol, in order to create more natural membrane surrounding for the overexpressed receptor. This approach resulted in even more receptor aggregation than usual.

In addition, both ectoins and hydroxyectoins were evaluated as buffer additives in the hope of stabilizing the receptor monomer form. These compounds are physical chaperones present in extreme halophilic bacteria. Ectoins are amphoteric, water-binding organic molecules that form large hydration shells and reportedly stabilize proteins and nucleic acids in extremophiles in their native state (Galinski, 1993). Therefore, they were added to all solutions used during receptor purification, to 1% w/v, but no profound effect on preventing receptor aggregation was observed.

Further experiments in this direction are required, in order to identify a substance or a mixture of substances which can stabilize the receptor in its monomeric form or aid in resolving the receptor aggregates once they form.

4.10 Receptor dimers?

With regard to the large amount of recently published data on GPCR homo- and heterooligomerization (see Introduction), the question of ET_B receptor oligomers emerged.

In this work, blue native electrophoresis of monomeric avidine eluate showed, apart from a portion of receptor aggregates which couldn't enter the gel, a prominent band with an apparent molecular mass corresponding to receptor dimer. However, further experiments are needed to obtain a definite proof of this finding, since the native electrophoresis could

produce artefacts in certain cases. Additionally, the use of non-membrane proteins as molecular weight markers for native electrophoresis of membrane proteins has to be validated.
It should be noted that it was not possible to obtain a reliable calibration curve for membrane proteins on a Superose 6 gel filtration column, where the precise relation of the Mw of a solubilized protein and its retention volume can be established. Whereas such a calibration curve for soluble proteins is easily obtained, it seems that for solubilized membrane proteins the size of the detergent micelle they are in is different for different proteins. So, the retention time on a gel filtration column depends for a membrane protein not only on the molecular weight, but also on the size of the detergent micelle. Therefore this method could not be reliably used for dimerization analysis of the ET_B receptor.
To date, there is no published data on endogenous homodimerization of the ET_B receptor, and it is believed that the functionally active form of the receptor is monomer. However, one report on ET_A-ET_B functional heterodimerization (Harada *et al.*, 2002) hints that new findings on ET_B receptor dimerization are to be expected.

4.11 Affinity purification of the endothelin B receptor

Affinity purification is the most commonly used purification method for GPCR proteins, and the most efficient one. There are two different approaches: affinity purification of the receptor based on receptor-ligand interaction, or based on affinity tags fused to the receptor protein. Using the first approach, so-called ligand affinity chromatography, Doi and co-workers purified the ET_B receptor in complex with biotinylated endothelin on avidine (Doi *et al.*, 1997). Because of the costly endothelin peptide, this kind of purification is not suitable for large-scale.
Two-step affinity purification based on affinity tags fused to the receptor protein is a more suitable approach for obtaining milligram quantities of pure protein. In this work, a combination of IMAC and monomeric avidin chromatography proved most appropriate for purifying large amounts of receptor protein.
Immobilized metal affinity chromatography is recommended as a first step in receptor purification, because of its robustness and low cost. Large amounts of membrane solubilizate can be loaded on this kind of matrix without a problem and the protein is eluted under physiological conditions with an excess of imidazole, which can be subsequently removed by

dialysis, if needed. In this work, Ni^{2+} as a metal ligand (compared to Co^{2+}, Fe^{3+} and Cu^{2+} ions), performed best in receptor purification.

Monomeric avidin was used for the second step in receptor purification. Avidin is a homotetramer, which binds biotin with very high affinity (Kd = 10^{-14}). This interaction is, in addition to that between streptavidin and biotin, one of the strongest known non-covalent interactions. This makes it an unsuitable candidate for protein affinity purification, since very harsh conditions have to be employed in order to disrupt the interaction and elute the protein. Avidin dissociation into monomers leads to a conformation change in each of the subunits, which lowers their affinity toward biotin (Kd = 10^{-7}), and makes it possible to employ mild elution conditions with a solution containing free biotin. In this work, a C-terminally fused Bio-tag containing *in vivo* biotinylated lysine enabled purification of receptor fusions on a monomeric avidin matrix. This purification step yielded receptor at greater than 95% purity as judged from silver stained gels. However, this is an expensive matrix, with a relatively low binding capacity and which can't be efficiently regenerated after its use for membrane protein purification in the presence of mild detergents.

Unfortunately, all attempts to find an alternative for the use of monomeric avidin (streptavidin, M1 anti-Flag antibody matrix) were not fruitful, mainly because of massive receptor aggregation that occurred during purification on these affinity matrices.

4.12 Double receptor band after heterologous expression in CHO cells

Immunoblot analysis of the ET_BHis10 receptor produced in CHO cells revealed a major double band with an apparent molecular weight between 40-50 kDa and one protein band of 80 kDa.

The minor band at 80 kDa most probably represents full-length receptor protein fused to capsid protein (Mw 30 kDa). Although the capsid protein undergoes autocatalytic cleavage upon expression of the fusion with receptor, it could be that the self-cleavage is not complete. A very similar result was obtained with the β_2 adrenergic receptor when produced using the same expression vector (Darui Huo, personal communication).

The major double band seen by Western blot analysis of His-tagged protein reveals the existence of two receptor species, one with a molecular weight of 50 kDa and one of approximately 40 kDa. The 50-kDa receptor band represents the full-length receptor, whereas the presence of the 40-kDa receptor band may indicate N-terminal proteolysis by proteases.

Although the protease cleavage site (R64 ↓ S65) in the receptor sequence was removed by mutating Arg64 to alanine, it is possible that the receptor still undergoes N-terminal degradation. This is in accordance with previously reported findings that this modification does not prevent receptor proteolysis in HEK293 cells and that the cleavage appeared to be independent of the receptor sequence (Grantcharova *et al.*, 2002). The physiological role of N-terminal receptor cleavage is as yet unclear. However, it has been reported that this proteolysis does not destroy receptor function or overall structure, as it is the case with the vasopressin V_2 (Kojro and Fahrenholz, 1995) or the β_2 adrenergic receptor (Jockers *et al.*, 1999).

Since no double band was observed at 80 kDa, it is possible that the fusion with capsid protein protects the receptor from proteolytic cleavage at its N-terminus.

The radioligand binding on the membranes of CHO cells infected with pSFV3CAPET$_B$His recombinant virus showed 7 pmol of active receptor binding/mg of membrane protein. The possible reason for such a low yield of active receptor in the CHO cells could be that the majority of produced receptor is in its inactive state, incorrectly folded, because of saturated protein processing and translocation machinery.

4.13 2D crystallization trials

The initial step in 2D protein crystallization is the reconstitution of the protein into lipid vesicles (Rigaud *et al*, 1995). For this to be accomplished, detergent removal is a prerequisite. Dialysis is the most often used method for detergent removal. However, because of its low critical micellar concentration (CMC), this method was not particularly suitable for n-dodecyl-β-D-maltoside used in this work for receptor solubilization. For this reason, removal of detergent by adsorption to Bio-beads was the method of choice. A disadvantage of this procedure for fast detergent removal is that the precise control of detergent removal or detergent concentration measurement is not possible. Therefore special effort was made to find a balance between the amount of the Bio-Beads used and the length of time they were present in the solution.

For the lipid reconstitution of the purified ET$_B$ receptor, vesicles were prepared using the polar *E. coli* lipids, DMPC and POPC. Although the freeze-fracture sections of the vesicles clearly showed protein incorporation, none of the vesicles showed crystalline pattern when investigated by electron microscopy.

Another approach to receptor 2D crystallization was crystallization of the receptor fusion FlagHisET_BBio with fluorinated Ni^{2+} lipids. These lipids have already been successfully used for the 2D crystallization of membrane proteins (Levy *et al*., 1999). Fluorinated Ni^{2+} lipids build a lipid layer on the surface of 2D crystallization probe, and are protected from detergent solubilization by fluorination. The receptor is "fished out" and bound to Ni^{2+} via its His-tag, and the detergent removed by the Bio-Beads. Although the initial attempts with this method were made, no lipid reconstitution of the ET_B receptor could be detected.

4.14 Outlook

The yeast *P. pastoris* was demonstrated to be a reliable expression system for the ET_B receptor, yielding large amounts of active protein. Milligram quantities of purified receptor were obtained after affinity purification combining Ni-NTA and monomeric avidine matrices. However, since the receptor aggregation problem was not completely solved, further efforts in this direction are needed.
Receptor production in insect cells may be more promising, since in this case the receptor was obtained in a homogeneous monomeric form after purification. However, this expression system is more costly and time-consuming than receptor production in yeast, and the overall receptor yield per liter of culture is smaller.
Receptor expression in mammalian cells needs to be improved. Although the N-terminal proteolysis of the receptor does not influence its activity, the heterogeneity of the sample (the existence of two receptor forms) poses a potential problem for structure studies. Nevertheless, transient expression of receptor in mammalian cells gives interesting possibilities for functional studies, since the receptor is produced in a native environment.
It would be interesting to address the question of receptor oligomerization by means of FRET (fluorescence resonance energy transfer) or BRET (bioluminescence resonance energy transfer) experiments, which have proven informative in the case of some other GPCRs (for review see Bouvier, 2002) and which could ultimately provide interesting insights concerning the endogenously active form and *in vivo* dimerization of the ET_B receptor.
To date, there is no test system for *in vivo* G-protein coupling of GPCRs produced in *P. pastoris*. This yeast has a very low amount of endogenously expressed G-proteins, and a very limited number of different G-proteins. One possibility would be to genetically modify a *P. pastoris* strain in order to produce either the promiscuous $Galpha_{16}$ protein or G_q and G_i, the

main coupling G_{α}–protein partners for ET_B, together with $G_{\beta\gamma}$ subunits. In this way it may be possible to directly test the signalling activity of the receptor in yeast spheroplasts.
This work has demonstrated that the amounts of receptor expressed in yeast and insect cells and the yield of active receptor, which can be isolated by purification, represent a good basis for starting 3D and continuing 2D crystallization trials.

5 Literature

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Appendix

I Abbreviations

AP	-	alkaline phosphatase
ATP	-	adenosine-triphosphate
cAMP	-	cyclic adenosine-monophosphate
cDNA	-	complementary deoxyribonucleic acid
BHK	-	baby hamster kidney
BSA	-	bovine serum albumin
CAP	-	capsid protein
CHO	-	chinese hamster ovary
Cpm	-	counts per minute
DMSO	-	dimethyl-sulfoxide
DNA	-	deoxyribonucleic acid
DTT	-	dithiothreitol
EDTA	-	ethylenediaminetetraacetic acid
FCS	-	fetal calf serum
GDP	-	guanine diphosphate
GPCR	-	G-protein coupled receptor
GTP	-	guanine-triphosphate
IgG	-	immunoglobulin G
LB	-	Luria-Bertani
LBD	-	ligand-binding domain
LD	-	lethal dose
NK	-	neurokinine
NMDA	-	N-methyl-D-aspartate
OD	-	optical density
PA	-	polyacrylamide
PAGE	-	polyacrylamide gel electrophoresis
PBS	-	phosphate buffered saline
PEG	-	polyethylene-glycol
PMSF	-	phenylmethylsulfonylfluoride
RNA	-	ribonucleic acid
RT	-	room temperature
SDS	-	sodium dodecyl sulphate
2D	-	two-dimensional
3D	-	three-dimensional
TBS	-	Tris-buffered saline
TCA	-	trichloroacetic acid
TGF	-	tumor growing factor
TMH	-	transmembrane helix

II Amino-acid sequences of affinity tags used in this work

Flag-tag	DYKDDDDK
His10-tag	HHHHHHHHHH

StrepI-tag AWRHPQFGG

StrepII-tag AWSHPQFEK

Bio-tag GGGTGGAPAPAAGGAGAGKAGEGEIPAPLAGTV SKILVKEGDTVKAGQTV**L**VLEAMKMETEINAPTD GKVEKVLVKERDAVQGGQGLIKIG

III cDNA and amino-acid sequence of the human ET_B receptor (*SRS-EMBL-EBI database, AC L06623; SWISS-PROT P24530)*

```
1      ATGCAGCCGC CTCCAAGTCT GTGCGGACGC GCCCTGGTTG CGCTGGTTCT
        M  Q  P   P  P  S  L   C  G  R   A  L  V   A  L  V  L

51     TGCCTGCGGC CTGTCGCGGA TCTGGGGAGA GGAGAGAGGC TTCCCGCCCG
         A  C  G   L  S  R   I  W  G  E   E  R  G   F  P  P

101    ACAGGGCCAC TCCGCTTTTG CAAACCGCAG AGATAATGAC GCCACCCACT
       D  R  A  T   P  L  L   Q  T  A   E  I  M  T   P  P  T

151    AAGACCTTAT GGCCCAAGGG TTCCAACGCC AGTCTGGCGC GGTCGTTGGC
        K  T  L   W  P  K  G   S  N  A   S  L  A   R  S  L  A

201    ACCTGCGGAG GTGCCTAAAG GAGACAGGAC GGCAGGATCT CCGCCACGCA
         P  A  E   V  P  K   G  D  R  T   A  G  S   P  P  R

251    CCATCTCCCC TCCCCCGTGC CAAGGACCCA TCGAGATCAA GGAGACTTTC
       T  I  S  P   P  P  C   Q  G  P   I  E  I  K   E  T  F

301    AAATACATCA ACACGGTTGT GTCCTGCCTT GTGTTCGTGC TGGGGATCAT
        K  Y  I   N  T  V  V   S  C  L   V  F  V   L  G  I  I

351    CGGGAACTCC ACACTTCTGA GAATTATCTA CAAGAACAAG TGCATGCGAA
         G  N  S   T  L  L   R  I  I  Y   K  N  K   C  M  R

401    ACGGTCCCAA TATCTTGATC GCCAGCTTGG CTCTGGGAGA CCTGCTGCAC
       N  G  P  N   I  L  I   A  S  L   A  L  G  D   L  L  H

451    ATCGTCATTG ACATCCCTAT CAATGTCTAC AAGCTGCTGG CAGAGGACTG
        I  V  I   D  I  P  I   N  V  Y   K  L  L   A  E  D  W

501    GCCATTTGGA GCTGAGATGT GTAAGCTGGT GCCTTTCATA CAGAAAGCCT
         P  F  G   A  E  M   C  K  L  V   P  F  I   Q  K  A

551    CCGTGGGAAT CACTGTGCTG AGTCTATGTG CTCTGAGTAT TGACAGATAT
       S  V  G  I   T  V  L   S  L  C   A  L  S  I   D  R  Y

601    CGAGCTGTTG CTTCTTGGAG TAGAATTAAA GGAATTGGGG TTCCAAAATG
        R  A  V   A  S  W  S   R  I  K   G  I  G   V  P  K  W

651    GACAGCAGTA GAAATTGTTT TGATTTGGGT GGTCTCTGTG GTTCTGGCTG
         T  A  V   E  I  V   L  I  W  V   V  S  V   V  L  A

701    TCCCTGAAGC CATAGGTTTT GATATAATTA CGATGGACTA CAAAGGAAGT
       V  P  E  A   I  G  F   D  I  I   T  M  D  Y   K  G  S
```

```
751     TATCTGCGAA TCTGCTTGCT TCATCCCGTT CAGAAGACAG CTTTCATGCA
         Y  L  R   I  C  L  L   H  P  V   Q  K  T   A  F  M  Q

801     GTTTTACAAG ACAGCAAAAG ATTGGTGGCT GTTCAGTTTC TATTTCTGCT
          F  Y  K   T  A  K   D  W  W  L   F  S  F   Y  F  C

851     TGCCATTGGC CATCACTGCA TTTTTTTATA CACTAATGAC CTGTGAAATG
        L  P  L  A   I  T  A   F  F  Y   T  L  M  T   C  E  M

901     TTGAGAAAGA AAAGTGGCAT GCAGATTGCT TTAAATGATC ACCTAAAGCA
         L  R  K   K  S  G  M   Q  I  A   L  N  D   H  L  K  Q

951     GAGACGGGAA GTGGCCAAAA CCGTCTTTTG CCTGGTCCTT GTCTTTGCCC
          R  R  E   V  A  K   T  V  F  C   L  V  L   V  F  A

1001    TCTGCTGGCT TCCCCTTCAC CTCAGCAGGA TTCTGAAGCT CACTCTTTAT
        L  C  W  L   P  L  H   L  S  R   I  L  K  L   T  L  Y

1051    AATCAGAATG ATCCCAATAG ATGTGAACTT TTGAGCTTTC TGTTGGTATT
         N  Q  N   D  P  N  R   C  E  L   L  S  F   L  L  V  L

1101    GGACTATATT GGTATCAACA TGGCTTCACT GAATTCCTGC ATTAACCCAA
          D  Y  I   G  I  N   M  A  S  L   N  S  C   I  N  P

1151    TTGCTCTGTA TTTGGTGAGC AAAAGATTCA AAAACTGCTT TAAGTCATGC
        I  A  L  Y   L  V  S   K  R  F   K  N  C  F   K  S  C

1201    TTATGCTGCT GGTGCCAGTC ATTTGAAGAA AAACAGTCCT TGGAGGAAAA
         L  C  C   W  C  Q  S   F  E  E   K  Q  S   L  E  E  K

1251    GCAGTCGTGC TTAAAGTTCA AAGCTAATGA TCACGGATAT GACAACTTCC
          Q  S  C   L  K  F   K  A  N  D   H  G  Y   D  N  F

1301    GTTCCAGTAA TAAATACAGC TCATCTTGA
        R  S  S  N   K  Y  S   S  S  *
```

Acknowledgements

I'm very much indebted to following people:

- Prof. Dr Hartmut Michel, for supervising my work, for discussions&useful suggestions and for always having time for me
- Prof. Dr Bernd Ludwig, for being my supervisor from the University and for personalizing a friendly face of the University
- Dr Helmut Reilaender: 1) for welcoming me to the GPCR group, supervising my work in the beginning and for his friendly attitude; 2) hadn't he left the Institute, I'd never have learned to work independently
- Dr Stephen Marino, for advice, patience&endless support
- Dr Christoph Reinhart, for answering my questions
- Dr Hilmar Schiller, from whom I "inherited" the project, for providing me with clones, tipps&literature at the beginning of my work
- Eva Molsberger and Gabriele Maul, for excellent technical assistance
- Dr Tassilo Krug von Nidda, for help with electron microscope, proofreading of „Zussamenfassung" , for telescope&tea and support
- Radostina Petrova and Dr Björn Mamat, my colleagues and friends, for making nice atmosphere in the Institute & for numerous discussions during tea&coffee breaks
- Barbara Schiller, for help in fighting Mac computers and her warmth&friendliness
- Rosemarie Schmidttel, for her kindness and efficiency in finding references&papers for my thesis
- Franziska Langheinrich, for her help with administrative hurdles in the beginning
- Dr Thomas Klabunde, for some valuable lessons
- My great serbian friends in Germany – Stefan Nemanja Radoš, Milutin Stefanovic and Ružica Vujovic, for colouring my life in Germany, help&support
- My family, for endless support
- All people from GPCR group&the Institute who wished me well

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Zusammenfassung

Zellen besitzen die Fähigkeit auf verschiedene Signale aus ihrer Umgebung adäquat zu reagieren und dadurch mit anderen Zellen zu kommunizieren. Die Signale werden durch eine große Anzahl unterschiedlicher Signalstoffe - von Proteinen und kleinen Peptiden über Aminosäuren, Nukleotide, Steroide und Fettsäurederivate bis hinzu gelösten Gasmolekülen wie Stickstoffmonoxid - vermittelt. Einige dieser Moleküle, die klein und hydrophob sind, dringen in die Zielzelle ein, indem sie durch die Lipiddoppelschicht der Zellmembrane diffundieren. Sie aktivieren intrazelluläre Rezeptoren, die dann direkt auf der Ebene der Gentranskription wirken. Die meisten extrazellulären Signalstoffe aber sind hydrophil und folglich nicht in der Lage in die Zelle durch Diffusion einzudringen. Stattdessen binden diese Botenstoffe an der extrazellulären Seite von integralen Membranproteinen, Membranrezeptoren genannt, und lösen eine Signalkaskade im Inneren der Zelle aus.

Die größte und pharmakologisch interessanteste Gruppe der Membranrezeptoren ist die Superfamilie der G-Protein-gekoppelten Rezeptoren (G-protein coupled receptors (GPCR)). GPCR-kodierende Gene machen zwischen ein und fünf Prozent des Wirbeltiergenoms aus (ca. 1% des menschlichen Genoms). Da G-Protein gekoppelte Rezeptoren der Angriffspunkt vieler Medikamente sind, sind sie von großer Bedeutung für die pharmazeutische Industrie. GPCRs besitzen sieben die Membran durchspannende Helices (sieben transmembrane Helices) und sind für die Übertragung des Signals aus der extrazellulären Umgebung in die Zelle verantwortlich. Die Signalkaskade hat folgenden Ablauf: der Ligand, der auf der extrazellulären Seite bindet, löst eine Konformationänderung des Rezeptors aus. Wenn der Ligand ein Agonist ist, wird diese Konformationsänderung auf die intrazelluläre Seite des Rezeptors übertragen, wodurch hetrotrimere G-Protein an ihn binden. Die Bindung an dem Rezeptor verursacht die Dissoziation des G-Proteinkomplexes in α und $\beta\gamma$ Untereinheiten, welche daraufhin verschiedenen Effektorsysteme aktivieren.

Um den Mechanismus der Signalübertragung zu verstehen und neue Angriffspunkte für diese medizinisch wichtige Proteinfamilie entwickeln zu können („structure-based drug design“), ist die dreidimensionale (3D) Strukturaufklärung notwendige

Voraussetzung. Bisher ist nur eine hochauflösende Struktur des Rhodopsin aus Rinder-Retina mit einer Auflösung von 2,8 Å gelöst worden. Die Struktur anderen GPCRs wurde daraufhin am Computer simuliert.
Der Endothelin-B-Rezeptor (ET_B), dessen Überproduktion, Solubilisierung und Reinigung das Thema dieser Arbeit ist, gehört zur Gruppe A („Rhodopsin ähnlich") der G-Protein gekoppelte Rezeptoren. Der ET_B Rezeptor wird vom Peptidligand Endothelin aktiviert, wodurch die Signalkaskade gestartet wird, die letztendlich zur Erweiterung der Blutgefäße führt. Der ET_B Rezeptor ist außergewöhnlich, weil er seinen Ligand fast irreversibel bindet. Deswegen und hinsichtlich seines pharmakologischen Wertes ist seine 3D-Struktur äußerst interessant.

Die Voraussetzung für die Aufklärung der 3D-Struktur von G-Protein gekoppelten Rezeptoren ist die Verfügbarkeit großer Mengen homogenen Proteins. Die benötigten Proteinmengen könnten nicht aus natürlichen Quellen gewonnen werden, da das Protein nur in geringen Konzentrationen im Gewebe exprimiert wird. Zusätzliche Probleme liefert die Heterogenität der Rezeptoren, da mit Hilfe von molekularbiologischen und pharmakologischen Techniken verschiedene Subtypen im Gewebe identifiziert werden konnten. Sie sind während der Reinigung sehr schwer oder überhaupt nicht voneinander zu trennen. Um ausreichende Mengen an dem gewünschten Rezeptor homogen zu produzieren, greift man auf geeignete Expressionssysteme für Heterologen Rezeptorproduktion zurück.
Jedoch ist die Wahl eines geeigneten Expressionssystems nicht einfach. Es ist zu bedenken, dass es sich bei G-Protein gekoppelten Rezeptoren um Membran-proteine handelt, deren hydrophobe Domänen in die Membran inseriert und richtig gefaltet werden müssen. Zusätzlich können sie eine Vielzahl von komplizierten post-translationalen Modifikationen tragen.
Die Eignung verschiedener Expressionssysteme – Hefe, Insekten- und Säugerzellen - zur funktioneller Überproduktion von ET_B Rezeptor wurde in dieser Arbeit getestet und anschließend die entsprechenden Solubilisierungs- und Reinigungsmethoden, die eine homogene Rezeptorpräparation zum Ziele hatte.
Hefen sind einzellige Eukaryonten, die den Vorteil eines eukaryontischen Wirtes, der die notwendigen post-translationalen Modifikationen an humanen Proteinen durchführen, mit sich bringt. Zusätzlich stellen sie ein kostengünstiges, leicht skalierbares Expressionssystem für die Produktion großer Proteinmengen dar. In

dieser Arbeit wurde *Pichia pastoris* als Wirt zur Produktion von ET_B Rezeptor verwendet. Sie ist eine methylotrophe Hefe, die fähig ist als einzige Kohlenstoffquelle Methanol zu nutzen. Die Hauptvorteile von *P. pastoris* sind wie folgt: 1) ist es möglich mit dem starken Genpromotor der Alkoholoxidase 1 (AOX1) ein hohes Niveau der heterologen Proteinexpression zu erzielen (30% des gesamten Zellproteins) 2) lässt sie sich gut zu hohen Dichten (bis 500g Feuchtgewicht/L) im Fermenter anziehen 3) wird das geklonte Konstrukt mit dem Rezeptorgen beständig in das Hefegenom integriert. Zusätzlich ist die Selektion von Klonen mit mehrfachen Geninsertionen möglich 4) ist *P. pastoris* zum Durchführen der post-translationalen Modifikationen fähig (Glykosylierung, Phosphorylierung, Palmitoylierung und Disulfidbindungs-anordnungen). 5) gibt es keine Hyperglykosylierung des produzierten Proteins, wie in dem Fall von *S. cerevisiae*.
Insektenzellen und Säugerzellen bieten die authentische Umgebung für die Expression von G-Protein gekoppelten Rezeptoren. Diese Expressionssysteme haben den eindeutigen Vorteil, dass die Faltung und alle post-translationalen Modifikationen am produzierten Rezeptor leistungsfähig durchgeführt werden. Eine effektive Methode zur transienten Expression von fremden Proteinen in Insekten- und Säugtierzellen bietet sich durch rekombinanten Viren. Eine hohe Infektionsfähigkeit in einer Vielfalt von Zellen, die infiziert werden können, ist der Hauptvorteil dieses Systems. Im Fall von Insektenzellen werden rekombinante Baculoviren (insekten-pathogene doppelsträngige-DNS Viren) genutzt. Rekombinante Alfaviren (kleine umhüllten Viren mit einem einzelsträngigen RNS-Genom) wie z.B. Semliki-Forest-Virus, werden zur transienten Expression des fremden Proteins in Säugerzellen verwendet. Liljestrom & Garoff entwickelten die SFV-Expressionvektoren mit dem Ziel der Produktion großer Mengen von rekombinantem Protein in Säugerzellen (Liljestrom&Garoff, 1991). Das SFV-Expressionssystem hat sich als besonders geeignet für die GPCR-Expression erwiesen (Lundstrom, 2000).

Der Endothelin B Rezeptor wurde während dieser Arbeit in unterschiedlichen Wirten produziert: in der Hefe *P. pastoris*, in Sf9-Insektenzellen und in CHO-Säugerzellen. Die höchste Ausbeute an aktivem Rezeptor wurde in Hefe erhalten; der Klon FlagHisET_BBio mit einer Expressionsrate von 60 pmol/mg, erbrachte 1,2 mg Rezeptor/L Schüttelkultur. Durch Fermentation des gleichen *P. pastoris* Klons im 10L-Fermenter, war es möglich, 3kg Hefezellen zu erhalten, die 20-39 mg des

aktiven Rezeptors enthielten. Die Menge an aktivem ET_B-Rezeptor, der in Insektenzellen mit einer maximalen Expression von 30 pmol/mg produziert wurde, war 0,7 mg Rezeptor/L Zellkultur. Die damit erreichte Menge stimmt mit den vorher berichteten Werten überein (Doi *et al.,* 1997). Verglichen mit der ET_B Rezeptor-produktion in Hefe, ist die Ausbeute an aktivem Rezeptor in Insektzellen niedriger und die Systembehandlung ist zeitaufwändiger, wird aber durch eine homogene Rezeptorpräparation nach der Reinigung belohnt. Die Expression des aktiven ET_B-Rezeptors von 7 pmol/mg in den CHO-Zellen ist niedriger als erwartet, wenn man in Betracht zieht, dass das Vektorkonstrukt pSFV3CAPET_BHis, der zur Rezeptorproduktion benutzt wurde, ein Gen für das Capsidprotein enthält. Dieses Gen hat ein „translation-enhancer“ Signal auf seinem 5’-Ende, das im Fall anderer GPCRs zu in einer mehrfach höheren Expression im Vergleich zum Konstrukt ohne das Capsidprotein führte (Lundstrom *et al.,* 2001). CHO-Zellen sind zweifellos der finanziell anspruchsvollste Wirt für die Rezeptorproduktion und kann mit einer in dieser Arbeit erzielter Expression von ET_B-Rezeptor von 7 pmol/mg nicht für eine Rezeptorproduktion in großen Maßstab empfohlen werden. Es sollte erwähnt werden, dass der am häufigsten genutzte Wirt zur Proteinproduktion, *E. coli*, als Wirt für die ET_B-Produktion in dieser Arbeit nicht ausgewertet wurde. Frühere Versuche in E.coli erbrachten 41 aktiv bindende ET_B Rezeptor/Zell (Haendler *et al.,* 1993), die erreichten Expressionsraten sind aber zu niedrig, um *E. coli* als konkurrenzfähiges System für die heterologe Produktion des ET_B-Rezeptors im großen Maßstab betrachten zu können.

Für die Überproduktion des ET_B-Rezeptors in Hefe und Insektenzellen wurde der Klon FlagHisET_BBio ausgesucht, der drei verschiedene Affinitätsanhängseln hat. Aufgrund dieser Affinitätsanhängsel war es möglich, einige Reinigungsstrategien zu testen und effizientestere zu bestimmen. Außerdem haben diese „Tags“ den immunologischen Nachweis von produzierten Rezeptorfusionen ermöglicht.

Der „Bio-Tag“ wird in *P. pastoris* und in den Insektzellen biotinyliert, was durch Immunoblotanalyse mit Streptavidin bestätigt wurde. Die Präsenz von „Bio-Tag“ wurde in dieser Arbeit zur Reinigung der Rezeptorfusion mit Avidin verwendet.

Auch wurde in dieser Arbeit der Rezeptor immer als Komplex mit dem Liganden ET-1 gereinigt, der an den Rezeptor fast irreversibel bindet und dadurch den nativen Rezeptor stabilisiert. Jedoch war der Nachteil dieser Reinigungsstrategie, dass die

Bestimmung der Rezeptorausbeute mittels Radioligandenbindung während der unterschiedlichen Reinigungsschritte nicht möglich war.

Der in Hefe überproduzierte Rezeptor wurde mit dem Detergenz n-Dodecyl-ß-D-Maltozid aus Hefemembranen solubilisiert, da in früheren Solubilisierungsstudien an aus Hefe gewonnenem ET_B-Rezeptoren dieses Detergenz die besten Ergebnisse erzielte (Schiller *et al.,* 2001).
Als effizienteste Rezeptorreinigung hat sich die zweistufige Affinitätschromatographie mittels Ni-NTA und Avidin-Matrix gezeigt. Der Rezeptor wurde zuerst durch die Ni-NTA-Matrix mittels seines His-Tags gereinigt. Nach diesem Reinigungsschritt waren immer noch Verunreinigungen auf dem Silber-gefärbten Gel des Eluats zu sehen. Die Avidin-Affinitätsmatrix wurde als zweiter Schritt in der Rezeptorreinigung eingesetzt. Dieser Reinigungsschritt erbrachte mehr als 95% reinen Rezeptor (durch Silberfärbung abgeschätzt) und meistens enthielt das Eluat nur eine Verunreinigungsproteinbande.
Die Versuche, den Rezeptor über andere Affinitätsmatrizen, wie M1-anti-Flag Antikörper und Streptavidin, zu reinigen, wurden wegen starker Aggregationsbildung aufgegeben.

Die Aggregationstendenz des in Hefe produzierten ET_B-Rezeptors stellte ein ernstes Hindernis für die Kristallisationsanforderungen dar. Zahlreiche Versuche wurden in dieser Arbeit unternommen, um eine Lösung für dieses Problem zu finden. Es gibt eine Vielzahl von möglichen Ursachen für die Rezeptoraggregation. In dieser Arbeit wurde eine Anzahl von Additiven zur Vermeidung dieses Verhaltens ausprobiert. Darunter waren kurzkettige Lipide (1,2-Dicaproyl-n-Glycero-3-Phosphocholine, 0,2%), physikalische Chaperone wie Ectoine und Hydroxyectoine (je 1%), Cholesterin-hemisuccinate (0,5%) und DMSO (2%). Keins dieser Additive konnte die monomere Form des Rezeptors stabilisieren und Bildung von Rezeptoraggregaten völlig verhindern.
Nach der Rezeptorproduktion in Baculovirus infizierten Insektenzellen wurde ein geeignetes Detergenz für die Solubilisierung ausgesucht. Die Rezeptoraffinitäts-messungen mittels radioaktivem [I^{125}] ET-1-Liganden zeigten, dass n-Dodecyl-ß-D-Maltosid (LM), Lauryl-Saccharose (LS) und Digitonin/Cholat (D/C) die größte Menge aktiven Rezeptor brachten. LM wurde für die weitere Arbeit verwendet. Das Konstrukt

FlagHisET$_B$Bio aus Insektenzellenmembranen wurde mit LM solubilisiert und in zwei Schritten mittels über Ni-NTA- und Avidin-Chromatographie analog zur Hefe-Prozedur gereinigt. In diesem Fall gelang es eine homogene Form des ET$_B$-Rezeptors zu erhalten, wie die Gelfiltrationsanalyse (Superose 6) zeigte.

Angeregt durch kürzlich erschienene Daten bezüglich der Homo- und Hetero-oligomerisierung von GPCRs, wurde der Oligomerisationszustand des ET$_B$-Rezeptors durch „native blue“ Elektrophorese untersucht. Eine deutliche Bande mit einem Molekulargewicht des Rezeptordimers konnte nachgewiesen werden. Jedoch sind weitere Experimente erforderlich, um zu einen definitiven Beweis für die Rezeptordimerisierung zu gelangen, da die native Elektrophorese Artefakten in manchen Fällen produzieren kann.

Die Hefe *P. pastoris* konnte als zuverlässiges Produktionssystem für den ET$_B$-Rezeptor mit hohen Ausbeuten an aktivem Protein etabliert werden. Milligrammmengen des gereinigten Rezeptors wurden nach der Affinitätsreinigung erhalten, die die Ni-NTA und Avidin-Matrizen kombinierte. Jedoch konnte das Problem der Rezeptoraggregation in diesem Fall nur teilweise gelöst werden. Weitere Bemühungen sind erforderlich. Die Rezeptorproduktion in den Insektzellen ergab weniger Rezeptor im Vergleich zu *P. pastoris*, ist jedoch vielversprechender, da in diesem Fall der Rezeptor in der homogenen monomeren Form nach der Reinigung erhalten wurde. Die in Hefe und Insektenzellen produzierten Mengen des Rezeptors und seine Aktivität stellen eine gute Grundlage für den Beginn der 3D- und zur Fortsetzung von 2D-Kristallisationsversuchen dar.

Obwohl die Expression des ET$_B$-Rezeptors in Säugerzellen mittels Semliki-Forest-Virus optimiert werden muss, bietet diese Art transienter Expression interessante Möglichkeiten für Funktionsstudien, da sich der Rezeptor hier in seiner natürlichen Umgebung befindet. Es wäre interessant, die Frage der Rezeptor-Oligomerisierung mittels FRET (Fluoreszenzresonanz-Energieübertragung) oder BRET (Bio-lumineszenzresonanz-Energieübertragung) zu untersuchen. Diese Methoden waren sehr informativ im Fall von anderen G-Protein gekoppelten Rezeptoren (Bouvier, 2002) und konnten interessante Einblicke über die endogene, aktive Form und die *in vivo* Dimerisierung des ET$_B$-Rezeptors geben. Bisher gibt es kein Testsystem für *in*

vivo G-Proteinkopplung von G-Protein gekoppelte Rezeptoren, die in *P. pastoris* produziert wurden. Diese Hefe hat sehr niedrige Menge an endogen exprimierten G-Proteinen und eine sehr begrenzte Zahl unterschiedlicher G-Proteine. Eine Möglichkeit wäre einen *P. pastoris* Stamm genetisch so zu verändern, dass er entweder das Protein G_{16} oder Gq und Gi, die Hauptpartner für die Kopplung mit dem ET_B, zusammen mit den Untereinheiten βγ produziert. Bei einem solchen Stamm wäre es möglich, die Rezeptorsaktivität im Hefesphäroplasten nach Ligandenzugabe direkt durch die Ca^{2+}-Konzentrationsänderungen zu messen.