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Constitutive expression of the human D_{2S}-dopamine receptor in the unicellular yeast *Saccharomyces cerevisiae*

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Abstract

The cDNA for the human D_{2S}-dopamine receptor has been functionally expressed in the unicellular yeast *Saccharomyces cerevisiae*. Two expression plasmids pRS421D2 (original D_{2S}-gene coding region) and pRS421D2S (the first 24 aa of the yeast *STE2*-gene are fused to the N-terminus of the D_{2S}-gene) were constructed and transformed into the protease deficient *S. cerevisiae* strain cI3-ABYS-86. Northern blot analysis of total RNA from transformed yeast clones revealed that for both constructs the D_{2S}-gene was constitutively transcribed from the plasmids *PMA1* promoter. Membranes prepared from recombinant *S. cerevisiae* exhibited saturable binding with the antagonist [^{3}H]methylspiperone. Competition studies revealed pharmacological properties for these sites which were comparable to those reported for the D_2-receptor heterologously expressed in mammalian cells. The expression of the receptor was monitored by Western blot analysis using an antiserum raised against a peptide from the third intracellular domain of the receptor protein and by ligand binding assay.

Key words: Gene expression; Radioligand binding; D_{2S}-dopamine receptor; Yeast

1. Introduction

Similar to other receptors cloned recently, the dopamine receptors mediate their function through interactions with heterotrimeric guanine nucleotide binding regulatory proteins (G-proteins) [1]. Members of this receptor family, named the G-protein coupled receptors, bear detectable sequence similarities with one another and all receptors of this family share similar hydrophobicity profiles consistent with the existence of seven transmembrane spanning regions [2]. Only two subtypes of dopamine receptors have been identified on the basis of pharmacological, biochemical and physiological studies whereas at least five to six subtypes of dopaminergic receptors have been identified by molecular cloning of their cDNAs and are referred to as D_{1A}, D_{1B}, D_2, D_3, D_4, D_5 (D_{1C}) [3–9]. The D_2-receptor was the first dopamine receptor to be cloned and therefore is the best characterized one [10]. The receptor exists in two isoforms (D_{2L} and D_{2S}) which are produced by differential splicing [11–13]. Activation of the receptor results in various responses, including inhibition of adenylyl cyclase, inhibition of phosphatidylinositol turnover, increase in K^+ channel activity and inhibition of Ca^{2+} mobilization [10,14–17].

Since there is no rich natural source of a homogeneous subtype of the dopamine receptors, we sought to produce the D_{2S}-receptor heterologously in the unicellular yeast *Saccharomyces cerevisiae*. In the past, *S. cerevisiae* has been sucessfully used for the heterologous production of several foreign membrane proteins (reviewed in [18]). Genetically *S. cerevisiae* is well characterized and many expression vectors are readily available. The yeast expression system also has the potential for producing large quantities of receptor proteins because *S. cerevisiae* can be grown inexpensively in large fermenters.

Abbreviations: BCIP, 5-bromo-4-chloro-3-indolyl-phosphate *p*-toluidinium salt; DMF, dimethylformamide; PMSF, phenylmethylsulfonyl fluoride; NBT, Nitroblue-tetrazolium chloride; mcs, multiple cloning site; BSA, bovine serum albumin; STE2, *STE2*-gene from *S. cerevisiae*.

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2. Materials and methods

2.1. Strains and transformations

E. coli strain XL-1 Blue (*rec*A1, *end*A1, *gyr*96, *thi*-1, *hsd*R17, *sup*E44, *rel*A1, *lac*$^-$, (F′*pro*AB, *lac*I^{q}Z-ΔM15, Tn10(tet^r)) (Stratagene) was used for propagation of the recombinant plasmids. For *E. coli* transformation, the $CaCl_2$ procedure was used as described [19]. *S. cerevisiae* strain cI3-ABYS-86 (MATα, *pra*1–1, *prb*1–1, *prc*1–1, *cps*1–3, *ura*3 – 5, *leu*2–3,112, *his*$^-$) [20] was kindly provided by Prof. Dr. D.H. Wolf (University of Stuttgart, Germany). Yeast cells were made competent for plasmid uptake by treatment with lithium acetate and poly(ethylene glycol) [21].

2.2. Recombinant DNA technology and construction of recombinant plasmids

DNA isolation, restriction enzyme analysis, agarose electrophoresis and cloning procedures were performed using established techniques [19,22]. All plasmid constructs were ensured by DNA sequencing using the dideoxy-termination method [23]. Oligonucleotides (Dop1: 5′-TCGACAAAAAATG-3′ and Dop2: 5′-GATCCATTTTTTG-3′) were synthesized using an Applied Biosystems DNA Synthesizer.

To facilitate the construction of yeast expression vectors containing the D_{2S}-gene, a 1451 bp *Bam*HI/*Kpn*I restriction fragment encoding the D_{2S}-receptor with the exception of the ATG-initiation codon was isolated from plasmid pD2 (kindly provided by Dr. M.G. Caron, Duke University, Durham, NC, USA) and subcloned into an appropriately digested vector pBS + (Stratagene). This construction (pBSD2) was used to optimize the initiation region of the D_{2S}-gene for the heterologous expression in *S. cerevisiae*. The two oligonucleotides Dop1 and Dop2 were annealed and ligated into *Sal*I/*Bam*HI restricted pBSD2 vector resulting in the DNA sequence 5′-AAAAAATG-D_{2S}-3′-gene (the D_{2S}-receptor start codon is underlined). To facilitate further cloning steps the resulting plasmid pBSD2Y was cut with *Kpn*I and *Eco*RI, the vector fragment was purified from the gel and a 173 bp *Eco*RI fragment including parts of the mcs of the vector pBluescriptII KS + (Stratagene) was subsequently ligated into the plasmid. From this plasmid, named pBSD2YSal the complete, adapted D_{2S}-coding region could be released by *Sal*I digestion as a 1512 bp DNA fragment.

Plasmid pBLUESTE2A (unpublished results) which bears the codons for the first 24 amino acids of the yeast STE2-gene was restricted with *Bam*HI/*Kpn*I and ligated with the 1451 bp DNA-fragment isolated from plasmid pD2. The different reading frames of the two genes in the resulting plasmid pBLUESTED2f were synchronized by restriction with *Bam*HI, subsequent blunting of the restriction site with Mung Bean Nuclease followed by religation. This plasmid now encodes the D_{2S}-receptor in fusion with the N-terminal region of the yeast STE2-receptor. This plasmid, named pBLUESTED2 was cleaved with *Xba*I and partially with *Kpn*I and the 1533 bp DNA fragment bearing the STE/D_2-fusion was isolated from the gel and ligated into appropriate digested vector pBS + . A mcs was

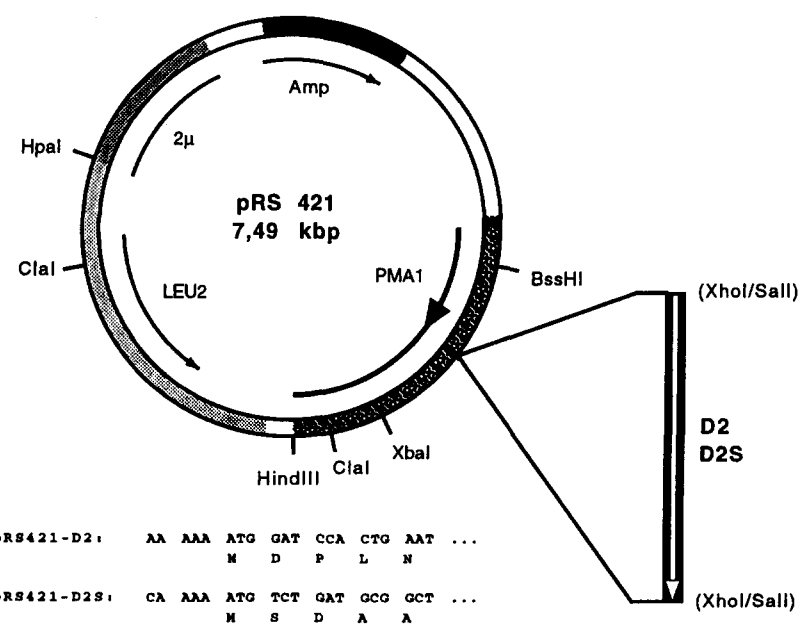

Fig. 1. Map of the yeast expression vectors pRS421D2 and pRS421D2S. In both plasmids the constitutive yeast *PMA1* promoter is used for heterologous expression.

introduced in the same way as has been described for plasmid pBSD2Y above, now allowing the release of a DNA fragment encoding the STE/D_2-gene fusion by a *Sal*I digestion.

For heterologous expression in *S. cerevisiae*, episomal vector pRS421 which utilizes the constitutive *PMA1* promoter [24] for the expression of a foreign gene was used (kindly provided by Dr. R. Serrano, Valencia, Spain). The *Sal*I-DNA fragments harbouring either the adapted D_{2S}-gene and the STE/D_2-fusion were isolated from the plasmids mentioned above and cloned into the *Xho*I site of vector pRS421 (Fig. 1).

2.3. Yeast culture

Non-transformed *S. cerevisiae* strains were kept in YEPD-medium (1% yeast extract, 2% bactopeptone, 2% glucose). Transformed yeast always was cultured under selective pressure in SM (per liter: 3.4 g yeast nitrogen base without amino acids and ammonium sulfate; 5 g $(NH_4)_2SO_4$; 2% glucose supplemented with respective amino acids) to counteract the loss of transformed plasmids. For the preparation of membranes 250 ml of selective medium were inoculated with a start titer of $1–2 \cdot 10^6$ cells/ml from a 30 ml overnight culture and incubated at 30°C under permanent oxygenation by rotation. After 16 h growth the cells were harvested by centrifugation and membranes were prepared as described below.

2.4. Northern blot analysis

Recombinant and non-recombinant yeasts were grown overnight at 30°C and total RNA was prepared as previously described [25]. Following electrophoresis on a 1% agarose gel in the presence of formaldehyde and transfer onto a nylon membrane, hybridization was performed with an in vitro synthesized ^{32}P-labelled D_{2S} specific anti-sense RNA. After several washes of the membrane at high stringency the blot was analysed by autoradiography.

2.5. Isolation of membranes from S. cerevisiae cells

250 ml cultures were harvested after overnight incubation by centrifugation. Cells were washed in water, centrifuged and the pellet was resuspended in 4 ml STED10-buffer (10 mM Tris-HCl (pH 7.6); 1 mM EDTA; 1 mM DDT; 10% sucrose) supplemented with protease inhibitors (5 μg/ml leupeptin, 5 μg/ml chymostatin, 5 μg/ml antipain, 1 μg/ml pepstatin, and 100 mM Pefabloc®). Glass beads (17 g; Mesh 400–600; Sigma) were added to the suspension and the cells were broken by vigorous vortexing at 4°C for 5 min. The homogenate was diluted with 15 ml of STED10 plus protease inhibitors and centrifuged at $5000 \times g$ for 10 min. Subsequently the membranes were pelleted at $35\,000 \times g$ for 45 min. The resulting membrane pellet was resuspended in 3–4 ml STED10-buffer and stored at −80°C for further experiments. The protein concentration of the membrane preparation was determined using the Bradford method with BSA as a standard [26].

2.6. Western blot analysis

For immunoblot analysis, yeast membranes were isolated as described above and membrane proteins were separated on a 10% SDS-polyacrylamide gel [27]. Thereafter, proteins were transferred onto polyvinyliden-difluoride membrane (Immobilon P; Millipore) and processed as described [28]. The primary antibody (directed against the receptor) was used at a dilution of 1:2000, the secondary anti-rabbit alkaline phosphatase coupled antibody at a dilution of 1:1000. Protein bands were visualized by enzymatic reaction in AP-buffer (100 mM NaCl, 100 mM Tris (pH 9.5), 5 mM $MgCl_2$) with BCIP (stock solution: 50 mg/ml DMF) and NBT (stock solution: 50 mg/ml 70%DMF) as recommended by the suppliers.

2.7. Radioligand binding to recombinant human D_{2S}-receptor

Saturation binding measurements on isolated membranes were carried out using antagonist [^{3}H]methylspiperone as a radioactive ligand (spec. activity 921.3 GBq/mmol; Amersham). Membranes equivalent to 10–30 μg protein were suspended in 500 μl binding buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl) and radioactive ligand was pipetted to the probe at a final concentration of 0.02–20 000 pM. Binding assays were incubated for 45 min at 30°C. Nonspecific binding was determined in the presence of non-labelled (+)-butaclamol (2 μM final concentration). Bound and free ligand were separated by rapid filtration (Whatman GF/F filters, soaked in 0.3% polyethyleneimine) and after three washes with ice-cold binding buffer, radioactivity bound to the filter was determined by liquid scintillation counting (rotiszint; Roth). K_D and B_{max} values were calculated by computer-aided nonlinear regression analysis of the binding isotherms under equilibrium conditions.

3. Results

S. cerevisiae has been used successfully for the functional expression of several different membrane proteins and, therefore, we chose this organism to examine the expression of the human D_{2S}-receptor. We constructed two recombinant plasmids (pRS421D2 and

pRS421D2S) for heterologous expression in *S. cerevisiae* (Fig. 1). The construction of both vectors was based on plasmid pRS421, where a foreign gene can be cloned under the control of the constitutive *PMA1* promoter for heterologous expression in yeast [24]. In plasmid pRS421D2 the initiation of translation was optimized for expression in yeast whereas the coding region of the D_{2S}-gene had not been altered during construction. Recently, it has been reported that a fusion of the β_2-adrenergic receptor to the N-terminal region of the yeast STE-protein resulted in an extremely high level of expression [29]. To test if an N-terminal fusion with the STE2-protein would also enhance the expression of the D_{2S}-receptor, we constructed plasmid pRS421D2S, where the D_{2S}-coding region is fused to the 24 amino terminal amino acids of the yeast STE2-gene product. The initial amino acid sequence in this construction is now: NH_2-**MSDAAP-SCSNLFYDPTYNPGQSTRI-**DPLNLS (the yeast STE2-receptor coding region is in bold-type, the D_{2S}-receptor is underlined). The vector pRS421 and the recombinant plasmids pRS421D2 and pRS421D2S were transformed into *S. cerevisiae*. To minimize proteolytic digestion of the heterologously produced proteins, we chose the protease-deficient *S. cerevisiae* strain cI3-ABYS-86 for the expression studies [20]. Visual examination of the resulting transformants revealed that yeast cells transformed with plasmid pRS421D2S had a changed morphology. Contrary to WT-cells or cells which had been transformed with plasmid pRS421D2, these had a swollen, balloon-like apppearance (data not shown) indicating that the expression of the fusion protein somehow affected the organism.

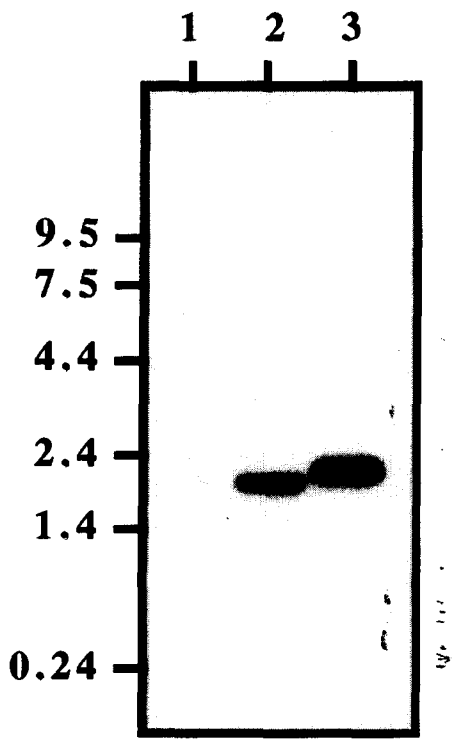

Fig. 2. High stringency Northern blot analysis of total RNA (10 μg per lane) from yeast (*S. cerevisiae* cI3-ABYS-86) transformed with pRS421 (lane 1), pRS421D2 (lane 2) and pRS421D2S (lane 3). Molecular size markers at the left are from a commercially available RNA ladder (BRL) that was run on the same gel.

The transcription of the D_{2S}-receptor and the STE/D_{2S}-receptor fusion was examined by Northern blot analysis. As shown in Fig. 2, D_{2S}-specific transcripts could be detected when yeast had been transformed with the plasmids pRS421D2 and pRS421D2S, whereas no signal could be observed in yeast cells which had been transformed with plasmid pRS421 only. As expected the transcripts from pRS421D2 and pRS421D2S differed in size by ≈ 100 bp due to the addition of the STE2-coding region in the pRS421 D2S-construct. Thus, both cloned genes are transcribed in recombinant *S. cerevisiae* from the PMA1-promoter of the vector pRS421.

A primary function of certain cell surface receptors is to recognize appropriate ligands. Accordingly, we determined ligand binding affinities to establish the functional integrity of the D_{2S}-receptor expressed in yeast. Recombinant yeast clones were tested for the presence of dopamine binding sites by radioligand binding assay using the antagonist [^{3}H]methylspiperone. Crude membranes prepared from *S. cerevisiae* cells transformed with pRS421D2 and pRS421D2S exhibited specific saturable binding of [^{3}H]methylspiperone with a K_D of 1.7 nM and 1.9 nM, respectively (Fig. 3). No endogenous expression of D_{2S}-receptor could be detected in the *S. cerevisiae* WT strain or in cells transformed with plasmid pRS421. Saturation studies performed on intact cells as well as cells where the cell wall had been permeabilized by three freeze-thaw cycles did not show specific [^{3}H]methylspiperone binding with either plasmid, suggesting that the receptor is not located in the plasma membrane. The K_D values and the B_{max} values were calculated from the saturation isotherms for the two constructs, and Scatchard transformation of the data indicated a single class of binding sites. Nevertheless, the K_D values determined for both constructions differ significantly from the K_D values previously described for the receptor in its natural environment or transient transfected mammalian cells. Possibly differences in the lipid environment or post-translational modifications of the heterologously produced receptor may influence its binding characteristics.

The density of dopamine binding sites in the yeast membrane preparations were estimated to 1–2 pmol/mg membrane protein. This value roughly corresponds to a receptor density of approx. 500–1000 receptors per yeast cell. In comparison the endogenous STE2-receptor of yeast has $\approx$ 3000–8000 copies per cell [30],

whereas a heterologously produced mACh-receptor has only ≈ 2–40 copies per cell [31]. The receptor level estimated in yeast membranes is comparable to those reported for transfected mammalian cells (0.6–1 pmol/mg) but is considerably lower when compared to the receptor level which can be reached in insect cells after infection with a recombinant baculovirus (≥ 6 pmol/mg membrane protein; manuscript in preparation). Nevertheless, the receptor level obtained in yeast membrane preparations is sufficient to start purification.

The pharmacological subtype characteristics of the two recombinant yeast lines were investigated with five subtype-selective dopamine ligands (spiperone, haloperidol, domperidon, (+)-butaclamol and apomorphin), that competed with [^{3}H]methylspiperone in the expected rank order at the two produced proteins [32] (Fig. 4). Displacement curves recorded with these com-

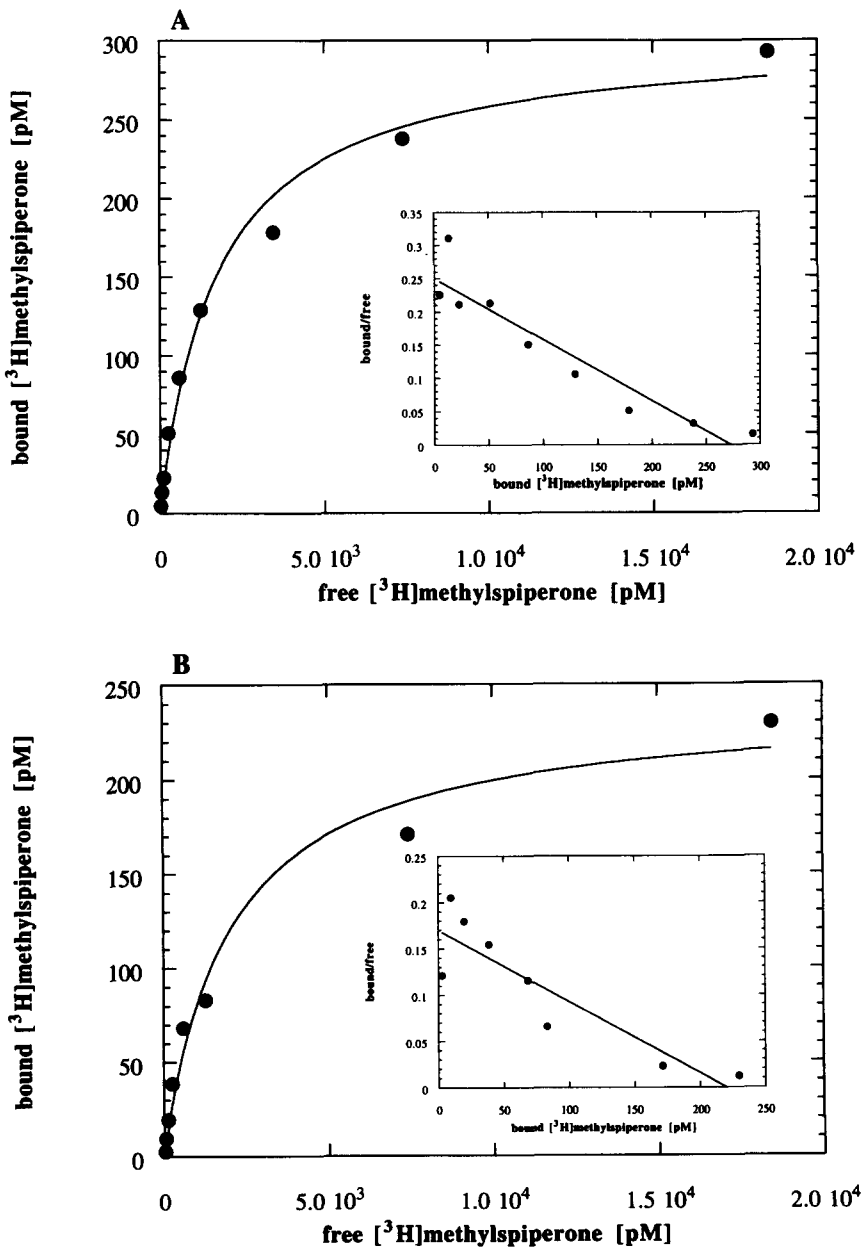

Fig. 3. Saturation isotherms for [^{3}H]methylspiperone binding and competition analysis of membranes prepared from yeast transformed with plasmids pRS421D2 (A) or pRS421D2S (B). [^{3}H]methylspiperone binding experiments were performed in triplicate on membranes as described previously. Nonspecific binding was determined with 1 μM (+)-butaclamol. Results from one of three independent experiments are shown. Control membranes from yeast transformed with non-recombinant plasmid did not show any detectable specific signal. Inset: Scatchard transformation of the data.

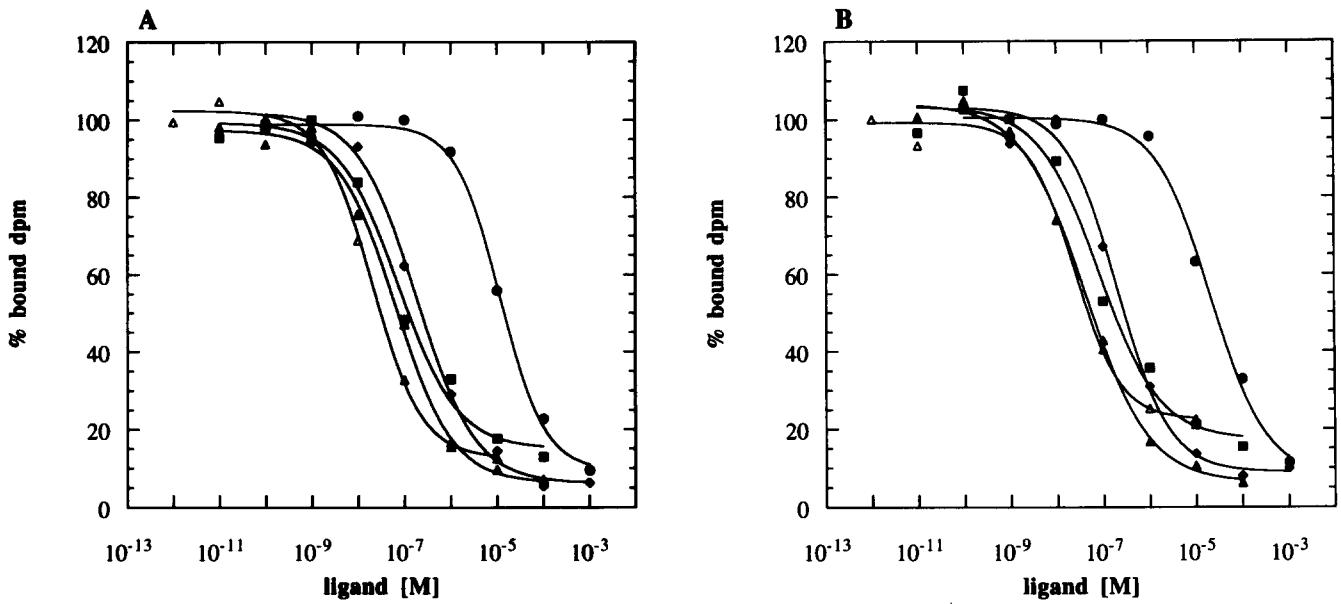

Fig. 4. Inhibition of specific [^{3}H]methylspiperone binding to yeast membranes. (A) Membranes from yeast transformed with plasmid pRS421D2. (B) Membranes from yeast transformed with pRS421D2S. Competition bindings were performed at a final [^{3}H]methylspiperone concentration about the K_D value determined on yeast membranes. The 100% value corresponds to specific binding in the absence of any competitor and the 0% corresponds to the nonspecific binding in the presence of 1 μM butaclamol. Shown are values obtained for apomorphin (●), butaclamol (■), domperidon (◆), haloperidol (▲) and spiperone (△). Data represent one of several independent experiments performed in triplicate.

pounds show the typical steepness with all the ligands, and the data for each of the ligands could be modelled by a one-site fit. The high affinity D_2-selective anatagonist spiperone is the most potent agent followed by haloperidol, (+)-butaclamol, domperidon and apomorphin. Dopamine is also able to inhibit [^{3}H]methylspiperone binding. The apparent dissociation constants estimated for the individual compounds from the curves (Table 1) are not consistent with values published for the receptor obtained from other sources. The ligands have dissociation constants which are in general worse than the data published earlier.

For estimation of the apparent molecular mass of heterologously produced D_{2S}-receptor, membrane proteins of recombinant yeast strains were probed with polyclonal antiserum RD2 raised against a peptide occurring in the third intracellular loop of the D_{2S}-receptor (NH_2-AARRAQELEMEML-COOH; position A243-L255 of the D_{2S}-receptor) [33]. As presented in Fig. 5, membrane prepared from a yeast clone bearing only vector pRS421 was absolutely devoid of immunoreactive protein. The antibody specifically stained a protein with an apparent molecular mass of $\approx$ 40 kDa in the yeast strain cI3-ABYS-86 transformed with plasmid pRS421D2. As expected, the yeast transformed with plasmid pRS421D2S which encodes for the STE/D_{2S}-fusion protein exhibits a band with a slightly higher apparent molecular mass ($\approx$ 3 kDa) than that of the clone with the D_{2S}-receptor alone. This increase in the apparent molecular mass is in agreement with the assumption that the STE/D_{2S}-fusion protein has an additional 24 amino acids at its N-terminus. The apparent molecular mass determined by SDS-PAGE differs significantly from the molecular mass which can be calculated from the cDNA sequence (45.5 kDa). It has been reported earlier that the apparent molecular mass of the isolated and deglycosylated rat D_{2S}-receptor is $\approx$ 7 kDa smaller than the molecular mass calculated from the corresponding cDNA sequence [34]. There are also some additional bands above the 40 kDa band which might represent different glycosylated forms of the receptor protein. This assumption is consistent because the amino acid sequence of the D_2-receptor contains three putative sites for N-linked glycosylation in the extracellular N-terminal region. In addition to the bands in the 40 kDa range, very strong specific signals also arise in the range of $\approx$ 90 kDa in both

Table 1
K_i values (nM) for membranes from *S. cerevisiae* transformed with either pRS421D2 or pRS421D2S

Strain	cI3-ABYS-86 pRS421D2	cI3-ABYS-86 pRS421D2S
Apomorphine	600	1160
Butaclamol	38	44
Domperidone	87	104
Haloperidol	36	24
Spiperone	8.2	10.7

The 50% inhibition values (IC_{50}) calculated in Fig. 5 were converted into K_i values. Results are geometric means of three experiments in which [^{3}H]spiperone binding was inhibited by various concentrations of unlabelled drug. The concentration of the radioligand is about the K_D value determined.

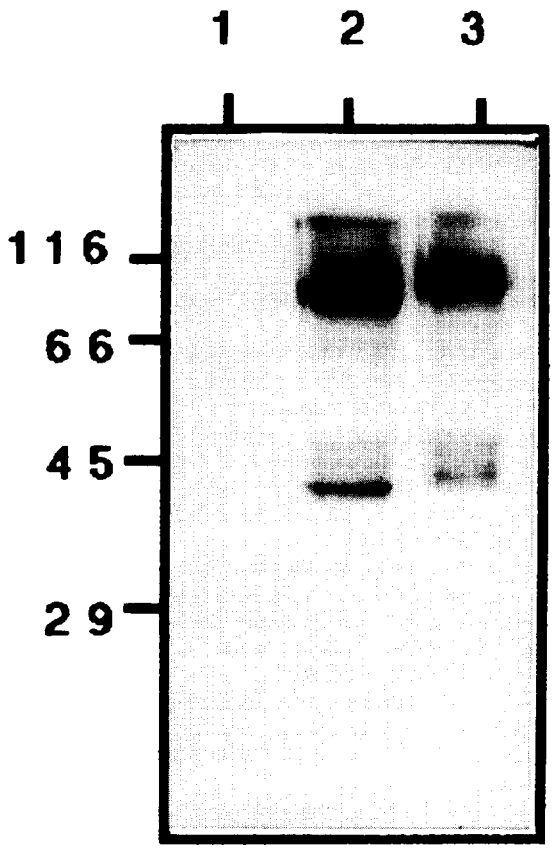

Fig. 5. Immunoblot analysis of membranes isolated from yeast. Cell membranes prepared from yeast (*S. cerevisiae* cI3-ABYS-86) transformed with pRS421 (lane 1), pRS421D2 (lane 2) or pRS421D2S (lane 3) were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose. Immobilized proteins were probed with a rabbit polyclonal serum specific for the D_{2S}-receptor. Immunostained proteins were visualized using an AP-coupled goat anti-(rabbit) IgG/M. One example of multiple experiments with similar result is shdwn.

lanes. We assume that these bands might represent aggregated protein produced in the cells. Although the same protein concentrations were applied to the SDS-PAGE, the construct with the STE/D_{2S} fusion induced lower levels of detectable protein.

4. Discussion

Until recently, attempts to study receptor function and structure have been hampered by the absence of pure populations of receptors. Natural sources in most cases represent a complex mixture of cell types and also receptor subtypes that make it impossible to characterize a specific receptor. In the light of this heterogeneity, it was clear that the only way to overcome this problem would be to express a certain receptor gene in host cells lacking endogenous receptors. *S. cerevisiae* has been described as an ideally suited system for the expression and subsequent characterization for many foreign genes. The system provides an established genetic background, easy manipulation and also fermentation in large scale is easier compared to higher eukaryotic cells.

In this communication we report the construction, transformation and preliminary characterization of the D_{2S}-receptor heterologously expressed in *S. cerevisiae*. Two plasmid constructs (pRS421D2 and pRS421D2S) from which the D_{2S}-receptor alone and a STE2/D_{2S}-fusion can be expressed, were examined. Northern blot analysis revealed that both constructs were transcribed from the plasmids *PMA1*-promoters. As expected the mRNAs synthesized from the two plasmids differ by $\approx$ 100 bp due to the fusion of the STE2-coding region in plasmid pRS241D2S. The integrity of the receptor proteins produced in recombinant yeast was followed by radioligand binding using membrane preparations of the transformed yeast cells. As could be measured with the antagonist [^{3}H]methylspiperone both plasmids induced the expression of specific, saturable D_{2S}-binding sites. We were not able to detect specific ligand binding on the intact yeast transformants. Most probably the heterologously expressed receptors remained in the internal cell compartments and were not transported to the plasma membrane. Preliminary electron microscopical studies of immunogold-labelled ultrathin sections of expressing yeast cells revealed that the heterologously expressed receptor could be detected in membrane vesicles within the vacuole of the cell (P. Sander, manuscript in preparation).

The K_i and K_D values obtained for several ligands in saturation or inhibition experiments using membrane preparations from the transformed yeast cells differed by a factor of 15–20 from the values previously reported for the receptor protein in its natural environment or heterologously expressed in higher eukaryotic expression systems. A possible explanation for this discrepancy could be the lipid composition of the membranes surrounding the receptor molecules. It has been well established that the lipid composition, especially the cholesterol concentration, is important for the activity of some membrane-bound hormone receptors like the nicotinic acetylcholine receptor and the β-adrenergic receptor [34,35]. Previously, an extensive study on the lipid composition of the subcellular membranes of the yeast *S. cerevisiae* reported that the ergosterol content of the internal membranes is very low, compared to the content of the external plasma membrane [36]. Ergosterol is the analogous sterol in yeast lipid bilayer membranes. So it could well be that the altered fluidity of the internal yeast membranes affect the ligand binding properties of the heterologously expressed D_{2S} dopamine receptor. A comparable shift of the ligand binding affinities was also monitored for the mouse $5HT_5$-serotonin receptor expressed in yeast cells. Here the receptor was targeted

to the inner membrane compartments of the expressing cell (M. Bach, manuscript in preparation).

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