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Structural genomics on membrane proteins: comparison of more than 100 GPCRs in 3 expression systems

Kenneth Lundstrom · Renaud Wagner · Christoph Reinhart · Aline Desmyter · Nadia Cherouati · Thierry Magnin · Gabrielle Zeder-Lutz · Melanie Courtot · Cécile Prual · Nicolas André · Gherici Hassaine · Hartmut Michel · Christian Cambillau · Franc Pattus

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Abstract Production of recombinant receptors has been one of the major bottlenecks in structural biology on G protein-coupled receptors (GPCRs). The MePNet (Membrane Protein Network) was established to overexpress a large number of GPCRs in three major expression systems, based on *Escherichia coli, Pichia pastoris* and Semliki Forest virus (SFV) vectors. Evaluation by immunodetection demonstrated that 50% of a total of 103 GPCRs were expressed in bacterial inclusion bodies, 94% in yeast cell membranes and 95% in SFV-infected mammalian cells. The expression levels varied from low to high and the various GPCR families and subtypes were analyzed for their expressability in each expression system. More than 60% of the GPCRs were expressed at milligram levels or higher in one or several systems, compatible to structural biology applications. Functional activity was determined by binding assays in yeast and mammalian cells and the correlation between immunodetection and binding activity was analyzed.

Keywords Expression vectors · G protein-coupled receptors · Structural genomics

K. Lundstrom · G. Hassaine
Bioxtal, Chemin des Croisettes 22, Epalinges CH-1066, Switzerland

R. Wagner · N. Cherouati · T. Magnin · G. Zeder-Lutz · M. Courtot · F. Pattus
UMR7175 Department of Receptors and Membrane Proteins, Illkirch, France

C. Reinhart · C. Prual · N. André · H. Michel
Max Planck Institute of Biophysics, Frankfurt, Germany

A. Desmyter · C. Cambillau
University of Marseille, Marseille, France

Present Address:
K. Lundstrom (✉)
Flamel Technologies, 33, Avenue du Dr. Georges Levy, Venissieux 69693, France

Introduction

G protein-coupled receptors (GPCRs) represent more than 50% of current drug targets, which generate more than $50 billion in annual sales. The functions of GPCRs are many and include various signal transduction pathways but also intercellular mechanisms (Lundstrom 2006). GPCRs can be activated through peptides, neurotransmitters, hormones, odors, ions, light, odorants, pheromones, amino acids, amines, nucleotides, nucleosides, prostaglandins and other low weight molecules. Due to the broad spectrum of functions, GPCRs play important roles in various indications such as cardiovascular, metabolic, neurodegenerative, neurological, psychiatric, viral diseases and cancers.

The success in obtaining high resolution structures of membrane proteins has in general been very modest. In comparison to the current number of more than 30,000 entries for soluble proteins in public databases, only 104 unique structures have been solved for membrane proteins (http://blanco.biomol.uci.edu/Membrane_Proteins_xtal.html). The situation is even worse for GPCRs for which only a single structure has

so far been published (Palczewski et al. 2000). Even so, to obtain the structure for the bovine rhodopsin was only possible when the receptor was isolated from cow retina in an inactive conformation. However, the high receptor density of rhodopsin is unique as GPCRs only occur at low abundance. The main bottlenecks for not being successful in obtaining high resolution structures for recombinantly expressed GPCRs are the following: First, the expression of quantitatively and qualitatively acceptable recombinant receptors has been difficult. Furthermore, the expressed GPCRs need to be subjected to solubilization procedures in the presence of detergents prior to purification. This step can substantially reduce the yields of active receptor. Moreover, the GPCRs are generally extremely sensitive to degradation during the purification step, which means that the stability of purified receptor material is poor and the shelf-life short. Finally, crystallization in presence of detergents is more demanding than for soluble proteins. GPCRs also present flexible regions thereby reducing the contact sites required for appropriate crystal formation.

Although the expression levels have been fairly moderate for many GPCRs tested, in some cases relatively high receptor levels have been obtained. For instance, the human adenosine A2a receptor was expressed as a fusion protein with the maltose binding protein (MBP) in *E. coli* membranes with a B_{max} value of 17–34 pmol per milligram (Weiss and Grisshammer 2002). Expression in bacterial inclusion bodies has generated large quantities of GPCRs, although the necessity of complicated refolding processes has hampered the success (Lopez de Maturana et al. 2003; Baneres et al. 2003). High yields and high density of functional GPCRs (25–40 pmol/mg) has been obtained in yeast expression systems (Reinhart and Krettler 2006), particularly in *Pichia pastoris* (Weiss et al. 1995; André et al. 2006). Expression in insect cells from baculovirus vectors has also been commonly applied for GPCRs resulting in milligram yields of receptors per liter culture (Mazina et al. 1994). Mammalian expression has been hampered by low yields, time-consuming production and high costs. Generally, studies have focused on a limited number of GPCRs at a time with the exception of a parallel study of 16 GPCRs in insect cells applying baculovirus vectors (Akermoun et al. 2005). For this reason, there has usually been neither any comparison between the expressability of individual receptors nor comparable studies between expression systems. The approach initiated by MePNet (Membrane Protein Network) to study 103 GPCRs overall in parallel in three well-established expression systems has therefore provided a unique opportunity to evaluate the expression properties of a large number of GPCRs. The selection of GPCRs was preliminary based on ligand availability to be able to monitor the functional activity of receptors expressed in yeast and mammalian cell membranes by radioligand assays and also later on to perform binding assays for purified and solubilized GPCRs as well as refolded receptors from *E. coli* inclusion bodies. Other criteria were to have representation of different classes and subtypes of GPCRs and to link the targets to human disease. In this context, the majority of GPCRs studied were from class A (95 GPCRs) receptors, which are represented by light (rhodopsin), adrenaline (adrenergic) and olfactory receptors. Only two members of the class B hormone and neuropetide receptors were included in the study. The class C GPCRs with the characteristically large extracellular N-terminal were represented by 4 members. Finally, two yeast receptors were from class D. Of the total number of 104 GPCRs in this study, 82 were of human origin, 9 from rat, 7 from mouse, 2 from bovine, 2 from yeast, 1 from pig and 1 from hamster.

The choice of expression systems was based on previous experience of convenience in handling a large number of clones in parallel, the potential of scale-up and obviously the possibility to obtain yields compatible to structural biology applications. *E. coli*-based expression was chosen because of the speed, the inexpensive process and the ease of use. The straight forward scale-up to fermentor cultures was also considered as an advantage. *E. coli* vectors chosen in this study were pET15 vectors with N- and C-terminally engineered 10-histidine tags, respectively. Fifty-five of the 103 GPCRs were also introduced into 6 Gateway vectors containing various fusion partners. Under the expression conditions used in this study, GPCRs were always produced as insoluble inclusion bodies in the cytoplasm of *E. coli*. It was decided to use *E. coli* vectors in this study that uniquely promote expression in bacterial inclusion bodies although relatively high expression have been reported for GPCR expression in bacterial membranes (Tucker and Grisshammer 1996, Weiss and Grisshammer 2002). However, recent improvement in refolding of GPCRs from inclusion bodies (Baneres et al. 2003) was encouraging and the extensive need for construct engineering (mutations, deletions, evaluation of different fusion partners and tags) required for membrane-based expression was not feasible for 100 GPCRs in parallel. For this reason, pET15 vectors with N- and C-terminally engineered 10-histidine tags, respectively, were employed. A large portion of the 103 GPCRs were also introduced into Gateway vectors

containing various fusion partners. *Pichia pastoris* vectors were applied for expression in yeast cells as a proven lower eukaryotic system. The *P. pastoris* system is well establish and fairly rapid to work with and generates large biomasses in fermentor cultures. Moreover, several GPCRs have been previously successfully expressed with this system (reviewed in Reinhart and Krettler 2006). Finally, the third system represents expression in mammalian cells. Semliki Forest virus (SFV) has previously been shown to be applicable to the expression of a large number of recombinant proteins, including many GPCRs (Lundstrom 2003). The expression levels have been very high with B_{max} values of 150–200 pmol/mg and large-scale bioreactor production in mammalian suspension cultures has been established for SFV (Blasey et al. 1997). The inclusion of members of all GPCR families and several subtypes provided additional interesting information in relation to expression patterns. The study of 103 GPCRs also allowed to describe which receptors were not successfully expressed and in which system. Finally, the expression evaluation study has provided a large number of well expressed targets that currently are subjected to further structural biology exploration. We have previously described the overexpression of 20 GPCRs from *P. pastoris* André et al. 2006) and 101 GPCRs from SFV-infected mammalian cells (Hassaine et al. 2006), but this is the first time a comparison of more than 100 GPCRs is presented for three expression systems.

Materials and methods

Cloning and subcloning of GPCR genes into expression vectors

Appropriate primers were designed for the 5′ and 3′ end coding regions of each GPCR, which were amplified from plasmid DNA when available or from commercial polyA$^+$ RNA preparations (Clontech, Palo Alto, CA, USA). In the latter case, RT-PCR reactions were performed to amplify the full-length ORFs using the Titan One-Step RT-PCR kit from Roche (Penzberg, Germany) according to the manufacturer's instructions. In both cases, the amplified fragments were subcloned into the pCR4®Blunt-TOPO® vector (Invitrogen, Carlsbad, CA, USA) and the accuracy of the sequence verified. In case of differences from the published sequence, the existence of potential polymorphism was investigated. Mutations introduced by the RT-PCR procedure were corrected accordingly. Orientation-defined GPCR fragments with 5′ end *Bam*HI and 3′ end *Spe*I sites were obtained using type IIS restriction endonucleases (*Esp*31 or *Bbi*I) or *Bam*HI–*Spe*I restriction enzymes when feasible. These fragments were then subcloned into the *Bam*HI–*Spe*I digested pET15N2, pET15C2, pPIC19KHisFLAGBio, pSFV2genB and pSFV2genC expression vectors (Fig. 1). For Gateway™ (Invitrogen, Carlsbad, CA, USA) cloning PCR fragments were amplified containing the respective attB1 and attB2 recombination sites into Gateway™ destination vectors. Six Gateway™ expression vectors were used (Fig. 1).

E. coli based expression in bacterial inclusion bodies

The bacterial expression vectors were transformed into four *E. coli* strains: BL21(DE3)pLysS and Rosetta(DE3)pLysS from Novagen and C41(DE3) and C43(DE3) from Avidis SA (Saint-Beauzire, France) (Miroux and Walker 1996). Freshly transformed bacteria were cultured in LB medium supplemented with 100 μg/mL ampicillin (34 μg/mL chloramphenicol for BL21(DE3)pLysS and Rosetta(DE3)pLysS) at 37°C until an OD_{600} value of 0.7–0.8 was reached. The expression of recombinant GPCRs was induced by addition of 1 mM IPTG (isopropyl-*β*-D-thiogalactopyranoside) at 37°C for 3 h or at 25°C or 15°C for 16 h. Cells were harvested at +4°C by centrifugation at 5000 × *g* for 20 min, lysed in 50 mM Tris–HCl pH 8.0, 300 mM NaCl, 0.1% Triton X-100, 1 mM EDTA, 0.25 mg/mL lysozyme and 1 mM phenylmethyl-sulfonyl fluoride and stored at – 20°C prior to Western blotting. The optimal growth conditions were applied to 1 L flask and 8 L fermentor cultures. Additionally, a number of GPCRs were analyzed for the localization of recombinant receptors in *E. coli*. Total lysates of bacteria were obtained by lysosome treatment and sonication. Inclusion bodies (pellet) were separated from soluble protein (supernatant) by centrifugation at 10,000 × *g* for 30 min followed by SDS-PAGE and Western blotting.

Yeast-based expression from Pichia pastoris vectors

The yeast expression vector constructs for the 100 GPCRs were linearized with *Pme*I or *Sac*I and the *P. pastoris* strain SMD1163 electroporated at 1500 V, 25 μF and 600Ω using a Gene Pulser I (Bio-Rad, Reinach, Switzerland). Selection of clones was done as previously described (Weiss et al. 1998, André et al. 2006). In conclusion, recombinant His$^+$ clones were first selected on MD agar plates (1.34% yeast nitrogen

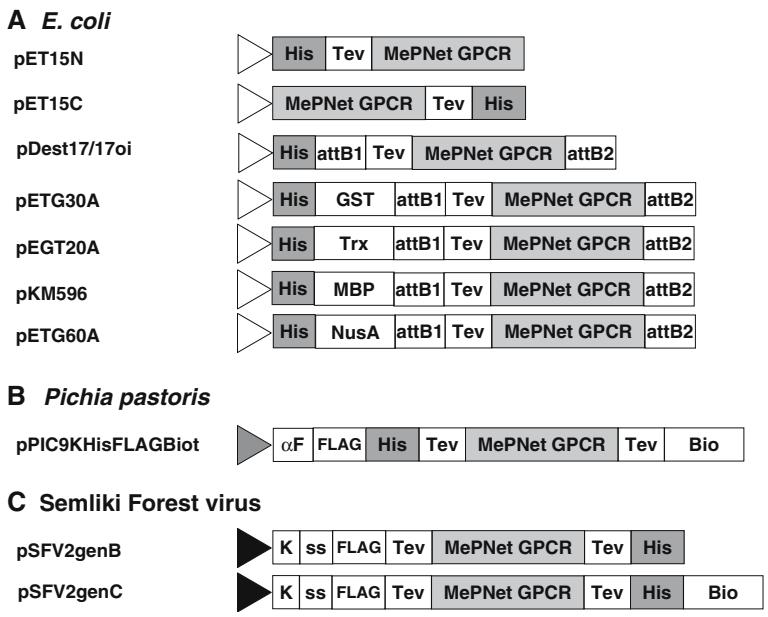

Fig. 1 Schematic presentation of expression vectors. α F, yeast α Factor; Bio, biotinylation domain from *Proprionibacterium shermanii*; FLAG, FLAG-tag; His, 10-histidine tag; GST, Glutathion-S-transferase; K, Kozak consensus sequence; MBP, maltose binding protein; NusA, transcription factor NusA; ss, signal sequence from influenza HA (hemagglutin) gene; Tev (Tobacco Etch Virus), Tev protease cleavage site; Trx, Thioredoxin; white triangle, T7 promoter; grey triangle, yeast AOX1 promoter; black triangle, SFV 26S subgenomic promoter

base without amino acids, 2% dextrose, 0.00004% biotin, 1.5% agar). In a second step the His^+ clones were cultured on YPD agar containing various concentrations of G418 (1% yeast extract, 2% peptone, 2% dextrose, 2% agar and 0.05–0.5 mg/mL G418) to obtain multicopy transformants.

For the production of GPCRs *P. pastoris* was precultured in BMGY medium (1% yeast extract, 2% peptone, 1.34% yeast nitrogen base without amino acids, 0.00004% biotin, 1% glycerol, 0.1 M phosphate buffer, pH 6) at 30°C and 250 rpm until an OD_{600} value of 2–6 was reached. The induction was carried out in BMMY medium (identical to BMGY except 0.5% methanol instead of 1% glycerol) at 30°C from an initial OD_{600} value of 1. After 18 h cells were pelleted at 3000 × *g* for 5 min and subjected to membrane preparation. Cells were washed once with ice-cold lysis buffer (50 mM sodium-phosphate buffer pH7.4, 100 mM NaCl, 5% glycerol, 2 mM EDTA, 1 mM PMSF) and resuspended to 30% wet weight. After 0.5 mm glass beads were added to the cell suspension, yeast cells were broken by vigorous vortexing at 4°C for 10 min. Breaking efficiency was inspected with a light microscope and was usually > 80%. Intact cells and cell debris were separated from the membrane suspension by a low speed centrifugation (3000 × *g*, 5 min, 4°C). Membranes were then pelleted using an ultracentrifuge (100,000 × *g*, 45 min, 4°C), resuspended in membrane buffer (50 mM Tris pH8.0, 120 mM NaCl, 20% glycerol, 1 mM PMSF) using a dounce homogenizer. Membrane proteins were quantified applying the BCA method (Pierce, Rockford, IL, USA), using BSA as a standard. Membranes were snap frozen in liquid nitrogen and stored at – 80°C.

Expression in mammalian cells applying Semliki Forest virus vectors

BHK-21 (baby hamster kidney), CHO-K1 (Chinese hamster ovary) and HEK293 (human embryonic kidney) cells were cultured in a 1:1 mixture of Dulbecco's modified F-12 medium and Iscove's modified Dulbecco's medium supplemented with 4 mM glutamate and 10% FCS (foetal calf serum). C8166 (human T lymphocyte) cells were grown in RPI Medium, 4 mM glutamate and 10% FCS. All cell culture reagents were purchased from Gibco BRL (Invitrogen, Carlsbad, CA, USA). Recombinant SFV particles were prepared as previously described (Lundstrom et al. 1994). Briefly, in vitro transcribed RNA from SFV expression vectors pSFV2genB and pSFV2genC, respectively, were co-electroporated with pSFV-Helper2 RNA (carrying the SFV capsid and envelope genes) into BHK-21 cells. Virus was harvested 24 h later, activated with α-chymotrypsin, aliquoted and stored at – 20°C (short term) or – 80°C (long term). Expression evaluation was performed on 12 and 24 well plates as follows. Adherent cells at 70% confluency were infected with recombinant SFV particles at various multiplicity of infection (MOI) values and cells harvested at different time points post-infection. Expression evaluation was done on cells lysed in 50 mM Tris–HCl, pH 7.6, 150 mM NaCl, 2 mM EDTA, 1% NP40 and 1 μg/μL PMSF. For membrane preparation all manipulations were performed at 4°C

as previously described (Hovius et al. 1998). Approximately 0.5 g of frozen cell pellets were resuspended in 10 mL of 10 mM HEPES containing 1 mM EDTA (pH 7.4) and homogenized for 10 s with an Ultra-Turrax T25 (IKA, Staufen, Germany) homogenizer. Membranes were pelleted by centrifugation at 27,000 × *g* for 30 min after a two-fold dilution and resuspended in 10 mM HEPES (pH 7.4).

Immunodetection methods

The expression evaluation was visualized by dot blots and/or Western blots. *Dot blots*. Membranes from *P. pastoris* cells expressing GPCRs were diluted in TBS buffer containing 0.2% SDS. PVDF membranes (Millipore, 0.45 μm) were washed with methanol and 38 mM Glycine, 10 mM Tris, 20% methanol in a 96 well microfiltration apparatus (Bio-Dot, Bio-Rad, Reinach, Switzerland) and 1 μg of total membrane was added. The PVDF membrane was blocked with 5% low fat powder in TBS for 60 min at room temperature, washed three times for 1 min with TBS and incubated with alkaline phosphatase-conjugated anti-FLAG M2 antibody (Sigma, Buchs, Switzerland). After three 15 min washes the blots were developed in AP-buffer (100 mM Tris pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) containing 330 μg/mL 5-bromo-4-chloro-indolylphosphate *p*-toluidinium salt and 165 μg/mL nitroblue tetrazolium chloride. For *P. pastoris* membranes, a standard curve for the β2 adrenergic receptor corresponding up to 25 pmol receptor per mg protein, was applied as a reference. In this context, signals of a lower intensity than observed for 1 pmol β2 adrenergic receptor were classified as low level (+), intensities corresponding to 1–10 pmol as medium (++) and > 10 pmol as high (+++). However, these extrapolations are only relative as only part of the signals obtained by immunodetection corresponds to functional receptors. *Western blots*. Samples were subjected to 10% SDS-PAGE and the proteins electrotransferred for 30 min to Hybond ECL nitrocellulose membranes (GE Healthcare, Uppsala, Sweden) and blocked with 5% milk in TBST (TBS with 0.1% Tween 20) at +4°C. The filter was then treated with primary antibodies (anti-FLAG 1:1000 dilution or anti-His 1:1000 dilution) (Sigma, Buchs, Switzerland), washed, and treated with the secondary anti-mouse antibody 1:2000 dilution (Sigma). The anti-His antibody is directly conjugated to peroxidase and requires no secondary antibody use. The visualization of GPCR-specific bands was done with the ECL chemiluminiscence kit from GE Healthcare (Uppsala, Sweden).

Radioligand binding assays

Membrane fractions were prepared from yeast (André et al. 2006) and mammalian (Hovius et al. 1998) cells as previously described. The protein concentration was measured applying the BCA method (Pierce, Rockford, IL, USA) with BSA as a standard. Aliquots of membranes were snap frozen and stored at – 80°C prior to use. Radioligand binding assays were established for individual GPCRs as previously described (André et al. 2006 and Hassaine et al. 2006). Briefly, membranes were incubated in triplicates in the presence of radioligand until equilibrium was reached. Non-specific binding was obtained by incubation with excess of non-radioactive ligand. Filtration in presoaked Whatman GF/B or GF/F filters in 0.3% polyethylenimine was followed by 3 washes and liquid scintillation counting. Single point binding assays were conducted at one non-saturating radioligand concentration. Saturation bindings were performed for certain GPCRs and the saturation curves were analyzed by non-linear regression applying Kaleidagraph (Synergy Software, Reading, PA, USA), which allowed determination of B_{max} and K_d values.

Results and discussion

Expression vectors

One hundred and three receptors representing the major GPCR families were subcloned after sequence verification into vectors representing the bacterial, yeast and mammalian expression systems of choice. The N-and C-terminally tagged pET15N2 and pET15C2 vectors were used for the expression evaluation of 101 and 18 GPCRs, respectively, and 6 Gateway vectors with or without fusion partners (Fig. 1) were tested for expression of 55 GPCRs in *E. coli* inclusion bodies. The *Pichia pastoris* expression vector used in this study has been previously described (André et al. 2006) and was designed on the basis of previous results obtained for GPCR production (Weiss et al. 1998). To facilitate purification efforts and to improve stability of recombinant GPCRs various tags and fusion partners were also introduced into the expression vectors as illustrated in Fig. 1. A signal sequence from the influenza HA (hemagglutinin) gene was further introduced into the SFV vector to promote transport and translocation to the plasma membrane as previously demonstrated for GPCRs expressed in insect cells from baculovirus vectors (Guan et al. 1992). The biotinylation domain from *Proprionibacterium*

shermanii (Bio-tag, Cronan 1990) was engineered into the *P. pastoris* and SFV vectors based on previous experience, which suggested that the biotinylation domain can improve the yields and stability of expressed GPCRs (Weiss et al. 1998, Grünewald et al. 2004, Reinhart and Krettler, 2006). Likewise, the human neurokinin-1 receptor was previously expressed in SFV-infected BHK and CHO cells as a fusion protein with the C-terminally tagged biotin (Bio-tag). In this case, the expression levels were monitored by metabolic labeling and saturation binding assays, which indicated similar levels of receptor density for wild type and NK1R-biotin domain constructs (Lundstrom et al. 1995). Furthermore in that study, functional coupling to G protein was demonstrated by measurement of intracellular Ca^{2+}-release, but no purification was performed to verify the stabilizing effect of the Bio-tag on the GPCR.

Expression evaluation by immunodetection

Here, the evaluation of expression levels was performed by dot blot and Western blotting techniques and four categories established as follows: high (+++), medium (++), low (+) and no (–) expression. The dot blots were particularly useful to verify the expression levels for individual yeast clones during the selection process, whereas Western blots revealed the size of the expressed GPCR. To qualify as a positive signal in Western blots, the corresponding immunospecific band needed to be in the approximate range of the calculated molecular weight and therefore targets demonstrating bands with significantly faster or slower mobility were considered as negative. The results of the expression evaluation are summarized in Table 1. In *E. coli* 101 GPCRs were evaluated for expression, of which 46 showed a positive signal by immunoblotting and 55 were negative (Table 1). Eighteen targets belonged to the group of high (+++), 12 to medium (++) and 16 to low (+) expression levels. Several factors significantly affected the expressability in bacteria. The choice of *E. coli* vector played a significant role. In this context, the Gateway vectors were superior to the pET15-based vectors. Expression from the N-terminally tagged pET15N2 vector resulted in positive signals in 28 of the 101 tested targets. Introduction of a deca-His tag at the C-terminus in the vector pET15C2 showed positive signals in only 2 of 18 GPCRs tested. In contrast, the success rate was much higher in Gateway vectors, demonstrating positive signals in 33 out of 55 tested targets. Overall, the success of expression of GPCRs in bacteria was relatively low. Characterization of the soluble and the cell pellet fractions in *E. coli* revealed that the recombinantly expressed GPCRs were uniquely located in inclusion bodies and not inserted into membrane structures, demonstrated by subjecting total bacterial lysates to lysosome treatment and sonication after which inclusion bodies (pellet) were separated from soluble protein (supernatant) by centrifugation and the expressed GPCR verified by SDS-PAGE and Western blotting. Other factors influencing the expression levels were the *E. coli* strain applied and the growth temperature of bacteria. In this context, the C43 strain was superior to BL1(DE3), Rosetta (DE3) and C41. The initial expression evaluation was carried out at 4 mL scale. Next, 23 well expressed GPCRs were selected for large-scale production in 1 L shake flask cultures. Among the 23 GPCRs 9 were expressed from the pET15N2 vector, 1 from pET12C2 and 15 from Gateway vectors. The yields in flask cultures varied between 2 mg/L and 40 mg/L and the recombinant receptors were only localized in inclusion bodies. Although the yields were relatively high the produced material was not sufficient for extensive purification and refolding exercises. For this reason, 7 of the 23 targets were further subjected to fermentation in 3 or 8 L bioreactors. The yields in the fermentor cultures were generally higher (75–350 mg/L) than in flask cultures and provided now sufficient material for the establishment of purification and refolding conditions.

Only a single vector construct was engineered for *P. pastoris* (Fig. 1B). Briefly, the vector contained an inducible AOX1 promoter followed by an α-factor signal sequence and FLAG- and deca-His-tags at the N-terminus. The GPCRs were introduced as *Bam*HI–*Spe*I fragments and flanked by Tev protease cleavage sites. These sites were engineered into the expression vector to allow removal of all tags after purification to avoid any interference of tags during the crystallization procedure. Furthermore, the Bio-tag was engineered at the C-terminal to potentially improve expression and stability of the recombinant GPCRs (Reinhart and Krettler 2006). After subcloning of the GPCRs into the *P. pastoris* vector pPIC9K, a high-throughput screening method based on in situ immunoblotting was established (Magnin et al. manuscript in preparation). This method facilitated significantly the clone selection process of the large number of GPCR constructs studied in parallel. The expression profile in yeast cells applying *P. pastoris* vectors was quite different from what was obtained in bacteria. The success rate was much higher, partly due to the clone selection process for each individual GPCR, which was established to obtain constructs with optimum number of copies integrated into the yeast genome (André et al. 2006).

Table 1 Expression evaluation by immunodetection

	MePNet#	*E. coli*	*P. pastoris*	SFV	Size	GC%
ACM1_HUMAN	23	–	+	+++	51.4	61
ACM1_MOUSE	24	–	+	+++	51.3	57
ACM2_PIG	26	–	+	+	51.7	47
ACM2_HUMAN	25	–	+	+	51.7	47
A1AA_HUMAN	104	–	+	+++	51.5	57
A1AA_RAT	14	–	+	+++	51.6	55
AA1B_HUMAN	15	nd	++	++	56.8	63
AA1B_MESAU	105	–	+	+	56.5	60
A1AD_RAT	106	–	+	+	59.4	62
A2AA_HUMAN	107	–	+	+++	49	69
A2AB_HUMAN	16	–	++	+	50	64
A2AC_HUMAN	18	–	++	+	49.6	66
B3AR_HUMAN	31	–	+	+	43.5	70
D2DR_HUMAN	43	–	++	++	50.6	57
D2DR_MOUSE	44	–	+++	+++	50.9	53
D3DR_HUMAN	45	+	++	+++	44.2	55
D4DR_HUMAN	46	+	++	+++	48.4	75
DADR_HUMAN	109	–	++	++	49.3	52
DBDR_HUMAN	110	–	++	+++	53	61
HH2R_HUMAN	61	+	+++	+++	40.1	57
HH2_RAT	62	+	nd	+++	40.3	54
5H1A_HUMAN	1	–	+++	+++	46.1	60
5H1B_HUMAN	2	+	+++	+++	43.6	57
5H1D_HUMAN	3	–	+++	+++	41.9	56
5H1E_HUMAN	4	–	++	+	41.7	51
5H1F_HUMAN	5	–	+++	+++	41.7	41
5H2A_HUMAN	6	++	++	+	52.6	46
5H2A_MOUSE	7	++	–	+	52.8	48
5H2B_HUMAN	8	–	+	+	54.3	43
5H2C_HUMAN	9	–	–	+++	51.8	45
5H4_HUMAN	144	–	++	+++	43.8	48
5H5A_HUMAN	10	–	++	+++	40.3	61
5H6_RAT	111	–	++	+++	46.9	64
5H7_HUMAN	12	+	+	+++	53.6	56
5H7_RAT	117	+++	++	–	49.8	56
AG2R_HUMAN	28	++	++	+	41.1	40
BRB1_HUMAN	32	++	+	+++	40.4	55
BRB2_HUMAN	33	–	+	+++	44.5	58
FSHR_HUMAN	131	–	+	+	78.3	59
IL8A_HUMAN	63	+	+	++	39.8	53
IL8B_HUMAN	136	–	+	+	40.8	53
CCR3_HUMAN	39	–	+	+++	40.7	64
CCR4_HUMAN	135	nd	nd	+++	39.7	50
CKR1_HUMAN	40	+	+++	+++	41.1	50
CKR2_HUMAN	41	+	nd	+	41.9	47
CKR6_HUMAN	42	+++	+	++	42.5	47
CKR7_HUMAN	145	+	+	++	42.9	56
CKRB_HUMAN	153	–	+	+++	39.9	41
CCKR_HUMAN	38	–	+++	++	47.8	54
GASR_HUMAN	57	–	+++	++	48.4	63
ET1R_BOVIN	50	+++	+++	–	48.5	47
ET1R_HUMAN	51	+++	+	+	48.7	45
ACTR_HUMAN	27	+++	++	+	33.9	51
ETBR_BOVIN	52	+	++	+	49.4	49
MC3R_HUMAN	64	++	+++	+	40	57
MC4R_HUMAN	65	+++	+++	+	37	46
MC5R_HUMAN	66	++	+++	+	36.6	53
NY1R_HUMAN	79	+++	+++	+++	44.4	40
NY2R_HUMAN	80	–	+++	++	42.7	50
NY4R_HUMAN	81	++	++	++	42.2	57
NY5R_HUMAN	82	–	+	nd	52	38
OPRD_HUMAN	83	–	+	+	40.4	65

Table 1 continued

	MePNet#	*E. coli*	*P. pastoris*	SFV	Size	GC%
OPRD_MOUSE	84	–	++	++	40.6	63
OPRK_HUMAN	85	+++	++	++	42.7	54
OPRK_MOUSE	86	+++	+++	+	42.7	49
SSR1_HUMAN	93	–	+++	+++	42.7	61
SSR2_HUMAN	94	+++	++	+++	41.3	52
SSR3_HUMAN	95	–	+++	+++	45.8	66
NK1R_HUMAN	72	+++	++	+++	46.3	55
NK1R_RAT	73	+	+++	+	46.4	55
NK2R_HUMAN	74	+++	++	+++	44.4	58
NK2R_RAT	75	++	+++	+++	43.9	57
NK3R_HUMAN	76	+++	+++	++	52.2	51
V1BR_HUMAN	103	++	+++	+++	47	63
GALR_HUMAN	54	–	++	+++	38.9	58
GALT_HUMAN	56	+++	+++	++	39.6	72
PAR1_HUMAN	99	–	+	–	47.4	50
MCR1_HUMAN	60	–	+++	++	46	57
LSHR_RAT	133	–	–	+++	78	49
FML1_HUMAN	139	–	++	++	39	51
TSHR_RAT	116	–	+	+	86.5	50
PE23_HUMAN	90	++	+	++	43.3	60
AA1R_HUMAN	19	+	+	++	36.5	60
AA2A_HUMAN	20	++	+	++	44.7	61
AA2B_HUMAN	21	+++	+	++	36.3	55
P2Y2_MOUSE	87	–	–	+	42.2	63
P2Y2_HUMAN	88	–	–	+++	39.4	36
P2Y6_HUMAN	137	–	++	+	36.4	63
P2YR_HUMAN	89	+++	+++	+++	42.1	51
CB1R_HUMAN	36	nd	+	nd	52.9	54
CB1R_MOUSE	121	+++	+	+	52.8	52
CB2R_HUMAN	37	+	+++	+++	39.7	56
EDG3_HUMAN	48	++	+	+++	42.3	58
CLT1_HUMAN	129	–	–	+++	38.5	39
CLT2_HUMAN	130	+++	++	+	39.6	45
CALR_HUMAN	34	–	+	–	57.3	47
GLR_HUMAN	59	+	+	++	54	63
MGR2_HUMAN	67	–	+	–	95.5	61
MGR3_HUMAN	68	–	+++	+	98.6	51
MGR4_HUMAN	69	–	+++	+++	101.9	61
MGR5_HUMAN	113	–	nd	nd	126.7	61
MGR7_HUMAN	114	–	+	+	102.3	51
STE2_YEAST	96	+	+	+++	47.8	38
STE3_YEAST	97	–	+++	+++	53.7	38

nd, not done; no expression, – (pink); low expression, + (light blue); medium expression, ++ (medium blue); high expression, +++ (dark blue)

Additionally, the eukaryotic status of yeast cells most likely contributed to the success in expression. Overall, 94 of 100 evaluated GPCRs showed a positive signal by immunodetection, of which 30 belonged to the group of high (+++), 27 to medium (++) and 37 to low (+) Table 1). Only 6 GPCRs showed no expression in *P. pastoris*. The expression pattern in yeast was also strongly affected by external factors. The cell culture temperature, addition of ligands and additive supplements to the yeast culture medium strongly influenced the expression levels. In this context, the lowering of the temperature from 30°C to 20°C, addition of specific ligand at concentrations close to 100 times the K_d value or providing additives such as 2.5% DMSO or 0.04 mg/mL histidine significantly increased the binding activity (André et al. 2006). The results, however, varied considerably from one GPCR to another. For instance, lowering of the temperature improved the binding activity for 10 GPCRs, had no significant effect on 9 receptors and for the human serotonin 5-HT1B receptor resulted in substantially reduced activity. Likewise, treatment with DMSO resulted in increased binding for 16 GPCRs whereas 4 receptors were unaffected. Addition of ligand also improved the binding activity for 18 receptors, but reduced B_{max} values were observed for the human serotonin 5-HT1B

and the rat neurokinin-2 receptors. The effect of histidine addition was favorable for 12 GPCRs and indifferent for 8. To further optimize the expression those parameters with a positive effect were combined, which resulted in doubled production levels for 13 GPCRs. The B_{max} values were more than 4 times higher for 5 receptors and the maximum increase of 8.7 fold was obtained for the human kappa opioid receptor. Interestingly, when the expression levels in Dot blots were compared to the B_{max} values, it was evident that optimization did not affect the receptor quantity (Fig. 2). It rather looked like the quality of the recombinant GPCRs was enhanced resulting in larger proportion of receptors with functional binding activity. The optimal expression conditions for individual GPCRs have been described in more detail previously (André et al. 2006).

Two expression vectors were designed for SFV-based expression (Fig. 1C). In the pSFV2genB vector a Kozak sequence was introduced downstream of the subgenomic SFV 26S promoter to optimize the initiation of ribosomes (Kozak 1986). A signal sequence from the influenza hemagglutinin (HA) gene was introduced to facilitate the translocation of the GPCRs to the plasma membrane. Furthermore, a FLAG tag was engineered at the N-terminus in front of the full-length GPCR flanked by Tev protease cleavage sites. The C-terminal contained a deca-His tag. The second SFV vector, pSFV2genC, was otherwise identical to pSFV2genB except for an additional biotin tag placed downstream of the His-10 tag. One hundred and one GPCRs were evaluated for expression in mammalian cells using SFV-infected host cells. Due to the broad host range of SFV, several mammalian cell lines can be efficiently transduced. However, dealing with such a large number of targets, it was decided to initially limit the expression evaluation to three cell lines, BHK-21 (baby hamster kidney), CHO-K1 (Chinese hamster ovary) and HEK293 (human embryonic kidney) cells, previously shown to express high levels of GPCRs (Lundstrom 2003). A few GPCRs were also expressed in the human T lymphocyte cell line C8166. The success rate for SFV expression was very high with 97 GPCRs showing positive signals and only 4 targets no detectable specific response in Western blots (Table 1). According to expression evaluation 44 GPCRs were classified as high (+++), 25 as medium (++) and 28 as low (+) expressers. Important factors in relation to expression levels were virus concentration (MOI, multiplicity of infection), and the duration of expression and host cell line. Taken together the expression results from the three systems demonstrated that positive signals were obtained for 102 GPCRs verified by immunodetection, of which 65 represented high (+++), 20 medium (++) and 19 low (+) expression levels. Only one GPCR (MGR5_HUMAN) was negative, although only tested for expression in *E. coli*. In fact, the human metabotropic glutamate mGluR5 has been previously successfully expressed from SFV vectors (Lundstrom 2000). Likewise, the glutamate receptor mGluR2 (Schweitzer et al. 2000) and the endothelin receptor (Cramer et al. 2001), both negative in this study when expressed from SFV vectors, demonstrated high specific binding and functional activity in mammalian cells. This discrepancy may be due to the use of the standard SFV vector in the previous studies, whereas here the vector contained the influenza HA signal sequence, which might interfere with the expression, folding and transport of the recombinant receptor.

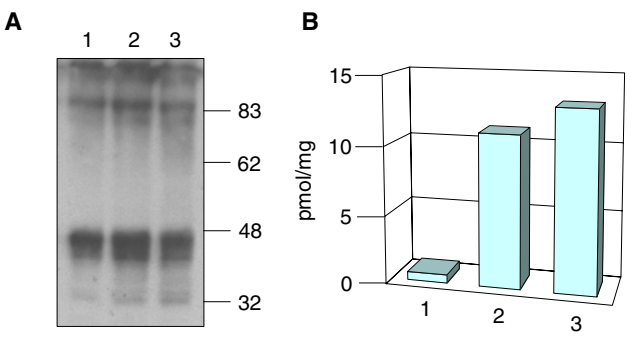

Fig. 2 Expression of the human NK2 receptor in *P. pastoris*: comparison of Western blots to binding activity before and after optimization. Sample 1, 30°C; sample 2, 20°C; sample 3, 20°C + 2.5% DMSO. (**A**) Western blots using anti-FLAG M2 antibody. Molecular weight marker indicated on the right. (**B**) Saturation binding using [^{3}H] SR48968

Expression comparison of GPCR families and subtypes

The comparison of expressability of GPCRs from the four major classes, A rhodopsin-like, B secretin-like, C metabotropic glutamate/pheromone and D fungal pheromone is not possible as the majority of GPCRs studied are class A receptors (95) and only very few representatives were from the other classes: B (2), C (4) and D (2). The reason for having such a high representation of class A receptors is their large number and their importance as drug targets. The expression pattern for the class A receptors showed a success rate of 48.4% in *E. coli*, 93.5% in *P. pastoris* and 96.8% in SFV evaluated by immunodetection. The expression pattern for the bacterial expression of the few targets of the other classes of GPCRs was similar except for

the class C receptors, where none of the four tested GPCRs gave a signal. Class C receptors in general and the four metabotropic glutamate receptors tested here are characterized by a large extracellular N-terminal domain. Whether the extracellular domain or the large size of the recombinant metabotropic glutamate receptors (95–102 kD) was the reason for failure of expression is discussed below under target size. The yeast expression showed 100% success rate for class B, C and D receptors and for SFV-based expression in mammalian cells most of the GPCRs were positive by immunodetection.

More interestingly, among the GPCRs studied several belonged to the same subfamily of GPCRs. For instance, 4 muscarinic, 4 α-adrenergic, 6 dopamine, 14 serotonin, 4 neuropeptide Y, 4 opioid, 5 neurokinin, 4 purinoreceptor P2Y and 4 metabotropic glutamate receptors were studied (Table 2). The expressability of the GPCRs was evaluated by immunodetection and grouped as previously into categories of no expression (–), low expression (+), medium expression (++) and high expression (+++). The serotonin receptors, the group with the highest number of subtypes, demonstrated no expression for 64.2% of the targets in *E. coli*, medium expression for 42.9% in *P. pastoris* and high expression of 57.1% in SFV. Overall, high expression levels were obtained for 31.0% of the serotonin receptors. Another subfamily of receptors that was well expressed for all three expression systems, were the neurokinin receptors. Of the 5 neurokinin receptors tested 60% were expressed at high levels in all three expression systems. None of the neurokinin receptors showed a negative signal. The 8 adrenergic receptors showed variable expression levels. None of them were successfully expressed in *E. coli*, the expression levels in yeast were either low or medium, whereas in mammalian cells 4 GPCRs showed low, 1 target medium and 3 targets high expression levels. Dopamine receptors were also problematic to express in *E. coli* as all 6 subtypes were negative. In *P. pastoris* the majority of dopamine receptors were expressed at medium levels, whereas for SFV 4 of the 6 GPCRs belonged to the group of high expressers.

Target size and GC content

The size of recombinantly expressed proteins might play an important role in relation to the expression levels obtained. In general, smaller proteins have given better expression yields especially in bacterial systems. In the case of the 100 targets studied here, they represented GPCRs ranging in size from a postulated molecular weight of 33.9 kD to 102.3 kD. Comparison of molecular weight to expression levels suggested that size played an important role in bacterial expression. In this context, the largest class A GPCR expressed successfully from *E. coli* had a postulated molecular weight of 53.6 kD. Similarly, the class B receptor with a size of 54.0 kD gave a positive signal whereas the GPCR of 57.3 kD was negative in Western blots. All 4 class C GPCRs were negative in *E. coli*. These metabotropic glutamate receptor are large (95–102 kD) and it is therefore not surprising that no expression was observed. Of the two class D yeast STE receptors, only the smaller STE2 (47.8 kD) was expressed, whereas the STE3 (53.7 kD) showed no signal. According to the expression results for the 93 class A GPCRs expressed in *E. coli*, 54 kD seemed to be the limit where positive signals were detected. In contrast to bacterial expression, when the 100 GPCRs were analyzed for expression in yeast and mammalian cells, no correlation could be observed between size and expressability. For example, all of the large size class C receptors were expressed in *P. pastoris* and 3 out 4 of them expressed in SFV.

Among factors influencing gene expression in general, the different codon usage in pro- and eukaryotes has been indicated as a cause. This issue has been addressed by developing special bacterial strains for recombinant proteins with poor codon usage for mammalian proteins (Kleber-Janke and Becker 2000). It has also been shown that the frequency of some codons and amino acids correlate with the GC content in the genome (Sueoka 1961). Recently, the trends in codon and amino acid usage in relation to GC composition have been studied (Knight et al. 2001). Here we have investigated whether the GC content has any effect on the expression levels of 103 GPCRs in bacterial, yeast and mammalian cells. The GC content for the 103 GPCRs varied between 36% and 75% for class A receptors. The two class B GPCRs had GC contents of 47% and 63%, respectively. Class C receptors showed a range of GC content from 51% to 61% and the two class D receptors had both a GC content of 38%. In none of the three expression systems was there any correlation between the expression levels and the GC content. Also the GPCRs that showed no signal showed a large variation in GC content.

Immunoblotting versus binding activity

GPCRs expressed in *E. coli* inclusion bodies are present in aggregates and require refolding before any functional activity can be restored. In contrast, expression of GPCRs in yeast and mammalian cells

Table 2 Expression comparison of GPCR subtypes

Nd	–	+	++	+++	
Muscarinic (4)					
0	4 / 100%	0	0	0	*E. coli*
0	0	4 / 100%	0	0	*P. pastoris*
0	0	2 / 50.0%	0	2 / 50.0%	SFV
0	4 / 33.3%	6 / 50.0%	0	2 / 16.7%	Total
Adrenergic (8)					
1	7 / 100%	0	0	0	*E. coli*
0	0	5 / 62.5%	3 / 37.5%	0	*P. pastoris*
0	0	4 / 50%	1 / 12.5%	3 / 37.5%	SFV
1	7 / 30.4%	9 / 39.1%	4 / 17.4%	3 / 13.0%	Total
Dopamine (6)					
0	4 / 100%	0	0	0	*E. coli*
0	0	0	5 / 83.3%	1 / 16.7%	P. pastoris
0	0	0	2 / 33.3%	4 / 66.7%	SFV
0	4 / 22.2%	0	7 / 38.9%	5 / 27.8%	Total
Serotonin (14)					
0	9 / 64.2%	2 / 14.3%	2 / 14.3%	1 / 7.1%	*E. coli*
0	2 / 14.3%	2 / 14.3%	6 / 42.9%	4 / 28.6%	*P. pastoris*
0	0	3/ 21.4%	2 / 14.3%	9 / 64.3%	SFV
0	11/26.2%	7 / 16.7%	10 / 23.8%	14/ 33.3%	Total
Neuropeptide Y (4)					
0	2 / 50.0%	0	1 / 25.0%	1 / 25.0%	*E. coli*
0	0	1 / 25.0%	1 / 25.0%	2 / 50.0%	*P. pastoris*
1	0	0	2 / 66.7%	1 / 33.3 %	SFV
1	2 / 18.2%	1 / 9.1%	4 / 36.4%	4 / 36.4%	Total
Opioid (4)					
0	2 / 50.0%	0	0	2 / 50.0%	*E. coli*
0	0	1 / 25.0%	2 / 50.0%	1 / 25.0%	*P. pastoris*
0	0	1 / 50.0%	3 / 50.0%	0	SFV
0	2 / 16.7%	2 / 16.7%	5 / 41.7%	3 / 25.0%	Total
Neurokinin (5)					
0	0	1 / 20%	1 / 20%	3 / 60.0%	*E. coli*
0	0	0	2 / 40%	3 / 60.0%	*P. pastoris*
0	0	1 / 20%	1 / 20%	3 / 60.0%	SFV
0	0.00%	2 / 13.3%	4 / 26.7%	9 / 60.0%	Total
Purinoreceptor P2Y (4)					
0	3 / 75.0%	0	0	1 / 25.0%	*E. coli*
0	2 / 50.0%	0	1 / 25.0%	1 / 25.0%	*P. pastoris*
0	0	2 / 50.0%	0	2 / 50.0%	SFV
0	5 / 41.7%	2 / 16.7%	1 / 8.3&	4 / 33.3%	Total
Metabotropic glutamate (5)					
0	5 / 100%	0	0	0	*E. coli*
1	0	2 / 50.0%	0	2 / 50.0%	*P. pastoris*
1	1 / 20.0%	1 / 20.0%	1 / 20.0%	1 / 20.0%	SFV
2	6 / 40.0%	3 / 20.0%	1 / 6.7%	3 / 20.0%	Total

Expression levels as for Table 1; highlighted in yellow; the highest number of targets expressed from individual expression vectors and in total

generate receptors located in cell membranes, which can be directly monitored for their functional activity by radioligand binding assays. Selected GPCRs were subjected to binding assays and the correlation between immunodetection and binding activity was evaluated (Table 3). A total of 51 GPCRs expressed in *P. pastoris* and 42 in SFV were assayed for specific binding. The GPCRs were divided into four groups indicating no (0), low (< 1 pmol/mg), medium (1–10 pmol/mg) and high specific binding (> 10 pmol/mg). Expression in yeast cells generated 7 targets with no, 15 with low, 13 with medium and 16 with high specific binding activity. The highest B_{max} value of 180 pmol/mg was obtained for the adenosine A2A receptor (AA2A_HUMAN). For SFV-based expression, 3 targets showed no, 7 low, 18 medium and 14 high specific binding. Again, the highest binding activity (287 pmol/mg) was obtained for AA2A_HUMAN. The correlation between immunodetection and specific binding activity was analyzed as shown in

Table 3 Immunodetection vs. binding activity of selected GPCRs

	MePNet#	Pichia ID	Pichia BA	SFV ID	SFV BA
ACM1_HUMAN	23	+	0.23	+++	2.44
ACM1_MOUSE	24	+	1	+++	2.93
ACM2_PIG	26	+	1	+	22
ACM2_HUMAN	25	+	1.5	+	6.1
A1AA_HUMAN	104	+	0.45	+++	0.25
A1AA_RAT	14	+	0.25	+++	4.7
AA1B_HUMAN	15	++	5	++	0
AA1B_MESAU	105	+	0.14	+	0
A1AD_RAT	106	+	0.21	+	0
A2AB_HUMAN	16	++	11	+	0.05
A2AC_HUMAN	18	++	0.25	+	0.65
B3AR_HUMAN	31	+	0	+	nd
D2DR_HUMAN	43	++	39	++	29.2
D2DR_MOUSE	44	+++	65	+++	21.8
D3DR_HUMAN	45	++	1.7	+++	1.8
D4DR_HUMAN	46	++	2	+++	3.47
DBDR_HUMAN	110	++	0	+++	nd
HH2R_HUMAN	61	+++	50	+++	20
5H1A_HUMAN	1	+++	0.9	+++	12.7
5H1B_HUMAN	2	+++	38	+++	19.2
5H1D_HUMAN	3	+++	102	+++	11
5H1E_HUMAN	4	++	0.8	+	0.75
5H1F_HUMAN	5	+++	0	+++	0.69
5H2A_HUMAN	6	++	1.8	++	8.5
5H5A_HUMAN	10	++	24	+++	31
5H6_RAT	111	++	1	+++	1.4
5H7_HUMAN	12	+	11.8	+++	2.2
5H7_RAT	117	++	1.5	–	2.18
AG2R_HUMAN	28	++	0	+	nd
BRB1_HUMAN	32	+	0.11	+++	0.19
BRB2_HUMAN	33	+	0.13	+++	7.9
ET1R_BOVIN	50	+++	3.5	–	nd
ET1R_HUMAN	51	+	0.1	+	nd
ETBR_BOVIN	52	++	0.4	+	nd
NY1R_HUMAN	79	+++	60	+++	2.3
NY2R_HUMAN	80	+++	0	+	1.85
OPRD_HUMAN	83	+	3	+	1.85
OPRD_MOUSE	84	++	3.7	++	4
OPRK_HUMAN	85	++	25	++	1.6
OPRK_MOUSE	86	+++	45	++	1
SSR1_HUMAN	93	+++	0.1	+++	nd
SSR3_HUMAN	95	+++	0	+++	nd
NK1R_HUMAN	72	++	15	+++	56.7
NK1R_RAT	73	+++	1.9	+	68.9
NK2R_HUMAN	74	++	39	+++	3.6
NK2R_RAT	75	+++	162	+++	46.4
NK3R_HUMAN	76	+++	23	++	26.5
V1BR_HUMAN	103	+++	0	+++	26.1
MCR1_HUMAN	60	+++	0.01	nd	nd
AA1R_HUMAN	19	+	0.8	++	0.4
AA2A_HUMAN	20	+	180	++	287

Expression levels as for Table 1 BA, binding activity; ID, immuno detection; nd, not done

Table 4. This comparison could obviously only be performed for GPCRs expressed in yeast and mammalian cells as the *E. coli* expressed GPCRs were located in inclusion bodies and required refolding to regain functional binding activity. In this case, there seemed to be no evident correlation between the intensity of the signals obtained in immunoblots and/or Western blots and the specific binding in agreement with previous observations (André et al. 2006). More than half (56.3%) of those targets that showed low expression levels by immunodetection also produced specific binding below 1 pmol/mg. 38.9% of the

Table 4 Correlation between immunodetection and specific binding

	+	++	+++
P. pastoris	16	18	17
0	1 / 6.3%	2 / 11.1%	4 / 23.5%
<1 pmol	9 / 56.3%	3 / 16.7%	3 / 17.6%
1–10 pmol	4 / 25%	7 / 38.9%	2 / 11.8%
>10 pmol	2 / 12.5%	6 / 33.3%	8 / 47.1 %
SFV	14	10	25
0	2 / 16.7%	1 / 10.0%	0 / 0%
<1 pmol	3 / 21.4%	1 / 10.0%	2 / 8.0%
1–10 pmol	3 / 21.4%	4 / 40.0%	11 / 44.0%
>10 pmol	2 / 14.3%	3 / 30.0%	11 / 44.0%
nd	4 / 28.6%	1 / 10.0%	3 / 12%

Expression levels as for Table 1; highlighted in yellow; the highest number of targets expressed from individual expression vectors and in total nd, not done

GPCRs demonstrating medium expression levels also showed medium binding activity (1–10 pmol/mg) although almost the same percentage was achieved for binding over 10 pmol/mg (33.3%). Almost half (47.1%) of the GPCRs which showed high expression levels by immunodetection also belonged to the group of targets with high binding activity. The situation for SFV-expressed targets was similar. Among the GPCRs with low expression levels in Western blots also 21.4% showed binding activities below 1 pmol/mg, 41.7% of this group produced specific binding values between 1 pmol/mg and 10 pmol/mg. Those targets that were expressed at medium level generated medium binding activity for 40.0% of the GPCRs. GPCRs with high levels of expression in Western blots, showed an equal distribution (44.0% each) of the specific binding in the groups for medium and high binding activity. However, some GPCRs with only weak signals in Western blots showed high functional binding activity. For example, the rat serotonin 5-HT7 receptor (5H7_RAT) generated no signal by immunodetection, but demonstrated a specific binding of 2.18 pmol/mg. This might be a reflection of the ratio between functional and non-functional receptors. A significant effect on the binding level was seen from the expression optimization efforts conducted in *P. pastoris*. Less efforts have so far been made for optimization of SFV expression. In this context, only limited studies in three cell lines (BHK-21, CHO-K1 and HEK293) were performed in parallel and also the optimal harvest time was established for each GPCR.

The two SFV vectors used in this study did not show any significant difference in expression levels for the GPCRs tested in parallel (Hassaine et al. 2006). It has also previously been demonstrated that a C-terminally fused Bio-tag had no effect on the expression levels and specific binding of the human neurokinin-1 receptor (Lundstrom et al. 1995). In contrast, some of the GPCRs expressed from the pSFV2genB and pSFV2genC vectors showed significantly lower specific binding activity than previously reported. For instance, the human α2-adrenergic receptor (α 2-AR) showed specific binding of only 0.05 pmol/mg in comparison to previously published values of 176 pmol/mg (Sen et al. 2003). Likewise, the human histamine H2 receptor (HH2R) generated 20 pmol/mg receptor compared to another SFV-based study of 100 pmol/mg (Hoffmann et al. 2001). However, in the case of the α 2-AR, an SFV vector with a translation enhancement signal from the SFV capsid vector was used. This has been demonstrated to increase the expression level by 5–10 fold (Sjöberg et al. 1994). Moreover, in the previously published study on the HH2R, ligand was added to the cell culture medium, which significantly increased the specific binding. Although these differences might explain the discrepancy in the results, also the presence of the influenza HA signal sequence as well as other tags in this study could have an effect. Interestingly, the adenosine A2A receptor generated extreme specific binding values (287 pmol/mg) from the vector with the signal sequence in comparison to 40 pmol/mg from the standard SFV vector (Lundstrom 2000).

Conclusions and future prospects

In summary, all three systems provided high expression levels of several GPCRs. The bacterial expression allowed production of large quantities of GPCRs albeit in inclusion bodies. This strategy was, however, justified by the positive development of refolding technology on GPCRs and also by the large number of targets evaluated. Expression of GPCRs in bacterial membranes requires substantial engineering of constructs and with the resources available, it was impossible to perform studies on more than 100 targets in parallel. For this reason, direct comparison between

the success of GPCR expression in prokaryotes and eukaryotes is inappropriate. Thus, not surprisingly, the yeast- and SFV-based systems generated a much higher number of successfully expressed GPCRs than *E. coli*. Moreover, the functional activity could be verified by radioligand binding assays at each step of expression and purification. The post-translational modifications and particularly glycosylation was observed in both yeast and mammalian cells, but the different glycosylation patterns in the two cell types were not studied here. The effect of glycosylation on crystallization will only be discovered later, but from this aspect it might be an advantage to express the non-glycosylated GPCRs in *E. coli*.

The current study has provided a massive amount of information on the expression of more than 100 GPCRs in three expression systems. Although only 50% of the GPCRs were expressed in *E. coli*, all the recombinant GPCRs could be located in inclusion bodies and for some targets huge amounts, up to 350 mg/L, could be produced in fermentor cultures. Plenty of material is therefore available for refolding studies and the development of improved refolding technologies. Although the purpose of this study was not to perform extensive studies on refolding, preliminary results from trials on several GPCRs suggest that receptor binding activity could be re-established, which will be communicated in future publications in detail. Similarly, several of the GPCRs expressed in *P. pastoris* were subjected to large-scale production in yeast cell cultures, which generated huge biomasses, providing material for further studies on solubilization, purification and crystallization. Likewise, well expressed GPCRs from SFV-infected mammalian suspension cultures have been subjected to solubilization and purification. Preliminary observations indicated that several GPCRs expressed both from *P. pastoris* vectors in yeast cells and SFV vectors in mammalian cells can be solubilized and purified to high homogeneity. Detailed results will be presented elsewhere shortly. Selected GPCRs have now been subjected to crystallization screening and optimization.

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