Comparative biochemical and pharmacological characterization of the mouse $5HT_{5A}$ 5-hydroxytryptamine receptor and the human β_2-adrenergic receptor produced in the methylotrophic yeast *Pichia pastoris*

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Over the last few years, *Pichia pastoris* has been developed into a powerful expression system for a multitude of foreign genes. Here, we demonstrate that the *P. pastoris* expression system has similar power to the baculovirus expression system in high-level production of two G-protein-coupled receptors, the mouse $5HT_{5A}$ 5-hydroxtryptamine receptor and the human β_2-adrenergic receptor. Different expression plasmids were constructed in which the cDNAs of the two receptors were cloned under the transcriptional control of the highly inducible promoter of the *P. pastoris* alcohol oxidase 1 (*AOX1*) gene. In three expression plasmids, the receptors were fused to the *Saccharomyces cerevisiae* α-factor prepropeptide and also to the c-*myc* tag or the FLAG tag to permit immunological detection of the receptors. After transformation into *P. pastoris* strains KM71 and SMD 1163, recombinant clones were selected and tested for the production of the $5HT_{5A}$ receptor and the β_2-adrenergic receptor by radioligand binding using [*N-methyl*-^{3}H]lysergic acid diethylamide and [5,7-^{3}H](−)CGP-12177 respectively. The production level of the $5HT_{5A}$ receptor was improved by a factor of three by fusion with the α-factor prepropeptide. Also, the higher gene dosage resulting from multiple insertions of the expression cassette led to an improvement in production by a factor of two for both receptors. The addition of the adrenergic antagonist alprenolol to the culture medium had a positive effect on the number of specific binding sites detectable in clones producing the β_2-adrenergic receptor. For the $5HT_{5A}$ receptor the addition of yohimbine resulted in a similar but smaller effect. Binding assays revealed that approx. 25 pmol of β_2-adrenergic receptor and approx. 40 pmol of $5HT_{5A}$ receptor per mg of membrane protein in crude membrane preparations were produced. The pharmacological profiles for the heterologously produced receptors, estimated by ligand-displacement analysis using certain adrenergic and serotoninergic agonists and antagonists, were comparable with those reported for the receptors expressed in mammalian systems. Immunoblot analysis of the $5HT_{5A}$ receptor revealed an apparent molecular mass about 20 kDa higher than expected from the amino acid sequence. Here, the Kex2 endopeptidase failed to process the α-factor leader correctly. Blocking glycosylation *in vivo* by tunicamycin or *in vitro* deglycosylation of membranes by endoglycosidase H resulted in correct processing. In contrast, the β_2-adrenergic receptor fusion to the α-factor leader was correctly processed by the internal Kex2 endopeptidase. The Kex2-processed β_2-adrenergic receptor was not glycosylated. In conclusion, the high-level production of the two receptors in *P. pastoris* will allow their purification in quantities sufficient for subsequent biophysical and structural studies.

INTRODUCTION

The mouse $5HT_{5A}$ 5-hydroxytryptamine and the human β_2-adrenergic receptors both belong to the superfamily of G-protein-coupled receptors (GPCRs). A multitude of pharmaceutical substances mediate their effects through binding to GPCRs and thus these receptors are in the focus of medical and pharmacological research [1]. The β_2-adrenergic receptor was the first GPCR to be cloned and sequenced [2]. Since then, a plethora of GPCR genes has been cloned, including the mouse $5HT_{5A}$ and $5HT_{5B}$ receptor genes [3,4]. According to their pharmacological profile these 5-hydroxytryptamine receptors probably define a new subtype of the 5-hydroxytryptamine receptor family, which is quite distinct from the other known mammalian 5-hydroxytryptamine receptor subtypes. Until now there is a lack of knowledge about the effector pathway of the $5HT_5$ receptors, which makes a definitive classification not yet possible.

In general, GPCRs have been analysed by techniques, such as site-directed mutagenesis, construction of receptor chimaeras and coexpression of receptors with different G-proteins, leading to an advanced understanding of structure–function relationships. The pharmacological relevance of GPCRs has led to an increasing interest in their three-dimensional structure. So far, no structural data are available for any of these receptors. Large quantities of homogeneous receptor protein are a prerequisite for structural studies. Natural sources cannot provide the amounts of protein needed for crystallization attempts and also they usually represent complex mixtures of cell types and receptor subtypes which do not allow purification of a certain receptor subtype to homogeneity. A way of overcoming this problem is to express the receptor gene in a suitable expression system, allowing the production of a homogeneous population of receptor protein [5,6].

Over the last few years, the methylotrophic yeast *Pichia pastoris* has been developed as a host for the efficient production of foreign proteins [7]. Interest in the *P. pastoris* expression system has grown, since this organism has the potential for high-level expression and rapid growth to very high cell densities on

Abbreviations used: GPCR, G-protein-coupled receptor; EndoH, endoglycosidase H; PNGaseF, *N*-glycopeptidase F; LSD, lysergic acid diethylamide.

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relatively inexpensive media [8,9]. A number of foreign genes have already been heterologously expressed using the strong promoter of the alcohol oxidase I (*AOXI*) gene of *P. pastoris*. The activity of this promoter is strictly regulated by the concentrations of carbon sources in the culture medium. Full transcriptional activity can be achieved by the addition of methanol when other carbon sources have been depleted. In the presence of glucose or high concentrations of glycerol, the promoter is completely repressed. Therefore fermentation of recombinant *P. pastoris* clones to extremely high cell densities without any detectable transcription of the heterologous gene is possible, and the switch to high-level expression can be achieved just by changing the carbon source [7]. It has been reported that, in some cases, heterologous production has yielded up to several grams of the desired recombinant protein per litre of culture [7]. Although the *P. pastoris* system has been successfully used for the production of many soluble proteins (reviewed in [7] and [9]), only recently have there been reports on the successful heterologous production of membrane proteins [10–14].

Here, we describe the comparative biochemical and pharmacological characterization of the human β_2-adrenergic receptor and the mouse $5HT_{5A}$ receptor functionally produced in *P. pastoris*.

EXPERIMENTAL

Strains and transformations

Escherichia coli strain XL-1 Blue [*rec*A1, *edn*A1, *gyr*96, *thi*-1, *hsd*R17, *sup*E44, *rel*A1, *lac*-, F′*pro*AB, *lac*I^{q}ZΔM15, Tn10(tet^r)] (Stratagene GmbH, Heidelberg, Germany) was used for propagation of the recombinant plasmids. For *E. coli* transformation, the $CaCl_2$ procedure was used as described [15]. *P. pastoris* strain KM71 (his4, Mut^s) and *P. pastoris* strain SMD1163 (his4, Mut$^+$, pep4, pbr) (Invitrogen, San Diego, CA, U.S.A.) were used. The transformation of *P. pastoris* was performed by electroporation using a Gene Pulser (Bio-Rad Laboratories GmbH, München, Germany) according to the protocol given by the Pichia Expression Kit Manual (Version B) but with a resistance of 400 instead of 200 Ω.

Recombinant DNA technology and construction of the expression plasmid

DNA isolation, restriction enzyme analysis, agarose-gel electrophoresis and cloning procedures were performed using standard techniques [15,16]. All plasmid constructs were verified by DNA sequencing using the dideoxy-termination method [17]. The plasmids used for the expression of the β_2-adrenergic receptor or the $5HT_{5A}$ receptor were pPIC9K-βARFlagBio, pPIC9-$5HT_{5A}$myc, pPIC9-$5HT_{5A}$FlagBio and pK-$5HT_{5A}$Bio (see Figure 2). Plasmid pPIC9-$5HT_{5A}$myc has already been described [10]. The basic vector for the other plasmids which encode a FLAG tag was pPIC9K-Flag (Figure 1) which was constructed as follows. The *Bam*HI site of plasmid pPIC9 (Invitrogen) was deleted by digestion with *mung bean* nuclease. A synthetic oligonucleotide pair encoding the FLAG® octapeptide (*N*-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-C) and the additional restriction site *Bam*HI was ligated into the *Xho*I and *Eco*RI sites of the multiple cloning site of the pPIC9 vector resulting in vector pPIC9-Flag. The oligonucleotides used for the insertion of the tag (Flagfor: 5′-TCGAAAAAAGAGACTACAAGGACGATGACGATAAGGATCCCGGGCCCTCGAG-3′; Flagrev: 5′-AATTCTCGAGGGCCCGGGATCCTTATCGTCATCGTCCTTGTAGTCTCTTTTT-3′) were synthesized using an Applied Biosystems DNA Synthesizer. To obtain vector pPIC9K-Flag (Figure 1), the 1 kbp *Sac*–*Not*I restriction fragment of vector pPIC9K (Invitrogen), which contains a kanamycin resistance gene, was exchanged against the corresponding fragment of pPIC9-Flag. For construction of plasmid pPIC9K-$5HT_{5A}$FlagBio, a 1.15 kbp *Eco*RI–*Bg*/II fragment isolated from the baculovirus transfervector pVLC4ΔK$5HT_{5A}$ containing the cDNA coding for the $5HT_{5A}$ receptor was ligated into the *Bam*HI–*Eco*RI-digested vector pPIC9KFlag. Afterwards, a small *Cpo*I–*Not*I fragment was removed from this vector and replaced by a *Cpo*I–*Not*I fragment from pVL925HT_{5A}CtermBio which encoded the 75 amino acids of the biotinylation domain from *Proprionibacterium shermanii* [18]. The 1.6 kbp *Pme*I–*Cpo*I fragment from plasmid pPIC9K-$5HT_{5A}$FlagBio was replaced by the 1.35 kbp *Pme*I–*Cpo*I from plasmid pHIL-D2-$5HT_{5A}$ [10] resulting in plasmid pK-$5HT_{5A}$Bio. The *Bam*HI–*Not*I DNA fragment bearing the coding region for the fusion of the β_2-adrenergic receptor and the *Pb. shermanii* biotinylation domain were cloned into appropriately digested plasmid pPIC9K-Flag. The resulting plasmid was named pPIC9K-βARFlagBio. All vectors encoding the $5HT_{5A}$ receptor gene with the respective tags and fusions were linearized with *Sal*I before electroporation, whereas plasmid pPIC9K-βARFlagBio was linearized with *Sac*I. All *P. pastoris* transformants analysed had the same methanol utilization (Mut) phenotype as the cells from which they were derived (Mut$^+$ in the case of *P. pastoris* SMD1163, Mut^s in the case of *P. pastoris* KM71).

Yeast culture

After electroporation, His$^+$ recombinant clones were selected on MD plates (1.34 % yeast nitrogen base without amino acids, 0.00004 % biotin, 2 % dextrose, 1.5 % agar). For secondary selection of multicopy transformants using Geneticin G418 (G418), His$^+$ clones were pooled, diluted in sterile water, and about 1×10^5 cells were spread on to YPD–G418 plates (1 % yeast extract, 2 % peptone, 2 % dextrose, 2 % agar and 0.025–0.2 % G418). For production of the receptors, His$^+$ yeast cells were grown in BMGY medium [1 % yeast extract, 2 % peptone, 1.34 % yeast nitrogen base without amino acids, 0.00004 % biotin, 1 % (v/v) glycerol, 0.1 M phosphate buffer, pH 6.0] to an A_{600} of 2–6. For induction, cells were harvested (2000 ***g***; 5 min; room temperature), and resuspended to an A_{600} of 1 (transformants of SMD1163) or 20 (transformants of KM71) in BMMY medium (same as BMGY with glycerol replaced by 0.5 % methanol). For the production of the β_2-adrenergic receptor, all media were buffered to pH 6.6 instead of 6.0. Tunicamycin (stock solution 1 mg/ml in 0.1 M NaOH) was added to the BMMY culture directly on induction to a final concentration of 15 μg of tunicamycin/ml of medium.

Analysis of copy number

Genomic DNA from recombinant *P. pastoris* clones was prepared with minor modifications as has been described for *S. cerevisiae* [16]. The concentration and purity of the genomic DNA was checked by UV spectroscopy. For hybridization, 15 μg of genomic DNA from each clone was dropped on to a nylon membrane, fixed by UV radiation and hybridized with respective receptor-gene-specific ^{33}P-labelled DNA probes, which were generated by a random-priming approach [16]. Afterwards, radioactivity bound to the filters was determined by liquid-scintillation counting (Canberra-Packard TRI-CARB 1500, Frankfurt/M, Germany). Genomic DNA of non-transformed *P. pastoris* clones and transformed clones not selected by G418 and therefore, probably bearing only one expression cassette were used as a standard for the determination of copy number.

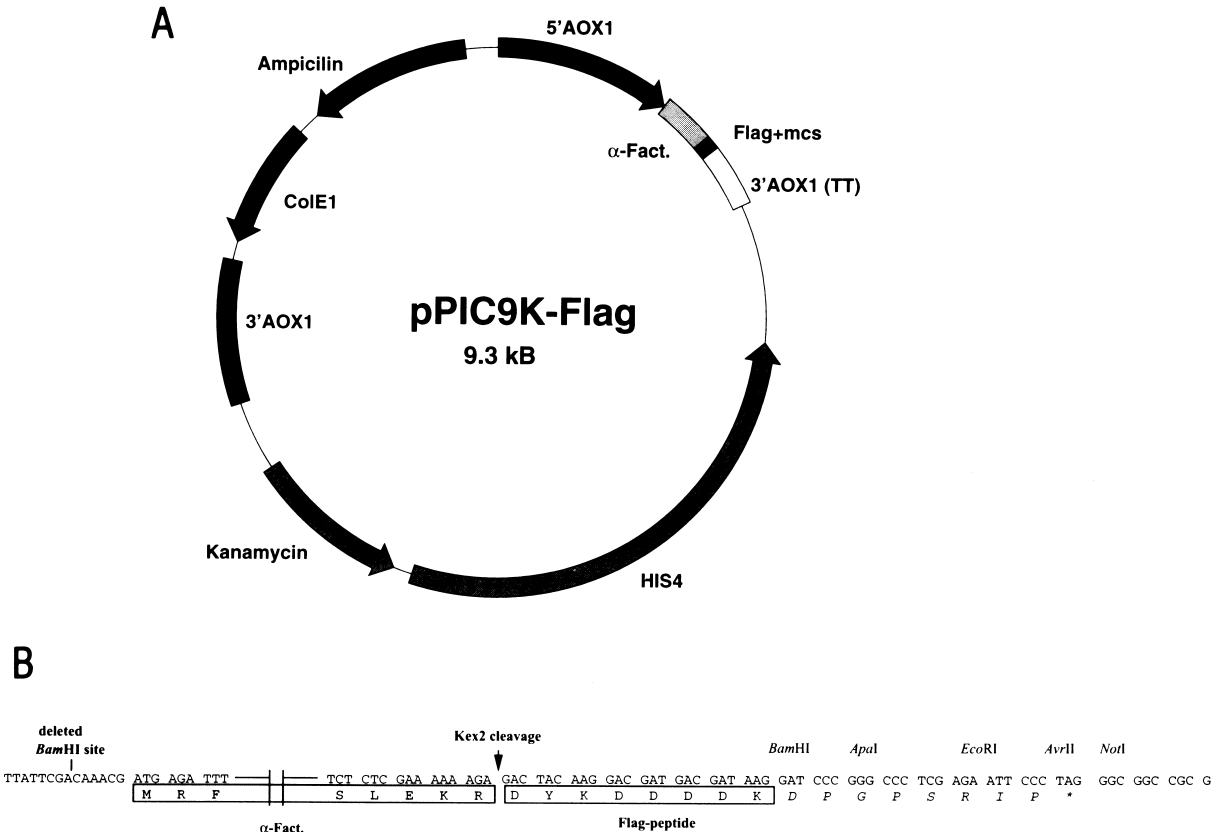

Figure 1 Map of the basic vector pPIC9K-Flag

(**A**) Map of the *P. pastoris* expression vector pPIC9K-Flag. The expression plasmid pPIC9K-Flag is essentially based on plasmid pPIC9K containing the coding region of the *S. cerevisiae* α-mating-type factor prepropeptide, the FLAG tag and a multiple cloning site. α-Fact., coding region for the prepropeptide of *S. cerevisiae* α-mating-type factor; 5′AOX1, promoter region of *P. pastoris* alcohol oxidase 1 gene (*AOX1*); Flag + mcs, coding region for the FLAG tag and multiple cloning site; 3′AOX1 (TT), transcription termination fragment from *P. pastoris* alcohol oxidase 1 gene; HIS4, coding region for *P. pastoris* histidinol dehydrogenase; Kanamycin, kanamycin-resistance gene; 3′AOX1, fragment from the 3′ flanking region of *P. pastoris* alcohol oxidase 1 gene; ColE1, ColE1 origin from *E. coli;* Ampicillin, ampicillin-resistance gene. (**B**) DNA sequence of pPIC9K-Flag flanking the *S. cerevisiae* α-mating-type factor prepropeptide and the FLAG tag. The amino acid sequence representing the FLAG tag that is recognized by the M1 and/or M2 antibodies is boxed. Unique restriction sites that can be used for the insertion of foreign genes are indicated above the DNA sequence.

Isolation of crude membranes from recombinant *P. pastoris*

Membranes were prepared from cells that had been grown for 24–30 h under induction conditions as described previously [10] with the following variations: 1 mM PMSF was used instead of Pefabloc®; the first centrifugation step after cell breakage was performed at 1800 ***g*** for 5 min at 4 °C; membranes containing the β_2-adrenergic receptor were suspended in 50 mM Tris/HCl, pH 8.0, containing 150 mM NaCl and 2 mM EDTA before snap-freezing. The protein concentration of the membrane preparation was determined using the BCA reagent (Pierce, Rockford, IL, USA) with BSA as a standard.

Immunoblot analysis and glycosylation studies

For immunoblot analysis, yeast membranes were isolated as described above. The membranes were diluted to a concentration of 2 μg/μl in appropriate buffer containing 1 mM PMSF, and an equal volume of 2 × SDS loading buffer was added. Proteins were separated by SDS/PAGE (10% gel) [19], electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) and processed as previously described [20]. For detection of *c-myc*-tagged recombinant protein, the primary antibody 9E10 (Cambridge Research Biochemicals, Northwich, U.K.) was used at a final concentration of 1.1 μg/ml [21]. Incubations with the primary and secondary antibody were for 1 h at room temperature each followed by three 15 min washes in PBS containing 0.1% Triton X-100. Anti-FLAG monoclonal antibodies M1 and M2 were purchased from Kodak (Kodak Scientific Imaging Systems, New Haven, CT, U.S.A.) and used according to the protocols provided by the manufacturer. In all cases, for visualization of the epitope-tagged proteins, membranes were incubated with goat anti-(mouse alkaline phosphatase)-coupled antibody (Sigma Chemie GmbH, Deisenhofen, Germany) which had been diluted 1:2000. Biotinylated receptor was traced directly with alkaline phosphatase-coupled streptavidin according to the product information (Promega, Madison, WI, U.S.A.). All blots were developed in 10 ml of AP buffer (100 mM NaCl, 100 mM Tris/HCl, pH 9.5, 5 mM $MgCl_2$) with the addition of 66 μl of 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidinium salt (stock solution 50 mg/ml of dimethylformamide) and 33 μl of NitroBlue Tetrazolium chloride (stock solution 50 mg/ml of 70% dimethylformamide).

Enzymic deglycosylation with endoglycosidase H (EndoH; Boehringer-Mannheim GmbH, Mannheim, Germany) was performed for 3 h at 37 °C. Samples with 2 mg/ml membrane

protein, 40 mM sodium citrate, pH 5.5, 0.5 % SDS and 2 mM PMSF were incubated with 0.14 unit/ml EndoH. Enzymic deglycosylation with *N*-glycopeptidase F (PNGaseF; New England Biolabs, Schwalbach, Germany) was performed for 30 min at 37 °C. Here, the samples which contained 3 mg/ml membrane protein in 50 mM sodium phosphate, pH 7.5, were treated with 100000 units/ml PNGaseF. Samples from deglycosylation experiments were placed on ice and PMSF was added to a final concentration of 1 mM before the addition of sample buffer. For subsequent immunoblot analysis, the samples were separated by SDS/PAGE, transferred to nitrocellulose and processed as described above.

Radioligand binding

Membranes prepared from *P. pastoris* clones transformed with plasmids containing the $5HT_{5A}$ receptor gene were assayed with [*N-methyl*-^{3}H]lysergic acid diethylamide (LSD) (2.5–3.1 TRBq/mmol; DuPont-NEN) as described previously [10]. The β-adrenergic ligand [5,7-^{3}H](−)CGP-12177 (1.65 TBq/mmol; DuPont NEN) was used in all studies. Non-specific binding was determined in the presence of 2 mM alprenolol. Experiments were performed with about 0.1 nM binding sites in buffer A (20 mM Hepes, pH 7.4, 100 mM NaCl, 12 mM $MgCl_2$) in a final volume of 1 ml at 30 °C for 30 min (for the saturation curve) or 60 min (for the competition studies). Bound and free ligand were separated by rapid vacuum filtration over Whatman GF/F filters (soaked in 0.3 % polyethyleneimine). Subsequently, filters were washed three times with cold water. Radioactivity levels on the filters were determined by liquid-scintillation counting. K_D and B_{max} values were calculated by computer-aided non-linear regression analysis of the binding isotherms under equilibrium conditions. Competition displacement was performed in the presence of [^{3}H]CGP-12177 at a final concentration of 0.8 nM and variable concentrations of the unlabelled drugs. For the initial screening of recombinant clones producing the β_2-adrenergic receptor, about 25 μg of membrane protein of the respective clone was incubated in a volume of 500 μl in the presence of 5 nM [^{3}H]CGP-12177.

Immunogold electron microscopy

Post-embedding immunostaining of *P. pastoris* cells was performed as described in [10]. Before, cells had been fixed in 4 % paraformaldehyde/2.5 % glutaraldehyde in PBS, enclosed in agar and post-fixed in 1 % OsO_4/0.1 M sodium cacodylate buffer for 1 h at 5 °C, treated with 2 % uranyl acetate overnight and then dehydrated in acetone. The yeast cells were embedded in Spurr's resin and heat polymerized (60–70 °C) in gelatine capsules [22]. Thin sections were cut with an Ultracut (Reichert), collected on Formvar®-coated nickel grids and processed as follows. For analysis with the anti-FLAG monoclonal antibody M1, the sections were incubated with Tris-buffered saline (TBS) containing 1 % milk powder, then with TBS plus 0.1 % milk powder and finally with TBS containing 1 mM $CaCl_2$. For analysis with monoclonal antibody M2, the sections were incubated with NaCl/P_i containing 1 % BSA, 0.5 % Tween 20 and 0.5 % Triton X-100 and thereafter with NaCl/P_i supplemented with 0.1 % BSA and 0.05 % Tween 20. Binding of the M1 and M2 (1:100) antibodies was performed for several hours or overnight. In the case of the M1 antibody, binding was in the presence of Ca^{2+} ions (1 mM). Unbound antibodies were washed off and secondary antibodies (goat anti-mouse IgG; 1:40; 90 min) coupled to 10 nm gold particles were applied. After refixation with 1 % glutardialdehyde in NaCl/P_i or TBS (10 min), sections were washed with water and the gold particles were enhanced using a silver enhancement kit (Inten SE™M; Amersham Buchler, Braunschweig, Germany). Before the sections were viewed in the electron microscope, they were double-stained with 2 % uranyl acetate and 1 % lead citrate.

The post-embedding labelling procedure with gold-coupled streptavidin was performed as described above for the anti-FLAG antibody M2. For pre-embedding labelling, fixed yeast cells were frozen, thawed and then enclosed in agar. From the material, about 50 μm sections were cut with a vibratome. The sections, after consecutive treatment with PBS + 2 % glycine (1 h), PBS + 1 % BSA + 0.5 % Tween 20 + 0.5 % Triton X-100 (15 min) and PBS + 0.1 % BSA + 0.05 % Tween 20, were incubated overnight with gold-coupled streptavidin (dilution 1:20; Amersham Buchler), washed with PBS and fixed with 1 % glutaraldehyde/PBS (1 h). After two wash steps with PBS and water, the gold particles were enlarged with a silver enhancement kit (Amersham), briefly osmicated (30 min; 1 % OsO_4), treated with water and incubated with 2 % uranyl acetate for 30 min. Afterwards the samples were dehydrated, embedded and polymerized in Spurr's resin.

RESULTS AND DISCUSSION

Processing of the α-mating-type factor–myc-tagged $5HT_{5A}$ receptor fusion protein

Previously, we reported the successful production of the mouse $5HT_{5A}$ receptor in the *P. pastoris* expression system [10]. In this recent study, plasmid pPIC9-$5HT_{5A}$myc (Figure 2) transformed into the protease-deficient *P. pastoris* strain SMD1163 exhibited high levels of production of more than 20 pmol of receptor/mg of membrane protein. The plasmid pPIC9-$5HT_{5A}$myc contained an N-terminal fusion of the receptor gene to the *S. cerevisiae* α-mating-type factor secretion signal as well as to the c-*myc*-tag (Figure 2). In comparison with construct without N-terminal fusion as well as a construct with fusion to the *P. pastoris* acid phosphatase signal sequence (*PHO1*), the fusion to the α-mating-type factor secretion signal improved production levels of the $5HT_{5A}$ receptor significantly. The c-*myc*-tag fused to the receptor allowed localization of the receptor within the yeast cells and should also allow detection of the recombinant protein in immunoblots. Therefore immunoblot analysis of membranes isolated from recombinant *P. pastoris* producing the myc-tagged $5HT_{5A}$ receptor with the monoclonal antibody 9E10 was performed. The antibody recognized a twin band with an apparent

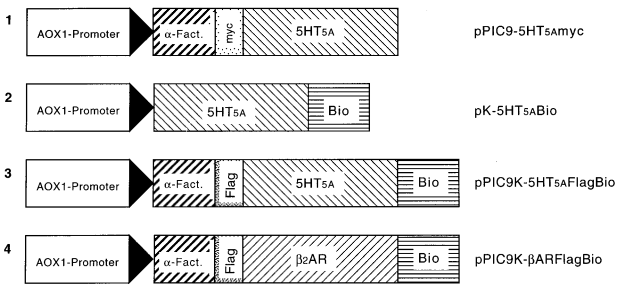

Figure 2 Expression constructs for the production of the mouse $5HT_{5A}$ and human β_2-adrenergic receptor in *P. pastoris*

AOX1-Promoter, promoter of the *P. pastoris* alcohol oxidase 1 gene; α-Fact., coding region for the prepropeptide of *S. cerevisiae* mating-type factor α; myc, coding region for the c-*myc* epitope; Bio, coding region for the biotinylation domain of the transcarboxylase from *Pb. shermanii;* Flag, coding region for the flag peptide; $5HT_{5A}$, gene of the mouse $5HT_{5A}$ receptor; β_2AR, gene of the human β_2-adrenergic receptor.

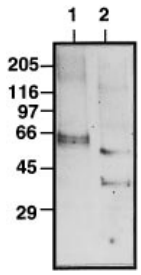

Figure 3 Immunoblot of membrane proteins prepared from *P. pastoris* cells (SMD1163) transformed with pPIC9-5HT$_{5A}$myc

Immobilized samples of crude membrane proteins were probed with monoclonal antibody 9E10 directed against the c-*myc* epitope. Each lane was loaded with 20 μg of membrane protein. Lane 1, mock-digested membranes; lane 2, PNGaseF-digested membranes. Enzymic deglycosylation was performed as outlined in the Experimental section.

molecular mass centred around 61 kDa (Figure 3, lane 1). This apparent molecular mass for the heterologously produced 5HT$_{5A}$ receptor was about 20 kDa higher than expected. From the amino acid sequence of the receptor a molecular mass of 38 kDa can be calculated. Since the 5HT$_{5A}$ receptor has two potential N-glycosylation sites (N-X-S/T), we investigated whether the difference in molecular mass might be due to hyperglycosylation of the recombinant protein. Therefore, membranes were treated with PNGase F which cleaves glycosamine linkages between oligosaccharides and the asparagine. As depicted in Figure 3 (lane 2), this treatment led to the disappearance of the immunostained twin band and at the same time two bands with molecular masses of 55 and 38 kDa appeared. In theory, the α-factor secretion signal should be removed in two consecutive steps from the receptor. First, the short signal peptide of the *S. cerevisiae* α-factor prepropeptide should be cleaved from the fusion protein by endoplasmic reticulum resident signal peptidase. Second, the specific Kex2 protease, located in the Golgi apparatus, should clip off the N-terminally fused α-factor prosequence. As predicted by the amino acid sequence, the α-factor prosequence has a molecular mass of 10 kDa and contains three putative glycosylation sites (N-X-S/T) which after glycosylation contribute another 7–10 kDa [23]. Therefore it is possible to ascribe the unexpected twin band at 61 kDa to the partially glycosylated unprocessed receptor fusion protein whereas after *in vitro* deglycosylation the lower band might correspond to the non-glycosylated receptor and the upper band most probably to the deglycosylated but unprocessed receptor fusion protein.

Construction of expression vectors for analysis of processing

In order to analyse further the N-terminal processing of the receptor fusion as well as to test affinity tags suitable for purification of the recombinant protein, we constructed the vector pPIC9K-5HT$_{5A}$FlagBio (Figure 2). For reasons of comparison, we also constructed plasmid pPIC9K-βARFlagBio which bears an equivalent fusion construct for the human β_2-adrenergic receptor. In addition, in these vectors the biotinylation domain of the *Pb. shermanii* transcarboxylase was C-terminally fused to both receptors [18]. This fusion should allow immunological detection as well as purification of the fusion proteins on monomeric avidin columns [24,25]. Both expression vectors also contained a kanamycin-resistance gene as a marker suitable for selection of recombinant clones bearing multiple copies stably integrated into the genomes [26].

In plasmid pK-5HT$_{5A}$Bio, the 5HT$_{5A}$ receptor gene was not N-terminally fused, but the vector also contained the kanamycin-resistance gene. We constructed this vector to distinguish between gene dosage effects and consequences of the presence of the α-factor leader peptide on the production of the 5HT$_{5A}$ receptor (Figure 4).

Transformation and selection of clones bearing multicopy insertions

The recombinant vectors were transformed into the His-deficient *P. pastoris* strains SMD1163 or KM71 after linearization, and His$^+$ yeast clones were selected. Recombinant yeast clones bearing vectors pK-5HT$_{5A}$Bio, pPIC9K-5HT$_{5A}$FlagBio and pPIC9K-βAR FlagBio, which contain a kanamycin-resistance gene, were further propagated on G418 plates for selection of clones with multiple copies of the expression cassette as described by Scorer et al. [26]. These authors reported that G418 concentrations of 0.25–4 mg/ml were required for selection. When we screened His$^+$ clones derived from strain SMD1163, no clones survived when the G418 concentration in the plates was higher than 0.25 mg/ml. This problem did not arise when strain KM71 had been used for transformation. As revealed by experiments using lower G418 concentrations, the *P. pastoris* strain SMD1163 exhibited higher sensitivity to G418 than had been reported for the strains SMD1168, KM71 or GS115. His$^+$ clones derived after transformation into strain SMD1163 which bore one gene copy only grew on plates supplemented with 0.025 mg/ml G418, whereas clones with multicopy insertions tolerated concentrations of 0.05 mg/ml and higher. This increased sensitivity of strain SMD1163 to G418 has to be taken into account when working with this protease-deficient strain.

By determination of the number of the expression cassettes in each individual clone it was possible to analyse the correlation of copy number to the production level and to distinguish between the effect of gene dosage and the effect that can be attributed to the α-factor prosequence. As shown in Figure 4, radioligand-binding assays performed on different recombinant clones producing the β_2-adrenergic receptor or the 5HT$_{5A}$ receptors with [^{3}H]CGP-12177 or [^{3}H]LSD respectively revealed enhanced production levels in the multicopy clones. Nevertheless, improvements were limited to a factor of about 2–3 even when the copy number was raised to 6 or above. We conclude from this result that gene dosage is not limiting for production of either receptor when the copy number of the expression cassette is higher than 2. In the case of a higher gene dosage, the bottleneck for production of functional receptor seems to be in the post-translational processing. This result is in agreement with data obtained for several secreted proteins, for which maximal production levels did not always correlate with the highest number of expression cassettes [9].

The addition of the antagonist alprenolol to the culture medium (3 μM) in the case of β_2-adrenergic-receptor-producing cells improved the production level of functional receptor by a factor of more than 3 (Figure 4b). In the case of 5HT$_{5A}$-receptor-producing cells, several ligands were also tested for their potential to improve production level. The best results were achieved with yohimbine (560 μM), which improved the receptor level by a factor of 1.5–2 (results not shown).

Processing

To test the assumption that the increased molecular mass of the 5HT$_{5A}$ receptor was due to lack of processing by Kex2 but was not due to hyperglycosylation, we used vector pPIC9K-5HT$_{5A}$

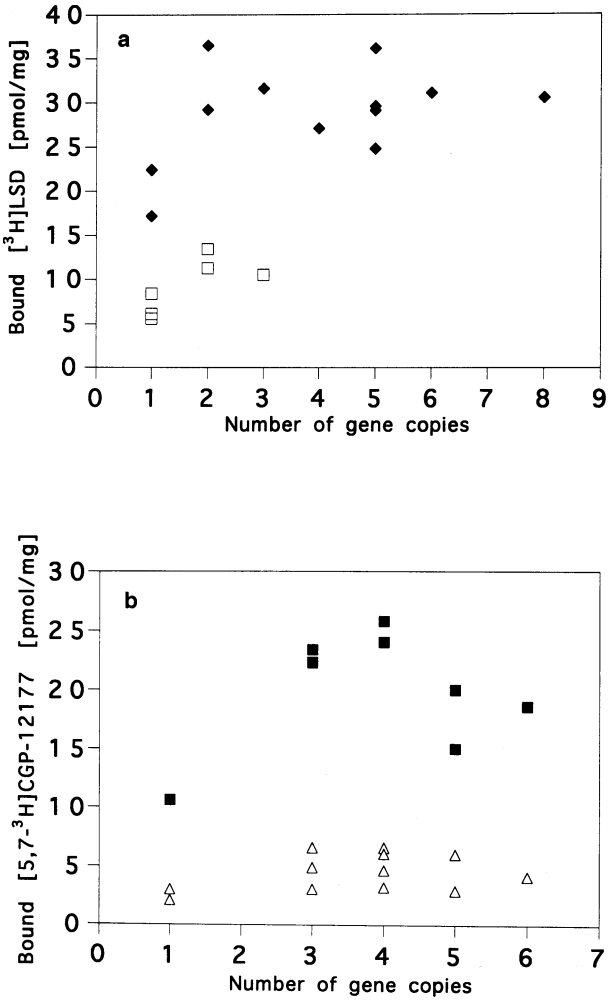

Figure 4 Correlation between gene dosage and production level

For different clones either producing the $5HT_{5A}$ (**a**) or the β_2-adrenergic (**b**) receptors, the number of gene copies per cell was determined as outlined in the Experimental section. Radioligand binding to respective membranes was performed at concentrations of 20 nM [^{3}H]LSD or 5 nM [^{3}H]CGP-12177 respectively. Cells (SMD1163) were transformed with pK-$5HT_{5A}$Bio (□), pPIC9K-$5HT_{5A}$FlagBio (◆) or pPIC9K-βARFlagBio (△ and ■), in the case of ■ alprenolol was added to the BMMY culture at a final concentration of 3 μM.

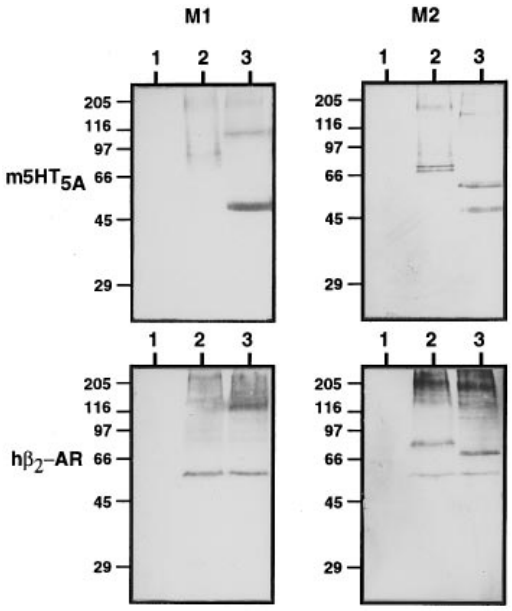

Figure 5 Immunoblot analysis of membrane proteins isolated from recombinant *P. pastoris* cells (SMD1163) producing the mouse $5HT_{5A}$ receptor (m$5HT_{5A}$; top) or the human β_2-adrenergic receptor (hβ_2-AR; bottom)

Immobilized samples of crude membrane proteins were probed with the monoclonal antibodies M1 (left) or M2 (right) which are both directed against the FLAG epitope. The M1 antibody recognized the epitope only when the Kex2 endopeptidase processing was correct. Each lane was loaded with 20 μg of protein. Lane 1, Yeast transformed with control plasmid; lane 2, yeast transformed with pPIC9K-$5HT_{5A}$FlagBio (above) or pPIC9K-βARFlagBio (below), both mock-digested; lane 3, same membranes as in lane 2 but with Endo H digestion. Enzymic deglycosylation was performed as outlined in the Experimental section.

FlagBio (Figure 2). This construct was based on vector pPIC9K-Flag (Figure 1), which bears the α-mating-type factor secretion signal as well as the FLAG tag. The FLAG tag in this vector was introduced by complementary oligonucleotides encoding the peptide sequence NH_2-DYKDDDDK-COOH [27]. The specific recognition sequence for the Kex2 endopeptidase which is located directly N-terminally to this tag was restored during these manipulations (Figure 1). For the detection of the FLAG tag as well as for purification of tagged recombinant proteins, two monoclonal antibodies, M1 and M2, are available. The M2 antibody recognizes the tag irrespective of its location within the protein. In contrast, the M1 antibody binds in a Ca^{2+}-dependent manner and only when the peptide recognized had a free N-terminus [27]. In the construct pPIC9K-$5HT_{5A}$FlagBio, the receptor was fused to the FLAG tag in a way that the translation product can be recognized by the M1 antibody only after cleavage by Kex2 protease. Therefore this construct should allow investigation of the processing by the Kex2 endopeptidase.

Immunoblot analysis of membranes prepared from cells transformed with pPIC9K-$5HT_{5A}$FlagBio using monoclonal antibodies M1 and M2 revealed that only the M2 antibody stained a double band with an apparent molecular mass around 70 kDa. This apparent molecular mass corresponds closely to the molecular mass of the glycosylated but not Kex2-processed receptor fusion protein as calculated from the amino acid sequence. The lack of a signal with the M1 antibody suggests that the α-mating-type factor prosequence was not removed from the receptor (Figure 5, lane 2). *In vitro* deglycosylation of membranes with EndoH, however, resulted in M1-immunostainable bands, suggesting that now correct processing of the $5HT_{5A}$ receptor fusion by the Kex2 endopeptidase had occurred in the isolated membrane (Figure 5, lane 3). The same result was obtained *in vivo* when glycosylation in cells producing the receptor was inhibited by tunicamycin [28]. As can be seen in Figure 6, recognition of the FLAG tag by the M1 antibody in post-embedding immunogold electron microscopy was absolutely dependent on the treatment with tunicamycin. Cells grown in the absence of tunicamycin were totally devoid of any M1-specific staining, although binding studies revealed the presence of active receptor protein. The addition of tunicamycin to recombinant yeast leading to inhibition of N-linked glycosylation *in vivo* resulted in M1-specific staining. Gold particles can be observed over the vacuole and to a lesser degree over the cell cytoplasm also. No distinct labelling of the plasma membrane was observed, although the receptor is a natural resident of the plasma membrane. Together these results indicate that, in the case of the

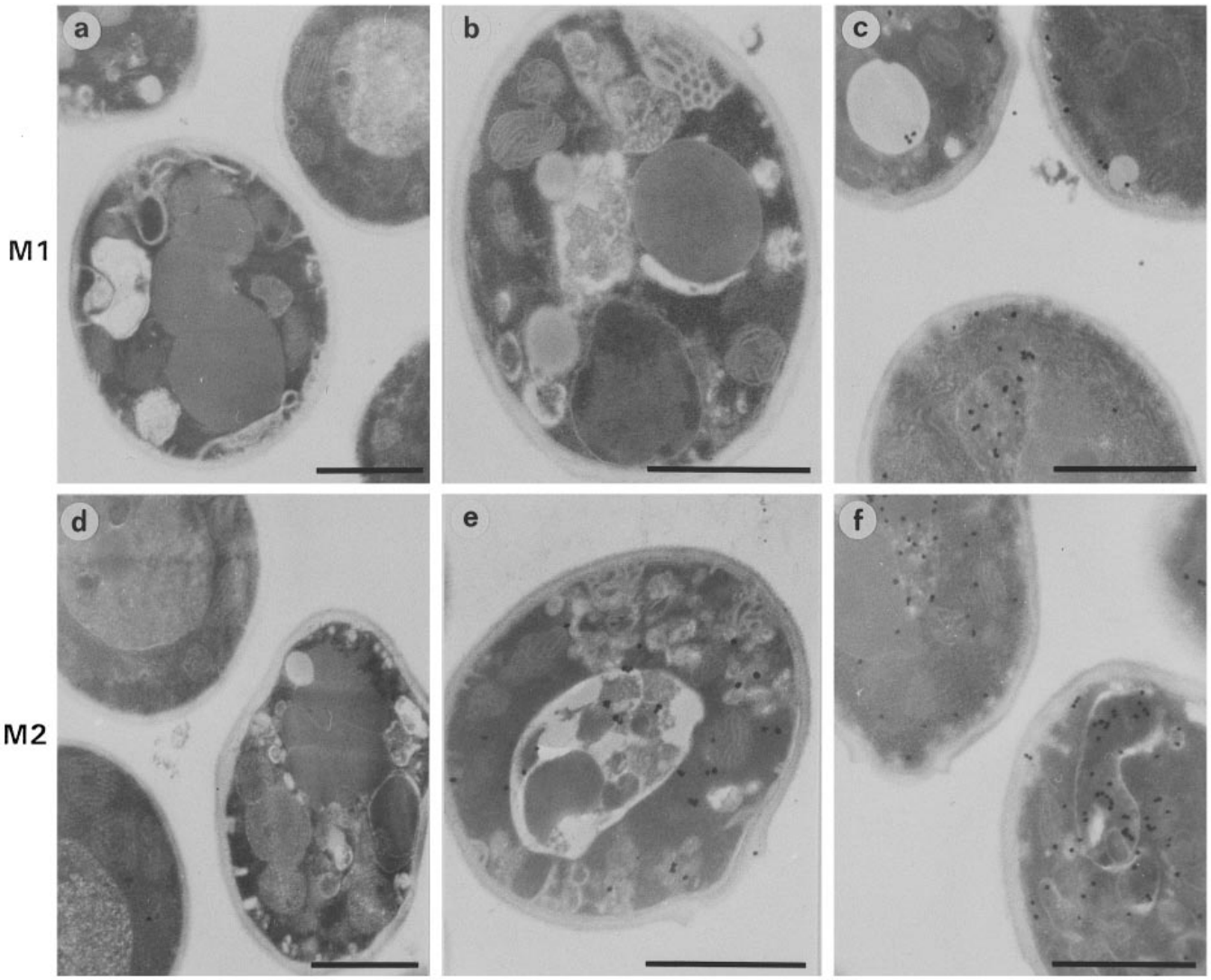

Figure 6 Cellular localization of the heterologously produced $5HT_{5A}$ receptor by immunogold labelling

Post-embedding immunogold staining of ultrathin sections from recombinant *P. pastoris* cells (SMD1163) transformed with pPIC9K-$5HT_{5A}$FlagBio grown in the absence (**b** and **e**) or the presence (**c** and **f**) of tunicamycin. Specific staining by monoclonal antibody M1 could only be seen when cells were grown in the presence of tunicamycin whereas in each case staining by monoclonal antibody M2 could be observed (**e** and **f**). In control cells transformed with pPIC9-$5HT_{5A}$myc, no labelling with the M1 and M2 antibodies was observed (**a** and **d**). Bars = 1 μm.

$5HT_{5A}$ receptor, the glycosylation specifically hinders correct cleavage by Kex2. The α-mating-type factor precursor was not removed from the glycosylated receptor regardless of whether the c-*myc* or the FLAG epitope was located adjacent to the Kex2 cleavage site. Nevertheless, pharmacological characterization performed with glycosylated receptor and therefore still containing the α-factor leader revealed that ligand binding was not affected (see Figure 8).

The β_2-adrenergic receptor has three potential N-glycosylation sites, of which two are located in the N-terminal region. In plasmid pPIC9K-βARFlagBio, the first of the two N-terminal sites was eliminated during the cloning procedure. As revealed by immunoblot analysis with the M1 and M2 antibodies, the Kex2 endopeptidase processed this receptor fusion protein correctly. With both antibodies, a 56 kDa band could be detected that corresponds to correctly processed receptor (Figure 5, lanes 2 and 3). Contrary to the situation described above, the correct processing of the fusion protein resulted in a free N-terminal FLAG–peptide which should be helpful during subsequent purification [29], and here, especially for separation of processed from unprocessed receptor. It is notable that the receptor released after Kex2 proteolysis was not glycosylated, which might be advantageous for structural studies.

Hyperglycosylation, which sometimes causes problems in *S. cerevisiae*, was less frequently detected in *P. pastoris* [7], which is in agreement with our results. The α-factor leader contains three potential N-glycosylation sites and, as can be seen in Figure 5, at least some of them were glycosylated in both receptor constructs. The fusion of the α-factor leader to the receptors resulted in an apparent molecular mass increment of more than 20 kDa when the glycosylated unprocessed species and the non-glycosylated processed species are compared (Figure 5). For both receptors, the apparent molecular-mass of the Kex2-processed and deglycosylated proteins was in good agreement with the molecular masses calculated from the amino acid sequences. It has already been reported that glycosylation of the β_2-adrenergic receptor is

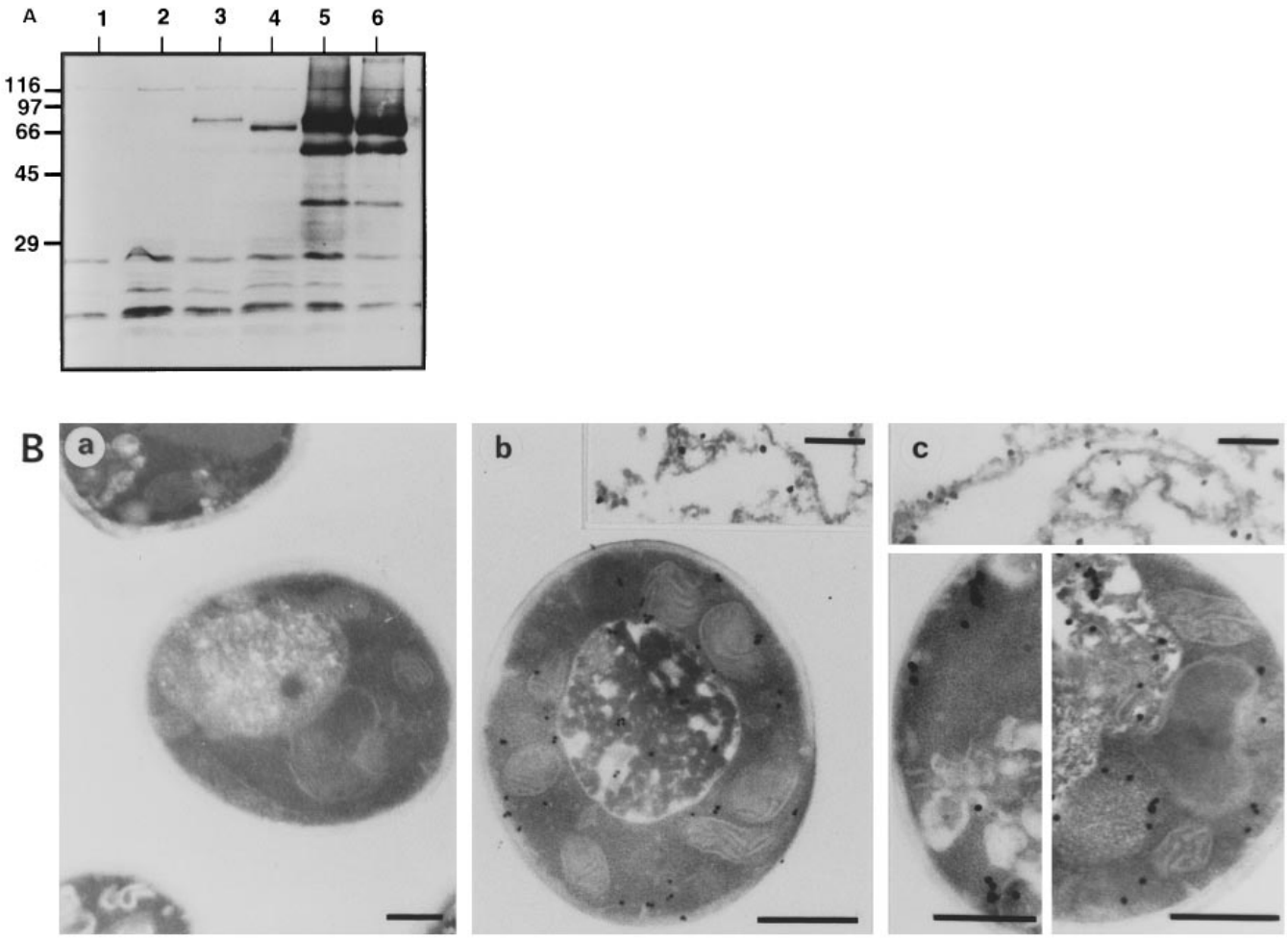

Figure 7 Immunological analysis of biotinylated receptors from recombinant yeast cells

(**A**) Immunoblot analysis: immobilized samples of crude membrane proteins isolated from recombinant yeast cells (*P. pastoris* strain KM71) which had been transformed with plasmid pPIC9K-βARFlagBio were probed with streptavidin–alkaline phosphatase conjugate. Each lane was loaded with 20 μg of protein. The differences in signal intensity between lanes 3 and 4 and 5 and 6 are due to a different expression level in different clones which might reflect a different gene dosage. In these clones, the exact copy number was not determined. Lanes 1, 3 and 5, mock-digested membranes; lanes 2, 4 and 6, membranes digested with PNGaseF; lanes 1 and 2, membranes of non-transformed KM71 cells; lanes 3 and 4, KM71 cells transformed with pPIC9K-βARFlagBio (clone 2); lanes 5 and 6, KM71 cells transformed with pPIC9K-βARFlagBio (clone 4). Enzymic deglycosylation was performed as outlined in the Experimental section. (**B**) Post- and pre-embedding labelling: post-embedding immunogold staining of ultrathin sections from non-recombinant *P. pastoris* cells (SMD1163) (**a**) or cells either transformed with pK-5HT$_{5A}$Bio (**b**) or with pPIC9K-5HT$_{5A}$FlagBio (**c**). Specific staining with gold-coupled streptavidin was only detected in recombinant cells (**b** and **c**). Pre-embedding immunogold staining with gold-coupled streptavidin resulted in the labelling of membranes from broken cells (insets in **b** and **c**). Bars = 0.5 μm.

not important for either ligand binding or coupling to G-proteins [30].

The *S. cerevisiae* Kex2 protease has been extensively characterized and is the prototype of a eukaryotic proprotein-processing protease that fulfils critical steps in the processing of many secreted peptides such as neuropeptides and growth factors [31–33]. Members of this family are Ca^{2+}-dependent serine proteases that cleave after dibasic sites (Lys-Arg or Arg-Arg). However, not every dibasic site is recognized and processed [34]. The influence of the amino acid sequence on the efficiency of the cleavage step has been extensively analysed, but the exact protein conformation might influence the specificity of the processing [34,35]. It should be mentioned that the amino acid sequence around the Kex2 cleavage site is identical for proteins expressed using the vectors pPIC9K-5HT$_{5A}$FlagBio and pPIC9K-βARFlagBio (Figures 1 and 2), indicating that the differences in processing are not due to the amino acid sequence. For the 5HT$_{5A}$ receptor, glycosylation might lead to a sterical hindrance which prevents access of the Kex2 protease. Since the α-factor leader is used as a secretion signal for many soluble proteins produced in *P. pastoris*, glycosylation might be what hinders secretion of incorrectly processed soluble proteins.

In vivo biotinylation of the bio-tagged receptor fusion

The rationale behind the construction of the bio-tagged receptor fusion proteins was that this tag would allow detection as well as purification of the receptors. Of course, a prerequisite for these approaches is that the fusion proteins are biotinylated *in vivo*. In Figure 7(A), we present the result of an immunoblot of membranes prepared from cells expressing the bio-tagged adrenergic receptor detected by alkaline phosphatase-coupled streptavidin. The same bands as with the M2 monoclonal antibody against the

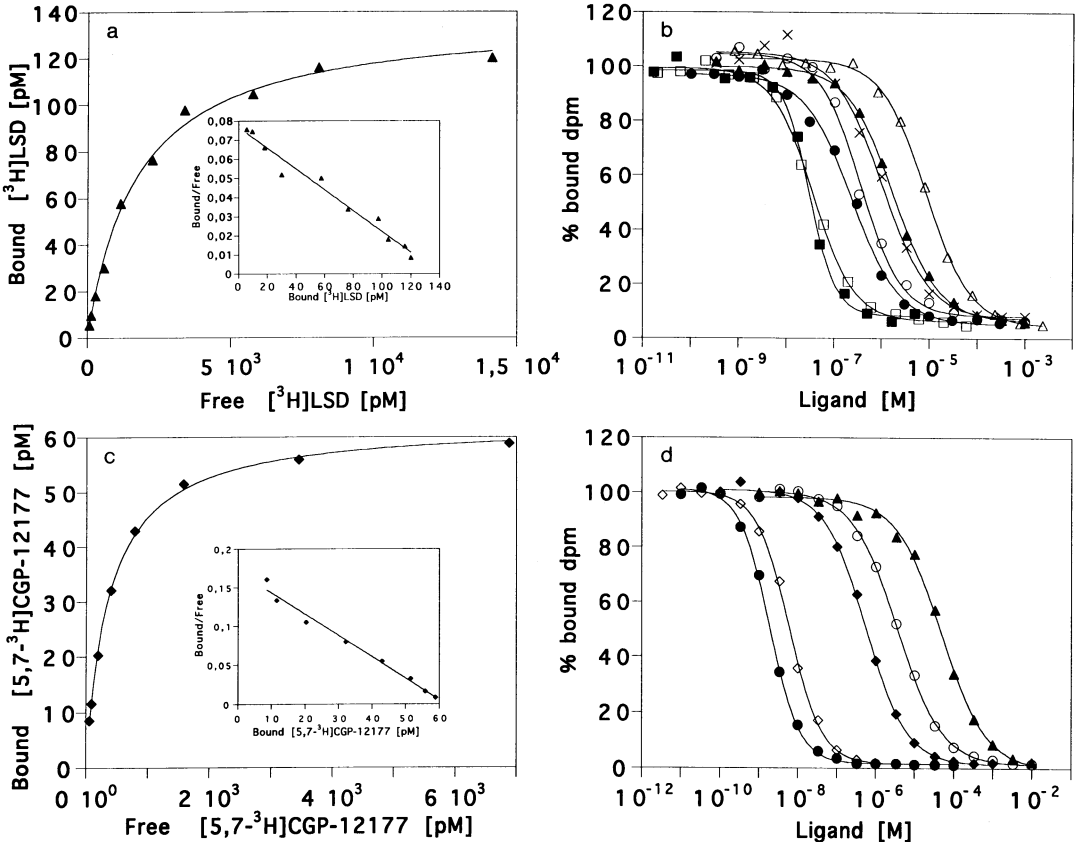

Figure 8 Pharmacological profiles for the mouse $5HT_{5A}$ receptor and the human β_2-adrenergic receptor heterologously produced in *P. pastoris*

Radioligand-binding studies on membranes of pPIC9-$5HT_{5A}$myc (top) or pPIC9K-βARFlagBio (bottom)-transformed *P. pastoris* strain SMD1163. Membrane preparations and binding assays were performed as outlined. (**a**) Saturation isotherm of [^{3}H]LSD binding. (**b**) Ligand-displacement curves: △, yohimbine; □, 5-carboxamidotryptamine; ○, 5-hydroxytryptamine; ▲, bufotenine; ×, lysergic acid; ■, ergotamine; ●, methysergide. (**c**) Saturation isotherm of [^{3}H]CGP-12177. (**d**) Ligand-displacement curves: ●, propranolol; ◇, alprenolol; ◆, isoprenaline; ○, adrenaline; ▲, noradrenaline. K_i values calculated from the graphs are given in Table 1. *Insets:* Scatchard transformations of the binding data.

FLAG tag were stained. Similar results were obtained with cells producing the bio-tagged $5HT_{5A}$ receptor (results not shown). As can be seen in Figure 7A, only a few bands were recognized in non-transformed *P. pastoris* cells which most likely result from labelling of internal biotinylated proteins. Also, the effect of multicopy insertions on the production level as revealed by the immunoblotting (Figure 7A, lanes 5 and 6) as well as the result of PNGaseF treatment (Figure 7A, lanes 4 and 6) is demonstrated. In agreement with the results of the immunoblot analysis, post-embedding labelling with gold-coupled streptavidin of yeast cells transformed with plasmids pK-$5HT_{5A}$Bio and pPIC9K-$5HT_{5A}$FlagBio resulted in specific staining comparable with that with the FLAG antibody (Figures 7B, b and c). Different targeting caused by the presence of the α-factor leader in cells transformed with plasmid pPIC9K-$5HT_{5A}$FlagBio in comparison with the construct without that fusion could not be observed. Non-transformed cells could not be stained because of the low abundance of biotinylated internal proteins (Figure 7B, a). As expected, pre-embedding immunogold labelling of recombinant yeast cells did not lead to staining of intact cells (results not shown). Only membranes originating from broken cells gave rise to specific staining, therefore confirming that the receptor is membrane bound (Figure 7B, b and c, inset). Together these data indicate that *in vivo* biotinylation of the *Pb. shermanii* transcarboxylase biotinylation domain fused to the receptors took place.

Ligand-binding studies of the $5HT_{5A}$ and β_2-adrenergic receptors produced in *P. pastoris*

For both receptors produced in *P. pastoris*, the K_D values for the respective radiolabelled ligands were determined in saturation experiments. The K_D for [^{3}H]LSD, which is a ligand of the $5HT_{5A}$ receptor, was determined to be 1.9 ± 0.3 nM, and the K_D for [^{3}H]CGP-12177, which specifically binds to the β_2-adrenergic receptor, was 0.5 ± 0.1 nM (Figure 8). Analysis of the binding data according to Scatchard indicated the presence of a single class of specific and saturable binding sites for both receptors. In the most efficiently producing clones, specific binding-site concentrations in crude membrane preparations of about 40 pmol/mg of membrane protein for the $5HT_{5A}$ receptor and 25 pmol/mg of membrane protein for the adrenergic receptor were detected. These values are considerably higher than those reported for the M_1 and M_5 muscarinic acetylcholine receptors [36,37], the D_{2S} receptor [38,39], the β_2-adrenergic receptor [40], the μ-opioid receptor [11] and the bovine rhodopsin [12] heterologously produced in *S. cerevisiae*, *Schizosaccharomyces pombe* and *P.*

Table 1 Pharmacological profiles of the $5HT_{5A}$ and the β_2-adrenergic receptor produced in *P. pastoris* (SMD1163) transformed with pPIC9-$5HT_{5A}$myc and pPIC9K-βARFlagBio respectively

Membranes prepared from *P. pastoris* producing the $5HT_{5A}$ or the β_2-adrenergic receptor at 22–30 h after induction were incubated with various concentrations of competing drugs in the presence of 1.6 nM [^{3}H]LSD or 0.8 nM [^{3}H]CGP-12177 respectively. IC_{50} values were determined using the four-parameter logistic function by KaleidaGraph and are reported as the mean and standard deviation of two independent experiments performed in duplicate. K_i values were calculated from the IC_{50} by the Cheng–Prusoff relation $K_i = IC_{50}/(1 + L/K_D)$, where L is the ligand concentration and K_D is the dissociation constant of [^{3}H]LSD or [^{3}H]CGP-12177 [43].

Ligand	K_i(M)
$5HT_{5A}$ receptor	
Ergotamine	$1.6(\pm 0.8) \times 10^{-8}$
5-Carboxamidotryptamine	$2.5(\pm 0.2) \times 10^{-8}$
Methysergide	$1.8(\pm 0.2) \times 10^{-7}$
5-Hydroxytryptamine	$4.5(\pm 2.6) \times 10^{-7}$
D-Lysergic acid	$7.4(\pm 0.2) \times 10^{-7}$
Bufotenine	$1.3(\pm 0.1) \times 10^{-6}$
Yohimbine	$5.5(\pm 0.6) \times 10^{-6}$
β_2-Adrenergic receptor	
S(−)-Propranolol	$8.0(\pm 1.0) \times 10^{-10}$
Alprenolol	$2.6(\pm 0.4) \times 10^{-9}$
R(−)-Isoprenaline	$2.2(\pm 0.1) \times 10^{-7}$
(−)-Adrenaline	$1.4(\pm 0.1) \times 10^{-6}$
(−)-Noradrenaline	$2.2(\pm 0.4) \times 10^{-5}$

pastoris. For the same receptors produced in *S. cerevisiae* similar results have been published: 16 pmol/mg for the $5HT_{5A}$ receptor [41] and 40 pmol/mg for the β_2-adrenergic receptor [42]. To determine the pharmacological profiles of the heterologously produced receptors, bound [^{3}H]LSD or [^{3}H]CGP-12177 was displaced with various serotonergic or adrenergic drugs respectively (Figure 8). The serotonergic compounds displayed the following rank order of potencies at the $5HT_{5A}$ receptor: ergotamine > 5-carboxamidotryptamine > methysergide > 5-hydroxytryptamine > lysergic acid > bufotenine > yohimbine, which is comparable with the data obtained for the $5HT_{5A}$ receptor expressed in COS-7 cells [3] as well as in *S. cerevisiae* [41]. The rank order of the adrenergic drugs was propranolol > alprenolol > isoprenaline > adrenaline > noradrenaline. Again, the pharmacological fingerprint was comparable with that obtained in other expression systems. The K_i values of the respective components are presented in Table 1. The absolute affinities for the ligands examined, which were calculated from the IC_{50} values, were a factor of 1.3–6 lower than the affinities for the receptors in their native environment or heterologously produced in mammalian cells, but they are in the same range as reported for these receptors heterologously produced in insect cells.

A possible explanation for this discrepancy could be the different lipid composition of the membranes surrounding the receptor proteins. It is well established that the lipid composition, especially the cholesterol concentration, is important for the affinity of some membrane-bound hormone receptors such as the nicotinic acetylcholine receptor and the β_2-adrenergic receptor [44,45]. In fact, the affinities of the transferrin receptor and the oxytocin receptor for their ligands could be modulated by changing the cholesterol contents of the surrounding lipid bilayer [46,47]. In yeasts, ergosterol is the analogous sterol in the lipid bilayer membranes. It could well be that the altered fluidity of the yeast membranes resulting from the insertion of ergosterol rather than cholesterol into the lipid bilayer or direct binding of the sterol affect the ligand-binding properties of the heterologously produced receptors.

Affinities for specific ligands usually decrease when receptors become solubilized because of the change of the receptor environment. Since for purification of a membrane-bound receptor solubilization is an absolute prerequisite, the absolute affinities of the receptors in the membrane will not be important for successful purification.

Conclusions

The use of protease-deficient strains for production, the fusion of the receptors to the *S. cerevisiae* α-factor preproleader peptide as well as selection of recombinant clones exhibiting a higher gene dosage can improve production levels of GPCRs in *P. pastoris*. Further improvements will probably result from optimization of conditions of large-scale culture. The production levels reported in this work for the two receptors are similar to or even better than those that can be obtained in baculovirus-infected insect cells {12–17 pmol/mg for the β_2-adrenergic receptor [48], 30–50 pmol/mg for the $5HT_{5A}$ (H. Reiländer, unpublished work)}. Handling and upscaling are much easier and less time consuming than for the baculovirus system. *P. pastoris* is very suitable for fermentation because it can grow to extremely high cell densities and it is genetically stable under induction conditions. In shake flasks the number of specific binding sites detectable corresponded to about 1.5 mg of receptor/litre of culture for the $5HT_{5A}$ receptor and about 1 mg/litre of culture for the β_2-adrenergic receptor. In some initial fermentation attempts with clones producing the $5HT_{5A}$ receptor, we obtained specific LSD-binding sites that corresponded to about 12 mg of receptor/litre of culture at a cell density of about 200 g wet weight/litre (results not shown). This demonstrates that the *P. pastoris* expression system could easily provide sufficient raw material for receptor purification. In the case of the β_2-adrenergic receptor, a purification system has already been established, and for the $5HT_{5A}$ receptor purification is in progress.

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