2 Heterologous expression of the ET_B receptor using the baculovirus/ insect cells system

2.1 Introduction

2.1.1 Heterologous expression of GPCRs

Information on GPCR-mediated signal transduction pathways are available. However, the ligand mechanism and the conformational changes upon binding of the ligand remain unexplored. These studies are mainly hindered by the difficulty of producing large amounts of homogeneous GPCRs which hampered the success of obtaining structural information for GPCRs. The lack of structural data on GPCRs mainly arises due to the membraneous nature of these proteins and their too low natural abundance to be successfully purified from native tissues. In order to gain structure-function information on GPCRs, heterologous expression is the most important step. There are some factors which need to be taken into consideration during the expression of GPCRs. Theoretically, the closer a receptor is to its native environment, the higher are the chances of expressing it properly. Post-translational modifications also play a critical role in the function of these receptors.

Several GPCRs such as the ET_B receptor (Haendler *et al*., 1993) has been expressed in *E. coli* at low levels. *E. coli* is an attractive expression system because of its ease of use and cost-effectiveness. However, there are some drawbacks due to its prokaryotic nature such as the thickness and composition of its membrane, which differ from the eukaryotic cell, e. g. no cholesterol is present in the bacterial inner membrane, possibly altering GPCR properties (Gimpl *et al*., 1995). It also lacks intracellular organelles such as the endoplasmic reticulum and the Golgi apparatus that are present in eukaryotes, which are responsible for post-translational modifications of proteins. In contrast, yeast expression systems offer several advantages. First, like bacterial expression systems, the growth of yeast is fast and inexpensive. Second, yeast cells are eukaryotes and perform most of the posttranslational modifications observed in mammalian cells, although N-glycosylation of mammalian membrane proteins in yeast seems to be inefficient. However, lack of glycosylation may not affect ligand binding (Schiller *et al*., 2000). Several mammalian cell lines such as COS, CHO, BHK21 and HEK293 have been used for the expression of GPCRs. High expression in stable cell lines has been reported for GPCRs, but could not be maintained in suspension culture. Large-scale expression in mammalian cells is, therefore, often difficult, expensive and time

consuming. An efficient alternative for producing mammalian receptors, based on the use of recombinant baculovirus, has achieved high expression of several GPCRs such as the ET_B receptor (Doi *et al*., 1997). Post-translational modifications in insect cells are identical to those observed in mammalian cells with the exception that GPCRs are often poorly glycosylated, although as for yeast and *E. coli* this may not be a concern when producing protein for structural studies.

2.1.2 Expression of GPCRs in baculovirus

2.1.2.1 Overview of the baculovirus expression vector system

The insect cell based baculoviral system has emerged as a popular system for rapid and high level expression of several GPCRs. In this system, the protein production results from infection of insect cells by recombinant viruses encoding the gene of interest. Unlike bacterial expression systems, the baculovirus-based system is an eukaryotic expression system and thus uses many of the protein modification, processing and transport systems present in higher eukaryotic cells. In addition, the baculovirus expression system uses a helper-independent virus that can be propagated to high titers in insect cells adapted for growth in suspension cultures, making it possible to obtain large amounts of recombinant protein. It expresses the majority of GPCRs in a functional form and at levels 10-100 fold higher than those observed in cells and tissues endogeneously expressing them (Bouvier *et al*., 1998).

Currently, the most widely used baculovirus expression system utilizes a lytic virus known as *Autographa californica* nuclear polyhedrosis virus (AcNPV; hereafter called baculovirus). The double-stranded circular AcNPV genome is approx. 128kb in size and replicated within the nuclei of lepidopteran species. Baculovirus gene expression takes advantage of some unique features of the viral life cycle, in which large amounts of proteins are synthesized late in the infection to promote virus survival in the environment. Due to the biphasic life cycle of baculoviruses two different types of virus particles are generated in the infection cycle (Fig. 2.1 A). The first phase (10-24 h postinfection) involves the formation of mature nucleocapsids that bud through the cellular membrane to form extracellular budded virus particles (BV). These BVs are infectious to neighbouring cells within the body of the host and serve to disseminate such an infection. At the second phase (24-72 h postinfection) enveloped nucleocapsids are occluded in the nucleus with crystalline matrices comprised of polyhedrin. Polyhedrin accounts for 50% of the total protein content of an infected cell and is responsible for embedding the mature virus particles within the cell nuclei, generating large viral occlusion bodies (occluded virus, OV). These OVs are released only after cell death and are

specialized for survival in the environment and for spread from insect to insect through oral ingestion. The spread of the virus within cell culture relies exclusively on the BV, which leaves the polyhedrin promoter free to allow high level expression of recombinant protein. In tissue (Fig. 2.1 B), this protein is non-essential for viral replication, and its gene can be exchanged with other genes to create a productive recombinant virus. For a review of the baculovirus expression system, see O' Reilly *et al*. (1992, 1994), and references therein.

2.1.2.2 Baculovirus expression system

The fact that polyhedrin is not essential for productive infection *in vitro* make baculoviruses desirable vectors for foreign gene expression. For recombinant protein production (Fig. 2.1 B), the polyhedrin gene of the viral DNA is substituted by the gene of interest, which is then expressed in high amounts. Since the AcNPV genome is too large for insertion of heterologous genes by *in vitro* ligation methods, homologous recombination *in vivo* is required. The different steps in the generation of a recombinant baculovirus include the construction of a suitable transfer vector, the co-transfection of this together with wild-type AcNPV DNA into insect cells and the isolation of recombinant functional baculovirus by means of plaque-assay. The transfer vector carries the gene of interest flanked by sequences homologous to the viral polyhedrin locus. Co-transfection of the plasmid and viral DNA in insect cells allows insertion of the gene of interest into the viral genome *in vivo* by homologous recombination. After that, the functional baculovirus is propagated in insect cells to generate high titer virus stocks that can be used for protein production. The first step in a production process is to grow the insect cells to a suitable cell density and then the virus is added at a predetermined multiplicity of infection (MOI). A MOI of 1 is equal to one added virus particle per cell. Different commercial systems provide transfer vectors and virus DNA such as Bac-To-Bac (Invitrogen), BacVector System (Novagen) and BaculoGold (Pharmingen) to mention a few.

This virus is able to selectively infect different insect cell types, among which the most frequently used are those of *Spodoptera frugiperda* (cell lines Sf9 and Sf21) and of *Trichoplusia ni* (cell lines Hi5 and MG1).

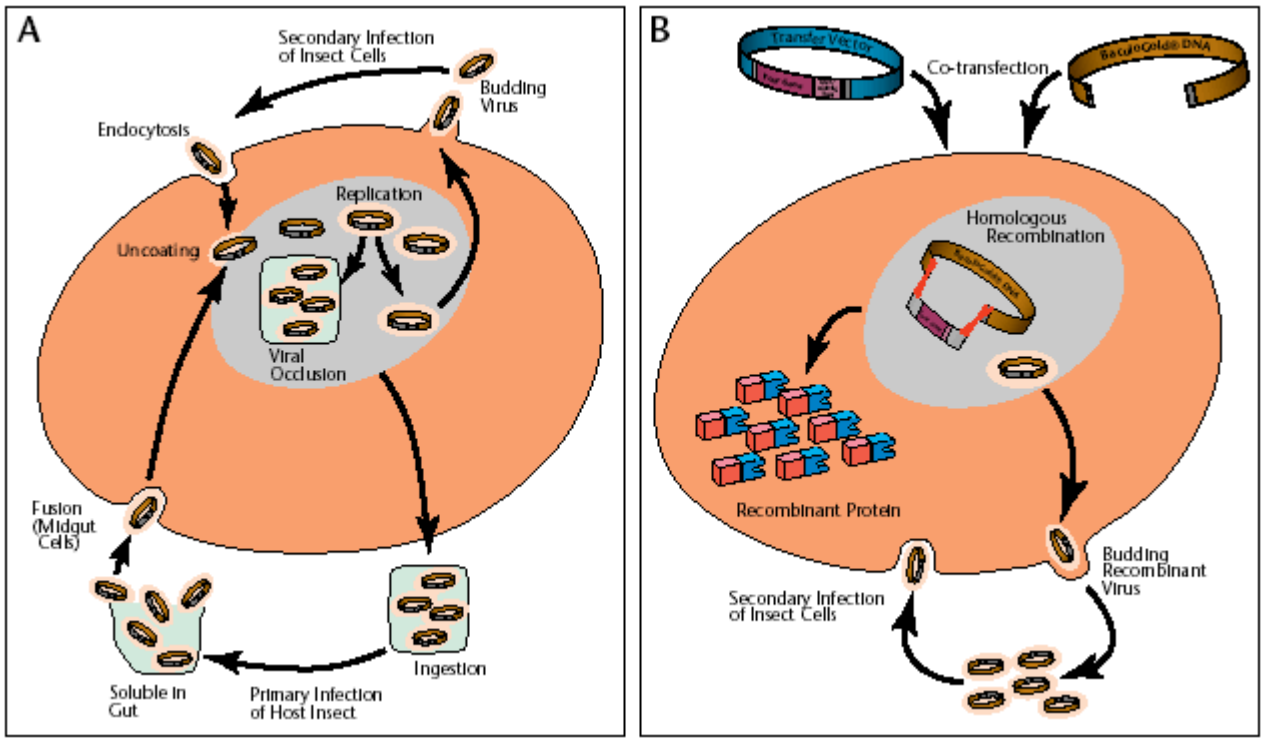

Fig. 2.1 The Baculovirus life cycle *in vivo* (A) and *in vitro* (B). (A) The viral life cycle is characterized by a temporal cascade of events that results in the generation of two virion phenotypes, budded virus (BV) and occluded virus. After entry into permissive cells by endocytosis, virions are uncoated in the nucleus and viral DNA is replicated. The late phase of the viral life cycle is initiated and enveloped progeny virus are released from infected cells by budding through the plasma membrane. BVs can infect additional cells and are the vehicles by which virus is transmitted within cell cultures. At a very late stage of infection, enveloped nucleocapsids are occluded in the nucleus with polyhedrin. Polyhedrin is the major protein of the matrix that surrounds the virus particles to form occlusion bodies. (B) Polyhedrin is essential for horizontal transmission of the virus, but not for replication and secondary infection, and is therefore dispensable under tissue culture conditions. This allows the replacement of the polyhedrin gene of the viral DNA with the gene of interest, which is then expressed in high amounts. The transfer vector carries the gene of interest flanked by sequences that allow homologous recombination event in the polyhedrin locus of the wild-type baculovirus DNA to form recombinant virus DNA. The proportion of recombinant viruses obtained from a cotransfection can be increased by the use of linearized virus DNA.

2.1.3 Previous isolation procedures for ET_BR

Enriched native membrane preparations was the primary source for the purification of endothelin receptors. Based on a knowledge of the extremely high affinity of ET-1 (or its derivatives, biotinylated or polyadenylated at Lys9) for its receptor ET_BR, affinity chromatography was used to purify the ET_BR from human placenta (Akiyama *et al*., 1991; Wada *et al*., 1990) and bovine lung (Kozuka *et al.,* 1991; Roos *et al*., 1998). However, either the receptor yields obtained were low, the purified receptor was inactive (Akiyama *et al*., 1991) or the preparations exhibited heterogeneity due to proteolysis and/or glycosylation (Kozuka *et al*., 1991). Additionally, ET_AR was copurified from the natural sources. Efforts have been made to overcome this problem by using heterologous organisms for protein

production. Several attempts at overexpression have been made, as can be seen in Table 2.1. While poor expression levels of ET_BR was obtained in *E. coli* (Haendler *et al*., 1993), the human ET_BR was successfully expressed in baculovirus-infected insect cells (100 ± 10 pmol/mg) (Doi *et al*., 1997; 1999) and COS cells (60 ± 10 pmol/mg) (Elshourbagy *et al*., 1993). To date baculovirus-infected insect cells are promising (Doi *et al*., 1997, 1999) and receptor purification from the insect cells in a ligand-free as well as a ligand-bound form was accomplished. The yeast *P. pastoris* expression system has also proved to be an efficient method for the high level expression of ET_BR (Schiller *et al*., 2000; 2001) with receptor levels of 20-60 pmol/mg. For stably transformed Ltk- cells (Koshimizu *et al*., 1995) a B_{max} of 596 pmol /mg wild type ET_BR and even higher production values were obtained for several C-terminally truncated ET_BR constructs (up to 2700 pmol/mg). In contrast, similar mammalian cell systems as COS-7, CHO and BHK cells show significantly lower production values.

Table 2.1. Heterologous production of the human ET_BR

Expression system	Expression level	Affinity	Reference
E. coli	41 sites/cell	K_D= 110 pM	Haendler *et al*., 1993
P. pastoris	20-60 pmol/mg	K_D= 41 ± 1.9 pM	Schiller *et al*., 2000
Sf9 cells	100 pmol/mg	K_D= 30 ± 5 pM	Doi *et al*., 1997
COS-7 cells	B_{max}= 1.02 pmol/mg	K_D= 8.8 pM	Stavros *et al*., 1993
COS-7 cells	0.5-1 x 10^4 sites/cell	K_D= 950 pM	Sakamoto *et al*., 1993
COS cells	B_{max}= 60±10 pmol/mg	K_D= 52 ± 4 pM	Elshourbagy *et al*., 1993
CHO cells	B_{max}= 0.36 pmol/mg	K_D= 80 pM	Chiou *et al*., 1997
CHO cells	B_{max}= 0.82 pmol/mg	K_D= 310 pM	Tagaki *et al*., 1995
BHK cells	1 x 10^5 sites/cell	K_D= 530 pM	Hechler *et al*., 1993
Ltk^- cells	B_{max}= 596 pmol/mg	K_i= 77 pM	Koshimizu *et al*., 1995

Several protocols have been developed for the purification of the ET_BR. In one of these studies, the purification procedure for ET_BR consisted of two steps: 1. solubilization with a detergent mixture and 2. affinity chromatography that utilized the strong biotin-avidin interaction. Solubilized receptors were first incubated with biotinylated ET-1 and the receptor-ligand complexes were adsorbed to avidin agarose. The bound proteins were eluted by cleaving the disulfide bond in the spacer arm with 2-mercaptoethanol. This resulted in the isolation of ET_BR with a yield of 200 μg of pure receptor from 3.5 kg bovine lung (Kozuka *et al.*,1991), showing the difficulty in purifying large amounts of the protein. In a related study, Doi *et al*. (1997) have also reported the purification of ET_BR from insect cells using the

biotin-avidin interactions. This protocol resulted in the production of milligram amounts of recombinant receptor.

2.1.4 Aim of study

The ET_BR cannot be purified in large amounts from natural sources. To obtain mg amounts of purified ET_BR for detailed structural studies by state-of-the-art biophysical techniques, a procedure for functional overexpression has to be developed. The insect cell baculovirus system should be exploited, that has been successfully used before for overexpression of the ET_BR and a variety of GPCRs in Sf9 cells. The availability of reasonable amounts of purified ET_BR from Sf9 cells, paving the way for the initiation of biophysical and structural studies, has been described (Doi *et al*., 1997; Satoh *et al*., 1997). The protocol of a purification scheme based on ligand affinity chromatography, allowing solubilization and purification of relatively large quantities of ET_BR produced in Sf9 cells has been reported. The aim has been to establish and optimize the receptor production in Sf9-baculovirus expression system. The receptor should be purified as inspired by previous reports.

2.2 Materials and experiments

2.2.1 Cloning and protein expression in baculovirus system

2.2.1.1 Insect cell culture

Sf9 insect cells were propagated in Sf900II medium supplemented with 50 µg/ml gentamycin as monolayer or suspension culture at 27 °C. Viruses were amplified, and virus titers were determined by end-point dilution as described in O‘ Reilly *et al.* (1992). The cells were passaged to a density of 0.5 x 10^6 cells/ml when the cell density reached 4 x 10^6 cells/ml. Cell density was determined using a hemocytometer and the viability was assessed by trypan blue exclusion.

2.2.1.2 Preparation of recombinant baculoviruses

Cloning into baculovirus was done in a two step process which involved cloning of the ET_BR gene into a transfer vector followed by homologous recombination between the transfer vector and viral DNA. The ET_BR construct encoded residues 1-424. The construct was subcloned into the baculovirus transfer vector pVL1393 (PharMingen) which was used in the

co-transfection of Sf9 cells with wild type *Autographa californica* nuclear polyhedrosis virus (AcNPV) DNA (linearized BaculoGold^{TM} baculovirus DNA; PharMingen) using the manufacturer's recommended procedure after verification by DNA sequencing (see appendix Table 2 for complete description of primers). Expression was under the control of the polyhedrin promoter. For a freshly seeded Sf9 cell monolayer (2 x 10^6 cells/ 35 mm plate), the following cotransfection mixture was used: 2 µg of recombinant transfer plasmid [pVL1393ET$_B$R], 0.5 µg of DNA, 0.5 ml of Sf900II medium and 10 µl polycationic lipid Lipofectamine (GIBCO-BRL). After 4 hours incubation at 27°C, the medium was aspirated and fresh prewarmed medium was added. After 4-5 days, the plate was checked for signs of infection under an inverse microscope. Infected cells appeared larger with enlarged nuclei, the transfection supernatants were then collected and used for the isolation of recombinant virus by limited dilution. First passage viruses were screened for ET$_B$R expression by ligand binding assays or PCR. Recombinant baculoviruses expressing ET$_B$R were passaged three times in Sf9 cells to obtain high titre stocks of 2-4 x 10^8 pfu/ml with virus titres being determined by end-point dilution assay. The virus stocks were stored at 4°C shielded from light and were then used for protein expression studies.

2.2.1.3 Determination of titer and end-point dilution assay

Budded virus titer was estimated using endpoint titration assay. The principle of the method is to obtain isolated viral plaques in a confluent cell monolayer, which can be counted for determination of virus titer. Plaques appear as circular regions in the monolayer and may show retarded cell growth. For performing end-point dilution, the virus stock samples were diluted serially as 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8}. After mixing 10 µl aliquots of each virus dilution with 100 µl aliquots of the Sf9 cell suspension (1 x 10^5 cells/ ml), each sample was seeded into 12 wells of 96-well plates. After incubating the plates at 27 °C for 7 days, each well was checked for virus infection either by differentiating from uninfected cells based on the enlargement of nucleus of infected cells (in case of ET$_B$R) or on the UV table (in case of ET$_B$RGFP, wavelength= 365 nm). Bright green light was seen in several wells. The green light, due to GFP expression, indicated virus infection. This method was also used to identify and isolate recombinant viruses following co-transfection.

2.2.1.4 Isolation of DNA from baculovirus and analysis of purified recombinant virus

To verify that the recombinant virus isolated by end-point dilution does indeed contain the inserted ET$_B$R gene, the presence of the gene was confirmed by PCR analysis. 750 µl of an occlusion negative cell suspension was transferred to a microfuge tube and centrifuged at

5000 rpm at room temperature. To the supernatant 750 µl of ice cold 20% PEG in 1 M NaCl was added and incubated on ice for 30 min. The viral particles were pelleted by centrifuging at maximal speed for 10 min at 4°C. Pelleted viral particles were centrifuged again, and the pellet of viral particles was resuspended in 100 µl of 10 mM TrisHCl, pH 7.6, 10 mM EDTA, 0.25% SDS and digested with 0.5 mg/ml proteinase K at 37°C for 1 h. 100 µl of phenol chloroform was added to the sample. The phases were separated by low-speed centrifugation and the lower organic phase was discarded. Viral DNA was precipitated with 0.1 vol of 3 M sodium acetate and 2 vol of ethanol at -20°C for at least 1 h and pelleted by centrifugation. The DNA was resuspended slowly in 15 µl of TE at 4°C overnight. Presence of the ET_BR gene was identified by PCR amplifying the isolated viral DNA with forward (pvl) and reverse (BS5r) primers (see appendix Table 2 for complete description). Generation of the ET_BRGFP virus used the same procedure, and its integrity was confirmed by DNA sequencing.

2.2.1.5 Cell culture and expression with recombinant baculoviruses

Test expressions were performed in 50 ml suspension cultures. After the determination of optimal conditions, large scale protein expression was carried out. Insect cells were grown in suspension in flasks up to a maximum volume of 500 ml in 2 l bottles at 27 °C with shaking at 150 rpm. Sf9 cells were grown in Sf900II medium. Cell density was determined by haemocytometer counts (Brand, Wertheim/Main, Germany). Cell viability was evaluated by the exclusion of 0.5% Trypan Blue dye in PBS. Once the cells reached a density of 3 x 10^6 cells/ml the cells were diluted by addition of fresh media at a ratio of 1:1 and upgraded to the next flask capacity. Cells were infected with recombinant virus for maximum expression as follows: when cultures had reached a density of 1.5-2 x 10^6/ml (Sf9), virus was added at a multiplicity of infection of 0.1-1. An equal volume of fresh medium was added immediately afterwards. Cells were harvested by centrifugation 96 h after infection and frozen at -80°C until further use.

2.2.2 Preparation of insect cell membranes

All membrane preparation were carried out with ice-cold buffers with the inclusion of protease inhibitor cocktail ‚complete' (Roche Diagnostics).

For small scale expression testing, cells were pelleted from 0.5-1 ml culture, broken by freeze-thaw (two cycles) or by homogenizing in a Potter homogenizer (10 strokes) and resuspended in 1 ml of Tris-BAME buffer (50 mM Tris pH 7.2, 0.02% bacitracin, 0.0015% aprotinin, 2 mM EGTA, 10 mM $MgCl_2$,). Membranes were first pelleted by centrifugation at

500 g for 10 min to discard cell debris, then re-centrifuged at 20.000 x g for 10 min to collect the membrane fraction.

For large-scale membrane preparation, infected cells from shake cultures (1 l total volume) were harvested by centrifugation at 2500 x g for 10 min. Cells were flash-frozen in liquid nitrogen and stored at – 80°C. Infected cells were thawed and suspended in 20 mM Tris/HCl, pH 7.5, 10 mM EDTA and protease inhibitor cocktail and disrupted at 1000 psi using a French pressure apparatus (SLM Amingo). The cell lysate was centrifuged at 800 x g for 10 min and the resulting supernatant was centrifuged at 25000 x g for 60 min. The membrane pellet was washed in 20 mM Tris/HCl, pH 7.5, 5 mM EDTA, 0.2 M NaCl and protease inhibitor cocktail, then with 20 mM Tris/HCl, pH 7.5, 5 mM EDTA and protease inhibitor cocktail. The pellet was suspended in the above buffer, frozen in liquid nitrogen and stored at –80 °C. Protein concentration was estimated using Bradford reagent with BSA as a standard.

2.2.3 Radioligand binding assays

The binding activity of the ET_BR or ET_BRGFP, respectively, was tested with ^{125}I-[Tyr13]ET-1 (NEN Life Science Products, Belgium). Saturation binding assays were performed with 1 μg total protein from whole cells or isolated membranes with ^{125}I-[Tyr13]ET-1 for 2 h at 25 °C in a total volume of 200 μl Tris-BAME buffer containing varying concentrations (50 to 1000 pM) of ^{125}I-[Tyr13]ET-1 in the absence (total binding) or presence (non-specific binding) of unlabeled ET-1 (1 μM), respectively. Membrane-bound ^{125}I-[Tyr13]ET-1 was separated from the free fraction by rapid vacuum filtration (Whatman GF/B glass filter sheets soaked in 0.3% polyethylenimine) using Brandel cell harvester. After three washes with PBS, the filter discs were transferred to polystyrene tubes. Filter-bound radioactivity was measured in a γ-counter. Nonspecific binding was estimated by incubating the ^{125}I-ET-1 in the presence of excess (1 μM) nonradioactive ET-1. Specific binding was defined as the difference between total and nonspecific binding. For displacement studies, various concentrations of unlabeled ET-1 and 100 pM ^{125}I-[Tyr13]ET-1 were used. All assays were conducted in duplicate. The results were analyzed using GraphPad PRISM (GraphPad Software, San Diego) or the RADLIG program. The B_{max} and K_d values were calculated from the results of saturation binding experiments.

Competition radioligand binding assays were performed to determine the affinity of IRL1620 for ET_BR. Membrane suspensions were incubated for 2 h at 25°C in 100 μl of Tris-BAME buffer containing 100 pM ^{125}I-ET-1 and progressively higher concentrations of IRL1620.

After incubation, free ^{125}I-ET-1 was separated from the bound ^{125}I-ET-1 using vacuum filtration.

2.2.4 Effect of cholesterol on the ligand binding of ET_BR

In order to test the effect of cholesterol on the ligand binding of the expressed ET_BR, the membranes of the infected Sf9 cells were preincubated with 0.1 mM soluble cholesterol-methyl-β-cyclodextrin (Sigma) in PBS for 10 min at 30°C. Then the membranes were centrifuged at 16000 g for 30 min at 4°C. The membranes were resuspended in PBS, and the ligand binding was performed as described.

2.2.5 Staining cells for fluorescence analysis

The procedure was performed essentially as described (Wiesner *et al*., 2005). In brief, cells were grown on coverslips in 24-well plates. Cells were diluted to 1.5 x 10^6 cells/ml and induced with virus one hour before adding to the coverslips. The infected cells were allowed to grow for 3-4 days. For live cell imaging, live cell markers were added to stain the plasma membrane or cellular organelles: cells were covered with PBS. For visualization of the plasma membrane, 20 µl trypan blue (0.1% in PBS) was added to the region of interest. For visualization of the nucleus, the bisbenzimide dye H33258 (final concentration 10 µg/ml), for the ER rhodamine 6G chloride (final concentration 5 µM) were added. The cells were visualized using a confocal laser microscope (LSM 510 META system, Zeiss).

2.2.6 Solubilization

All procedures were done at 4 °C. Membranes were solubilized for 2 h with end-to-end gentle rotation at 3 mg/ml membrane protein in 20 mM Tris/HCl, pH 7.5, 5 mM EDTA, 0.3 M NaCl, protease inhibitors ‘complete’ (Roche), supplemented with detergent as indicated. Residual membrane material was removed by centrifugation at 100000 g for 1 h. The resulting supernatant was measured with a fluorescence spectrometer LS-50B.

2.2.7 Electrophoresis and Western blotting

Proteins were resolved on 10% SDS-PAGE and then transferred onto the nitrocellulose membrane with the aid of semi-dry electroblotting apparatus. Protein transfer from the gel onto the nitrocellulose membrane was carried out at 110 mV of constant current for one hour. Non-specific binding sites for immunoglobulins on the membrane were blocked with skimmed milk powder. Immunodetection was carried out with polyclonal anti-rGFP antibody

(1/1670 dilution in 5% skim milk, Clontech). The membrane was incubated with secondary horseradish peroxidase-conjugated anti-mouse IgG (dilution 1/2000). Peroxidase activity was assayed by the SuperSignal Kit for horseradish peroxidase (Pierce).

2.2.8 Bradford assay

The concentration of proteins in solution was estimated with the assistance of the Bradford reagent.

2.3 Results

The goal was to develop an expression system that could produce mg amounts of functional ET_BR for subsequent purification. For that purpose, the recombinant virus stocks containing the ET_BR and ET_BR-GFP genes downstream of the polyhedrin promoter were generated and used for test and large-scale expression. The GFP was chosen as a multi purpose tag for characterizing the expression system. The concentration of the virus stocks were determined using endpoint titration assays. Allowing for the inaccuracy of the endpoint titration assay (Nielssen *et al*., 1992), the maximum virus titers were MOIs of 10^8 pfu/ml (plaque forming units/ml). Sf9 cells were cultivated in suspension at the 0.5-L scale using flasks with a medium volume/ flask volume ratio = 1/5.

2.3.1 Synthesis of ET_BR in Sf9 cells and optimization of expression

Two major factors, that determine the volumetric yield of recombinant protein, are the maximal obtainable cell density and the yield of recombinant protein per cell. Both of these parameters are likely to be influenced by the composition of the culture medium. For economic reasons, commercially available serum-free culture medium was used [Sf900II powder medium (Gibco-BRL)]. Cells adapted to this medium had a doubling time of approx. 24 h and could reach a maximum of 4 x 10^6/ ml. Special attention was given to the density at which cells were infected and the amount of virus used for infection of the cells. High level expression of integral membrane proteins challenges the cell's secretory route (e. g. endoplasmic reticulum or Golgi). Processes such as membrane translocation, folding, N-glycosylation and palmitoylation probably determine the rate of recombinant protein production and hence affect expression levels. Therefore optimal conditions for infection with regard to cell density, virus-to-cell ratio (multiplicity of infection, MOI) and time of harvest for ET_BR had to be established. Commonly, infection of Sf9 cells is performed in the early

exponential phase of growth at high MOI (at least 10) to ensure a synchronous infective process throughout the entire culture. However, because earlier analyses had already indicated that the effect of raising the MOI from 1 to 10 does not have a marked effect on the production level of recombinant rhodopsin (DeCaluwé *et al*. 1993), also very low MOI ranges (0.00005-0.1) were examined. The total cell numbers and production levels were checked over a period of 4-5 days after infection. The expression of ET_BR as a single protein and as a fusion protein with GFP was investigated in shaking cultures. The result of an expression experiment is shown in Figure 2.2 C. Expression of the receptor was monitored by ligand binding. When cells were infected in their early-mid-exponential phase of growth, their maximal recombinant ET_BR production level was relatively unaffected by the MOI (0.1-10). However there was a clear difference in the onset of ET_BR production. With a MOI of 1, 5 or 10, ET_BR production was first detected at approx. 2 days after infection and reached a maximum at approx. 3 days after infection. At MOI values below 1 the average onset of ET_BR synthesis was delayed because it took some time for the initial infection to spread throughout the entire culture. During this period, uninfected cells were still able to divide, resulting in higher total cell counts (Fig. 2.2 A). This also benefited the volumetric yield of recombinant ET_BR because larger numbers of cells could contribute to the production process. Final production levels did not vary significantly and reached approx. the same levels independently of the amount of virus (MOI 0.1-10) used to infect the cells, but lagged behind at a MOI < 1. This probably indicates that there is a maximum amount of recombinant membrane protein that a single cell can accommodate and that this level can be reached as long as the cells remain viable. With a lower MOI uninfected cells are still able to divide, thereby increasing the total number of cells ultimately included in the production process. Production of the ET_BR (Fig. 2.2 C) and ET_BR-GFP were maximal 3-4 days after infection and decreased slightly at later times. The lag could be decreased somewhat by increasing the MOI. This time course is typical for genes under the control of the polyhedrin promoter.

The total cell densities (Fig. 1.2 A) remained constant at the cell density at time of infection confirming that virtually all cells were infected shortly after the addition of the virus stock. As expected, due to the low MOI (=0.1), the cells still grow after infection reaching a maximum cell yield of 2.8×10^6 cells/ml.

Figure 2.2 B shows the viable cell concentration at different times post-infection for culture infected with baculovirus at 1.5×10^6 cells/ml and a MOI of 0.1-10 pfu per cell. The viabilities of infected cells decreased after 2 days post infection (dpi). Thereafter, cell lysis increased rapidly and the viability dropped at a constant rate. Again, there were virtually no

difference in the timing and rate of the decreasing viability observed between the different MOIs.

Production of human ET_BR-GFP usually exceeded 7 pmol/ mg protein (66 pmol/ liter culture). Purified and solubilized plasma membranes from natural sources of these receptors have specific activities of about 4 pmol/ g lung (Hick *et al*., 1995).

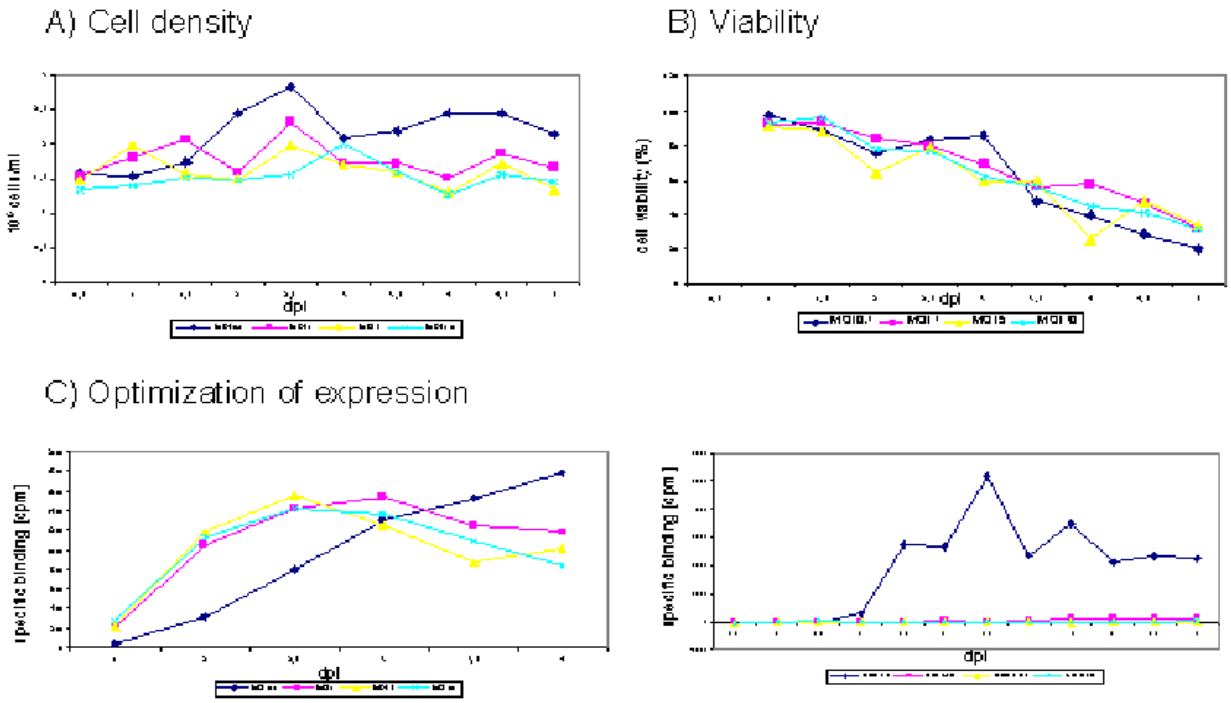

Fig. 2.2. (A) Time course of total cell density and (B) time course of cell viability after infection. (C) Time course of ET_BR expression in suspended Sf9 cells. Sf9 cells (1.5 x 10^6 cells/ml) in shaker culture were infected at a multiplicity of infection (MOI) of 0.1-10 and 0.00005-0.05, respectively, with a recombinant baculovirus that contained the cDNA for the ET_BR. At various time, 0.5-ml aliquots were removed and the expression levels were determined on intact cells. The concentration of ET_BR was measured according to binding activity.

2.3.2 Ligand binding characteristics of ET_BR expressed in Sf9 cells

Previously, expression levels of the constructs ET_BR and ET_BR-GFP were optimized as a function of time and MOI. These determined culture conditions yielding optimal volumetric receptor production in 100 ml shaker cultures were directly scaled up to 500 ml level. Large scale flask cultivation was performed for the ET_BR-GFP construct using MOI 0.1, as a low MOI requires less viral inoculate, which is an advantage for large-scale cultures. Cells were routinely harvested at 4 dpi.

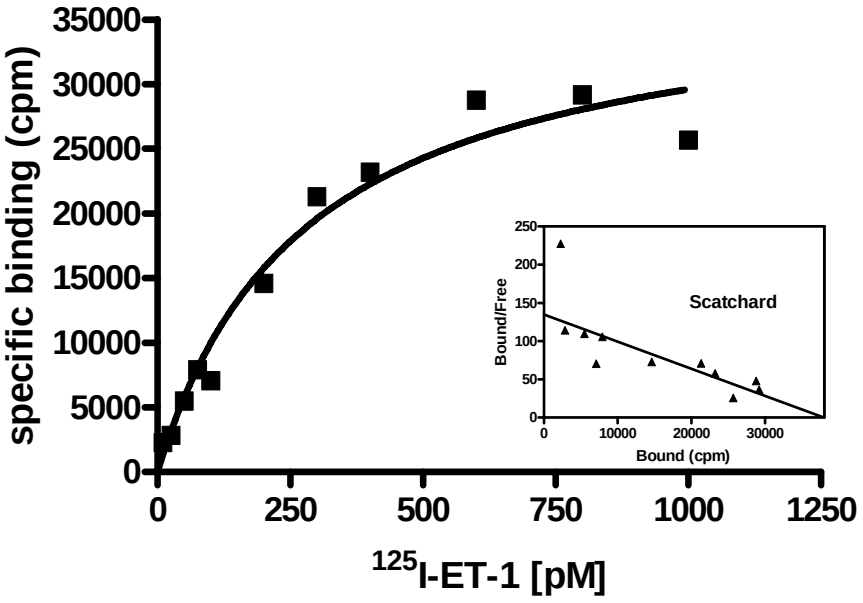

Fig. 2.3. Saturation analysis of ET_BR-GFP in membranes of Sf9 cells. The Sf9 cells were infected by baculovirus encoding the ET_BR-GFP at a MOI of 0.1. At 4 d post infection the cells were harvested and membranes were prepared. For saturation analysis the membranes (1 μg) were incubated with increasing concentrations of ^{125}I-ET-1. Each point is the average of double measurements. Nonspecific binding was determined in the presence of 1 μM ET-1. Binding data was plotted versus total ligand and analyzed by Scatchard plot.

To examine if the ET_BR-GFP produced in Sf9 cells has the same property as the ET_BR extracted from human placenta, a series of binding experiments were performed. ET_BR-GFP was able to bind radiolabelled ligand in a specific and saturable manner. Saturation receptor binding studies and the linear Scatchard plot, respectively, demonstrated that membranes from the baculovirus-infected Sf9 cells contained a single class of binding sites having high affinity for ^{125}I-ET-1 (K_D= 69 pM ± 7.2, Fig. 2.3). Thus, the GFP-tag did not affect its ligand-binding properties. The maximum receptor binding (B_{max}) to the Sf9 membranes was 66 pmol/mg of membrane protein. This level of ligand binding corresponds to approximately 0.04 mg active receptor/liter culture. The B_{max} value deduced from cell membrane experiments only represent the functional receptor sites located at the cell surface. Therefore, it is likely that the real amount of expressed receptors is underestimated.

The pharmacological properties of the expressed ET_BR-GFP was analyzed by the ability of the agonist IRL1620 to compete for the ^{125}I-ET-1 binding. In Fig. 2.4, a displacement curve is shown. Similar EC_{50} values for IRL1620 displacement of ET-1 to the one obtained here (EC_{50}= 34 nM) has been reported for ET_BR (EC_{50}= 3.9 nM) (Nambi *et al.*, 1997).

These results demonstrated that ET_BR-GFP generated in Sf9 cells represent the binding features of ET_BR occurring naturally.

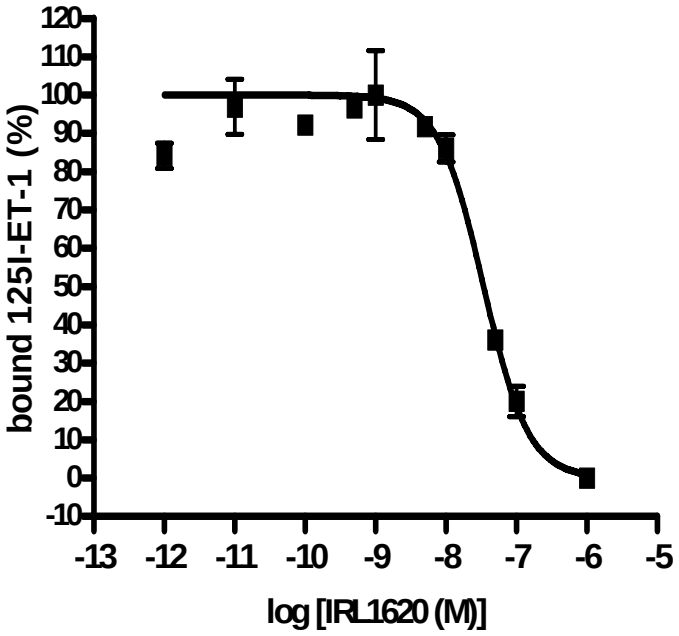

Fig. 2.4. Pharmacological characterization of recombinant ET_BR-GFP. Competition of ^{125}I-ET-1 binding with IRL1620 on ET_BR-GFP baculovirus infected Sf9 membranes.

2.3.3 Modification of membrane cholesterol on ligand binding

The role of cholesterol in the function of GPCRs has been reported: for some receptors including oxytoxin receptors (Klein & Fahrenholz, 1994; Gimpl *et al*., 1995), cholesterol has been reported to be required for retention of ligand binding activity or for increasing the affinity of receptors for ligands. The membrane of Sf9 cells contain little cholesterol. Little is known regarding the ET_BR's lipid environment or its functional or structural dependence on that environment. Therefore, it was examined if cholesterol can influence the binding of ET-1 to ET_BR in membranes of infected Sf9 cells. Ligand binding properties were compared between preparations of the ET_BR and the ET_BR with cholesterol, respectively. Incubation of the cell membrane with 0.1 mM cholesterol significantly increased the B_{max} by 55% without significantly changed the value of K_D (Table 2.2).

These results show that the ET_BR possesses the same ligand binding activity as the ET_BR expressed in mammalian cells (K_D= 52 pM; Elshourbagy *et al*., 1993). It seems that overexpression in Sf9 cells increases the amount of ET_BR produced in a nonactive conformation by reducing the overall availability of less abundant membrane cholesterol. Addition of cholesterol leads to an increase of ET-1 binding sites.

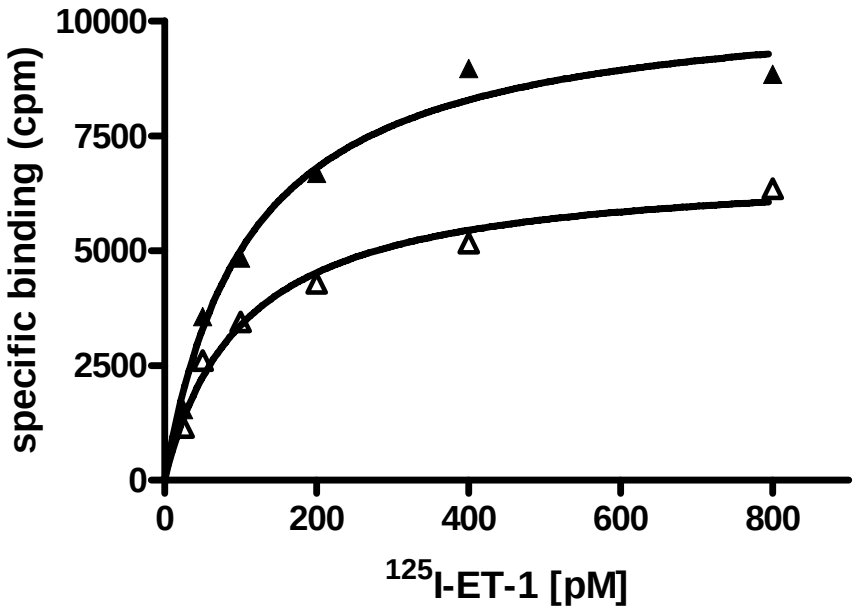

Fig. 2.5. Saturation analysis of ET_B receptors in membranes of Sf9 cells the absence or presence of cholesterol. Membranes of Sf9 cells (0.5 µg) were incubated with increasing concentrations of ^{125}I-ET-1 in the absence or presence of 0.1 mM cholesterol.

Table 2.2 Effect of cholesterol enrichment on ^{125}I-ET-1 binding

	Control	Treated
B_{max} (cpm/µg protein)	6841±382.6	10584±628.8
K_D (pM)	102.9±18.3	111.5±20.5

Treated cells was incubated with cholesterol before binding studies.

2.3.4 Solubilization from Sf9 membranes

Solubilization from membranes is necessary for purification of the receptor. Solubilization of the ET_BR in an unliganded state was studied using fluorescence of the C-terminal GFP for detection of the receptor. To obtain solubilizations, the experiments were performed 2 h at 4 °C. Following ultracentrifugation in order to sediment unsolubilized membranes, the GFP fluorescence (excitation wavelength 488 nm; emission wavelength 509 nm) of the supernatants from different solubilization samples was taken as a measure for solubilized receptor. Although the results of this experiment were not quantitative, it should give an idea which detergents would be worth further testing. A screening for the most effective detergent for solubilization was not performed. Instead digitonin alone and in combination were tested, which had been used to solubilize the ligand-free ET_BR preserving its activity in previous studies (Doi *et al*., 1997). It was used at the indicated concentration: 1% digitonin and 0.2%

CHAPS; 0.4% digitonin. Fluorescence measurement and Western blot analysis of detergent extracts (Table 2.3 and Fig. 2.6) from Sf9 membrane showed that the total amount of solubilized protein did not differ between the detergent mixtures. In cells harvested up to 4 days after infection, the ET_BR-GFP was detected at the expected size of 72 kDa and therefore represents monomeric intact receptor. Immunoblotting indicated that the receptor C-terminus was sensitive to proteolysis upon solubilization and that a significant amount of ET_BR-GFP remained insoluble. Proteolysis could not be reduced by the use of 'complete' protease inhibitor mix (Roche). Solubilized ET_BR-GFP revealed one major band at 40 kDa of strong intensity and two minor bands at M_r= 35 kDa and M_r= 45 kDa . These contaminating bands around 40 kDa in the insoluble and soluble fractions most likely represent proteolytic cleavage previously reported at the N-terminal domain at Arg64 in human placenta (Akijama *et al*., 1992). Cleavage of ET_BR-GFP in the last extracellular loop would result in a 35 kDa fragment that contains GFP. No intact immunoreactive receptor was detected. Additionally, a band corresponding to aggregated form of the receptor produced by solubilization and with a molecular weight of 120 kDa was visible by Western blot.

In the absence of ligand bands with lower molecular weights were detected, indicating degradation of the ET_BR-GFP, and is consistent with previous findings that the ET_BR is highly susceptible to proteolytic cleavage in the absence of ET-1 (Doi *et al*., 1997).

A range of other detergents was tested (Table 2.3) for comparison. They all exhibited poor solubilization efficiency (20-30%). The efficiency of solubilization with respect to total protein and NaCl concentration was not optimized due to the presence of a large amount of higher molecular weight aggregates and due to the idea that sufficient receptor quantity cannot be solubilized in the absence of ET-1.

Table 2.3 Solubilization efficiency studies of ET_BR-GFP from Sf9 membranes

Detergent	Protein concentration [µg]	Fluorescence intensity
0.5% n-dodecyl-β-D-maltopyranoside	640	43.3
0.2% CHAPS, 0.4% digitonin	1260	28.8
0.3% SDS	1240	24.3
1% SDS	1020	25

2700 µg membrane protein were incubated with detergent and solubilized with the indicated concentration. The amounts of ET_BR-GFP in the solubilized fractions were measured.

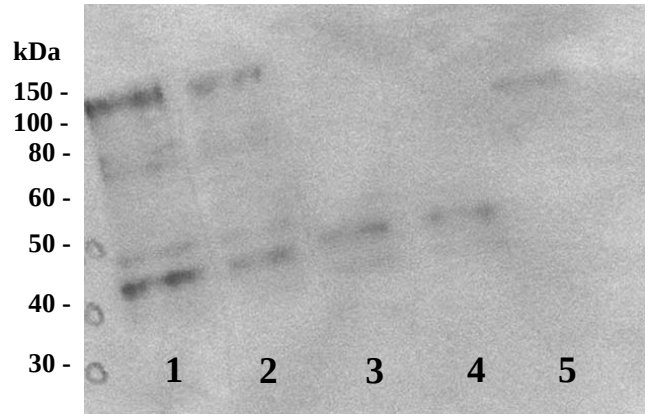

Fig. 2.6. Solubilization of ET_BR-GFP. Sf9 cell membranes containing ET_BR-GFP were solubilized with detergents. After ultracentrifugation, the supernatants were measured for fluorescence. Supernatants after ultracentrifugation analyzed by 10% SDS-PAGE and immunoblotting. Lane 1-2: insoluble fractions (solubilized with digitonin and digitonin/CHAPS), lane 3-4: soluble fractions (solubilized with digitonin and digitonin/CHAPS), lane 5= start material.

2.3.5 Cellular localization of the ET_BR-GFP heterologously expressed in Sf9 insect cells

To reveal the cellular localization of the heterologously expressed ET_BR-GFP within recombinant Sf9 insect cells, laser scan microscopy analysis was performed. The receptor was tagged C-terminally with the autofluorescent GFP to allow visualization. It was shown that a GFP moiety at this position does not influence cell surface expression or the pharmacological properties of the ET_BR (Oksche *et al*., 2000). To assess the expression and intracellular transport of the ET_BR, the GFP fluorescence signals of ET_BR-GFP were localized in living Sf9 cells by laser scan microscopy (Fig. 2.7 A, B, F). The GFP fluorescence is distinct from the blue H33258 fluorescence of the nucleus. This intracellular signal is confined in punctual structures close to the cells periphery (Fig. 2.7 A, E). The green receptor seems to be mainly evenly distributed at the periphery of the cells, either near or at the cell surface membrane. The cell surface of the same cells was visualized by the use of trypan blue (Fig 2.7 D, F). Receptors at the plasma membrane can be identified by computer overlay (Fig. 2.7 F). However, no overlap of GFP and trypan blue fluorescence was observed. Thus, GFP signals was located inside the cell. The signal obtained with ET_BR-GFP colocalized with the fluorescent probe rhodamine 6G, which is an ER marker (Fig. 2.7 A), presumably representing transport intermediates en route to the cell surface or a receptor population that has become trapped as a consequence of overexpression and saturation of the transport

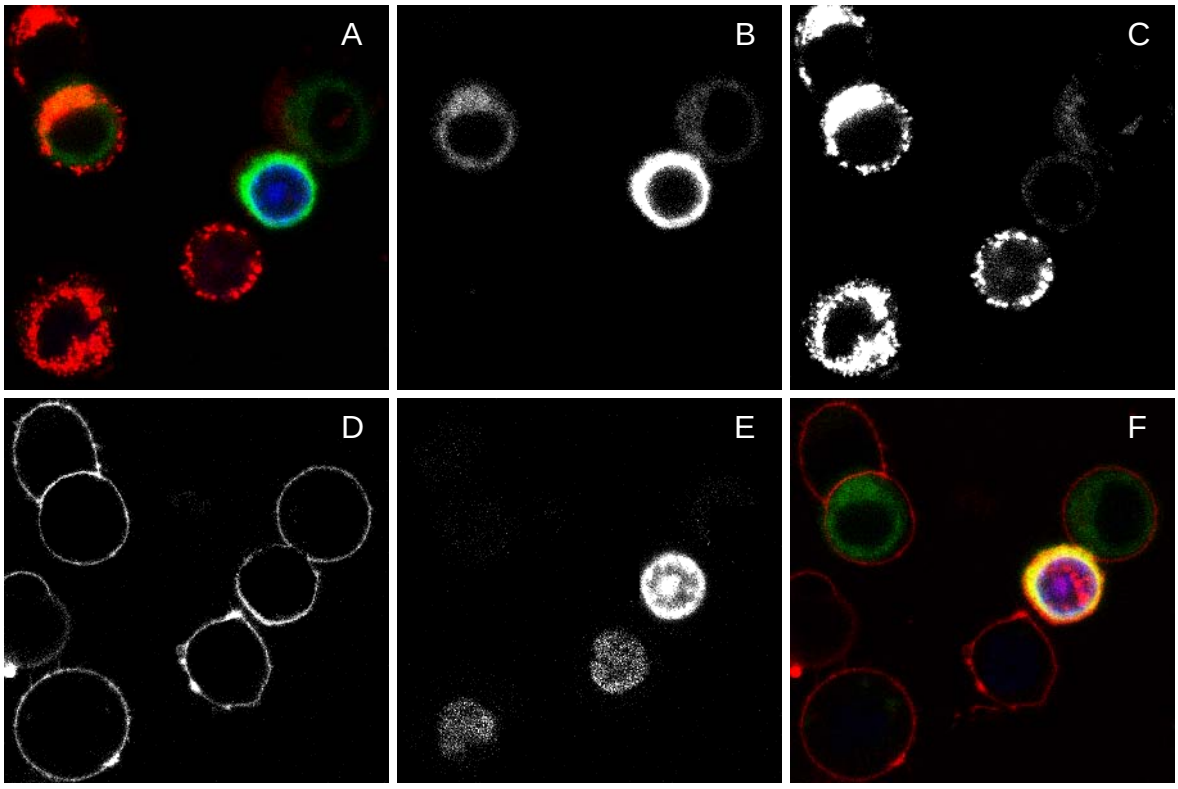

Fig. 2.7. Localization of ET_BR-GFP protein expressed in Sf9 cells (MOI 0.1, 4 dpi) by laser scanning microscopy. (A) The localization of ET_BR-GFP was examined by GFP fluorescence. The nucleus was stained with H33258. The comparison of the cells by two fluorescence probe shows that the receptor is mostly peripheral and intracellularly but not in the nucleus. (B) ET_BR-GFP fusion protein. GFP fluorescence is detectable only in case of infected cells. (C) Staining of the ER with rhodamine 6G. (D) Staining of the plasma membrane with trypan blue. (E) Staining of nuclei with H33258. (F) Confocal image shows the colocalization of ET_BR-GFP with the ER marker rhodamine 6G in Sf9 cells, which is shown in yellow.

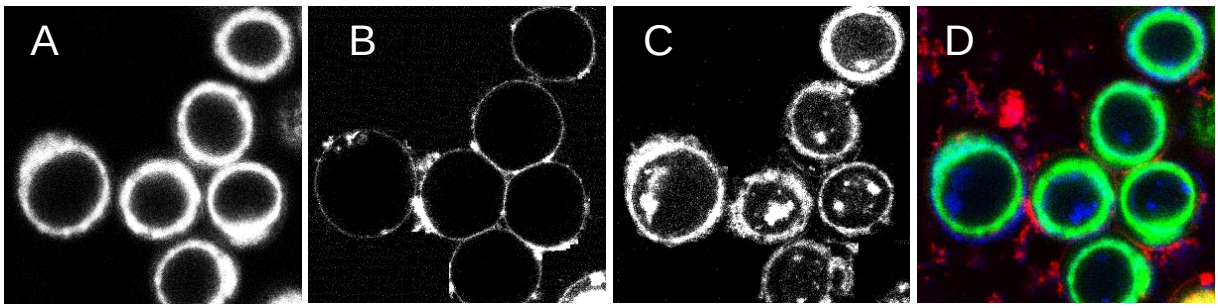

Fig. 2.8. Localization of ET_BR-GFP protein expressed in Sf9 cells (MOI 0.5, 4 dpi) by laser scanning microscopy. (A) ET_BR-GFP was examined by GFP fluorescence. (B) Staining of the plasma membrane with trypan blue. (C) Staining of nuclei with H33258. (D) The comparison of the cells by two fluorescence probe shows that the receptor is mostly peripheral and intracellularly but not in the nucleus.

system. The intracellular retention of the receptor may be a consequence of a folding defect that is recognized by the quality control system of the ER, which ensures the export of

correctly folded proteins. The GFP signal overlapped the ER rhodamine 6G signal as indicated by the yellowish colour (Fig. 2.7 F) which indicated that the receptor is retained in the ER.

Together, confocal laser microscopy data indicated that even increasing the MOI of 0.1 to 0.5 (Fig. 2.8) most of the recombinant ET_BR-GFP is found intracellularly, i. e. presumably within the ER and Golgi compartments, as seen by the green fluorescence bulge (Fig. 2.8 D), and that the receptor is efficiently incorporated into the plasma membrane.

2.4 Discussion

2.4.1 Expression of ET_BR-GFP in Sf9 insect cells

In the present study, the expression of human ET_BR and ET_BR-GFP in Sf9 insect cells under the control of a strong polyhedrin promoter is described. With the ET_BR-GFP construct the GFP gene was added to the C-terminal part of ET_BR in order to detect the expression of the protein by fluorescence measurements and by Western blotting. For this purpose, the ET_BR gene was cloned into the commercially available standard transfer vector pVL1393. Finally, we obtained the pVL1393ET_BR and pVL1393ET_BR-GFP, and have verified that the C-terminal GFP marker does not affect the pharmacological properties of the receptor. As already mentioned, one of the advantages of the recombinant baculovirus/insect cell expression system is, that culture volumes can be scaled up. The optimal conditions derived from small-scale experiments with the ET_BR construct (Sf9 cells in Sf900II medium, infection in logarithmic growth phase, MOI=0.1, cells harvested at 4 dpi) was directly adapted to large-scale suspension culture (0.5 l) using the ET_BR-GFP construct.

Our expression level obtained (7 pmol/mg) was lower than those of ET_BR so far reported (ranging from 30 to 100 pmol/mg) (Doi *et al*., 1997). The mutant protein [H57-H62, G63-G65]ET_BR, carrying a His6 tag in the N-terminal tail, could be purified most effectively and the yields were 0.3 mg ligand-free ET_BR and 0.5 mg ligand-bound ET_BR from Sf9 membranes derived from 3-l cultures (Doi *et al*., 1997). Our ET_BRGFP construct was functionally expressed in the same range in Sf9 cells, compared with the turkey β-adrenergic receptor or the human muscarinic cholinergic receptor, which were expressed in Sf9 cells at 5-30 pmol/mg protein (Parker *et al*., 1991). The reasons for differences in expression among different laboratories are unclear, but may be related to the nature of the DNA constructs employed. For example, small changes in nucleotide sequences, particularly near the initiation

codon, can have a marked effect on protein expression. The composition of the growth medium may also have a significant impact on protein production. Quantification of GPCR levels using B_{max} measurements is also complicated since the quantity of receptor detected by radioligands is influenced by receptor G-protein coupling, ion concentration and the presence or absence of guanine nucleotides.

2.4.2 Time course study and optimization of ET_B receptor expression

Given the insect cell line, the virus construct, and the medium, the key optimization parameters are time of infection (TOI, here measured as cell density at infection) and MOI. To achieve the optimal expression efficiency, we varied the MOI. While a theoretical study suggested, that low MOI's (around 0.1) could improve the yield of recombinant protein (Licari & Bailey, 1992), the large majority of baculovirus literature uses MOI's in the range 1-10. Accordingly, Doi *et al*. (1997) used a MOI of 1-2 and TOI of 2 x 10^6 cells/ml. We also evaluated MOI's in the range of 0.00005-10. Varying the MOI between 0.1 and 10 only moderately affected the ET_BR expression, but indeed slightly higher binding activity at lower MOI's was observed. This is largely due to the increase in cell density, since at a low MOI cellular division still can proceed for some time. The net cellular production capacity appears to be quite independent of the MOI. It needs to be emphasized, that the use of a low MOI leads to an asynchronous infection process originating multiple cell populations types that coexist simultaneously, this further increases the complexity in the understanding and thus controlling the system. Asynchronous infection occurs when cultures are infected with an MOI < 1. Under this condition only a fraction of cells become infected by the virus added to the culture (primary infection). The non-infected cells continue to grow and will become infected during the secondary infection by budded viruses released from the cells infected during the previous round. Therefore, individual cells within the same culture become infected at entirely different times. Analysis of cells expressing ET_BRGFP under the fluorescence microscope revealed a great variability of the fluorescence level reflecting varying expression levels between cells.

At high MOI (>5) essentially all cells will be infected immediately (O'Reilly *et al*., 1994) and the infection process will be synchronous. For a MOI of 5, the final infection is also the primary infection and receptor production commenced at the onset of the very late phase, 24 h post infection (Fig. 2.2 C). Cell growth ceased shortly after infection and thus the cell yield was close to the cell density at TOI (cell yield concept). Thus, to achieve optimal production in our case according to the cell yield concept, cells should be infected at a cell density (TOI=

2 x 10^6 cells/ml for MOI= 5) close to the optimal cell yield (see Fig. 2.2 A). The potential higher volumetric productivity would be ascribed to the higher number of productive cells contributing to the final production.

Our study was also extended to MOIs as low as 0.00005 (Fig. 2.2 C). For a MOI of 0.05 only 5% of the cells are infected in the primary infection. The secondary and final infection appears to have occurred around 24 h post infection, leaving another 24 h until the commencement of the major release period. Similarly for the MOI= 0.005, two and three round of infections seems to have preceded the major final infection. The significant differences between these both profiles are a delay in the onset of major production and the maximum receptor level. For MOIs < 0.05, the appropriate TOI is still to be determined to achieve optimal expression.

Experimental variation must also be considered. It is important to ensure that the initial conditions (TOI and MOI) are very controlled. Variation in initial conditions, may explain the difference in yield and onset of production observed in the two shaker flask infection experiments (Fig. 2.2 C) for MOI=0.1 and MOI=0.05 at TOI of 1.5 x 10^6 cells/ml. To achieve reproducible initial cell density, it may be necessary to rely on a Coulter counter rather than on the relatively inaccurate hemocytometer cell counts. Given the inaccuracy of virus assays, the virus stock must also be carefully titered.

The ability to apply low MOI values to large-scale cultures has a clear advantage over high MOI values because a much smaller viral inoculum is required. For instance, infection of 10-litre culture at 2 x 10^6 cells/ml with an MOI of 10 would require 2 litres of high-titer viral inoculum (typically 10^8 pfu/ml). Assuming that the virus stock is stored in 10 ml lots, the virus inoculum could be produced through two or three fermentations. In other words, a process relying on synchronous infection will consist of two parallel scale-up processes: one for cells and one for virus. Our results show that the amount of virus can be decreased by a factor of 100 without compromising total recombinant protein yield. The low required amount of virus makes the large-scale production problem more manageable, which encourages the use of low passage number, thus avoiding the problem of defective particles (Wickham *et al*., 1991).

2.4.3 Other factors influencing overexpression

The expressed receptor was localized in the intracellular compartments in addition to the plasma membrane, as visualized using GFP. The main reason for this phenomenon is probably saturation of the transport machinery due to the overexpression of the receptor. This

has also been observed with other GPCRs (Lundström *et al*., 2001). The large number of receptors in the internal compartments could also correspond to those in the exocytic pathway on the their way to the plasma membrane, or to receptors which have already reached the plasma membrane and in a second step have entered the endocytic pathway (Perret *et al*., 2003). The presence of a signal sequence and many potential glycosylation sites in the ET_BR, should favor the translocation of the receptor to cell membranes. A caveat of the insect cell system is the expression of a significant proportion of inactive receptors, as indicated by laser scan microscopy. However, it remains difficult to estimate the actual fraction of nascent receptors that is correctly folded, processed and transported to the plasma membrane.

In several studies, immature and/or incompletely glycosylated forms of GPCRs (Parker *et al*., 1991) have been found in crude cell membrane fractions of Sf9 cells. These unglycosylated and presumably misfolded receptors are unable to bind ligands. As a consequence, Doi *et al*. included a step based on the biological activity of the receptor, such as ligand affinity chromatography, in the receptor purification scheme in order to avoid heterogeneous preparations contaminated with non-functional and misfolded receptors that biases structural studies.

In an attempt to get rid of immature forms of receptors, Loisel *et al*. (1997) coinfected Sf9 cells with two population of viruses. One encoded the β2AR, the other the P55Gag protein of the HIV type 1. The rationale of this approach was based on previous studies showing that plasma membrane proteins from Sf9 cells infected with a P55Gag baculovirus can be recovered in virus-like particles released in the extracellular medium. Interestingly, such immature receptors are not present in the extracellular baculovirus particles released from baculovirus infected cells. This may offer an advantageous source of receptor.

Efforts can be made to improve the level of expression attained in the insect cells to increase even further the amount of starting material for purification. For some GPCRs, such as oxytocin (Gimpl *et al*., 1995), the $5HT_{5A}$ serotonin receptor (Lenhard *et al*., 1996) and the D_{2S} dopamine receptor (Grünewald *et al*., 1996a, b) the addition of a cleavable prepro-melittin signal sequence to its N-terminus enhanced the level of expression, thus significantly augmenting the amount of receptor that can be purified.

The effect of cholesterol enrichment was investigated, which is known to affect the activity of GPCRs in other systems, on the binding of ^{125}I-ET-1 in Sf9. Cholesterol enrichment significantly increased the B_{max}, but there was no significant change in the mean value of K_D following cholesterol treatment. To confirm the observation that membrane cholesterol enrichment induced a significant increase of the number of receptor binding sites, which may

be the result of the reduction of membrane fluidity, experiments with phospholipids-treated cells have also to be performed. The increase of B_{max} following lipid incubation would appear to be specific for cholesterol when liposome made up with pure phospholipids does not alter binding. The physiological role of the increase of receptor binding sites induced by membrane cholesterol-enrichment is not clear but ET_BR has been reported to be located in cholesterol-rich caveolae (Oh & Schnitzer, 2001).

2.4.4 Conclusions and outlook

In conclusion, with our ET_BR construct and the expression conditions, the expression level corresponds to approximately 0.05% of the total membrane protein, which is not a good starting point for scale up and production. Still further increases of ET_BR expression are necessary to reach levels at least comparable to that of Doi *et al*. (1997).

Because of the virus infection nature, it kills significant percentage of cell population and therefore produces a mixture of intracellular precursors and extracellular mature receptors without signal peptides. Therefore, it is supposed that the discrepancy Doi *et al*. reported may be caused by the different MOI's and cultivation equipment used, as well as by the different expression strength of the constructs.

An expression level of at least 0.1 mg ET_BR/l culture would have been satisfied for large-scale production and to start to find optimal conditions for the solubilization and purification of the ET_BR, to minimize working volumes and to make procedures as efficient as possible. A high receptor concentration in the membrane would be efficient because solubilization of the receptor from the membrane at membrane suspensions of a relatively low concentration would allow the quantitative extraction of the receptor with moderate detergent concentrations. For large-scale purification procedures, the extraction volume is an important parameter because it determines for example the quantity of chemicals required and the time needed for centrifugation and column loading. With membrane suspensions containing low amount of receptor, the receptor can still be extracted but now required more detergent to achieve the same yield.

It is hypothesized that the modest amount of ER and Golgi apparatus in insect cells, combined with the overexpression obtained by baculovirus are reasons for low expression, as the little membrane volume gets aggregated with recombinant receptor. The detergent extraction also indicates that the receptor is already aggregated. Lack of glycosylation has been implicated as a cause for lack of expressed membrane protein activity (Tate *et al*., 2003). Further studies will be required to determine if (correct) glycosylation of ET_BR is required for expression.

Attempts should be made to improve ET_BR plasma membrane insertion by the replacement of the ET_BR signal sequence for a presumably more efficient signal sequence whether derived from the *Apis melifera* melittin, the influenza hemagglutinin (Guan *et al*., 1992) or the baculoviral gp64 (Massotte *et al*., 1999). Apart from glycosylation, lack of cholesterol could explain or add to the low activity. This study is not exhaustive of all the parameters that could be changed in the quest for obtaining high receptor level.

There are some observations that various cell lines yield different levels of recombinant proteins. And to optimize the production, it is worth investigating the kinetics and expression level in a range of host cell lines (Sf21, High Five) under identical conditions.