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Expression and pharmacological characterization of the human M1 muscarinic receptor in *Saccharomyces cerevisiae*

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The yeast *S. cerevisiae* has been examined as a heterologous host for the expression of mammalian neurotransmitter receptors which couple to guanine nucleotide regulatory (G) proteins. A cloned gene encoding the M1 subtype of human muscarinic receptor (HM1) was transformed into *S. cerevisiae* on a high copy plasmid under the control of the promoter for the yeast alcohol dehydrogenase (ADH) gene. Northern blotting demonstrated the presence of HM1 transcripts in transformants, and crude membranes prepared from these cells showed saturable binding of the muscarinic antagonist [^{3}H] *N*-methyl scopolamine with a K_d of 179 pM and B_{max} of 20 fmol/mg protein. Competition binding studies revealed pharmacological properties for these sites which were comparable to those reported for the M1 site in mammalian tissues. Yeast expressing HM1 did not exhibit high affinity agonist binding or cell-cycle arrest in the presence of muscarinic agonists, indicating that the mammalian receptor did not couple to the endogenous yeast G protein.

Gene expression; Radioligand binding; Acetylcholine receptor; Yeast; G protein

1. INTRODUCTION

The plasma membrane receptors for a wide variety of neurotransmitters and biologically-active peptides function through interactions with intracellular guanine nucleotide regulatory (G) proteins (reviewed in [1]). The application of molecular genetic techniques has provided cloned DNA and protein sequences for many receptors of this class; all are single polypeptides which are predicted to fold, like bacteriorhodopsin, into 7 membrane-spanning α-helices [1]. Heterologous expression of the cloned genes encoding these receptors in mammalian or amphibian host cells provides a powerful tool to investigate signalling mechanisms and receptor structure-function (reviewed in [2]). Such systems are also expected to generate the quantities of these rare proteins necessary for structure determination, and to provide novel reagents to the pharmaceutical industry for drug-screening applications [3].

The ability to use microorganisms as hosts for the expression of mammalian receptors with 'native' properties would provide easily manipulated, low-cost alternatives to current systems for use in large-scale drug screening programs, analysis of receptor mutants and high level production. The eukaryote *S. cerevisiae* is a particularly attractive candidate for such applications, since it possesses signal transducing machinery with remarkable similarities to mammalian G protein coupled systems. Specifically, the receptors for the peptide mating pheromones α- and a-factors, termed STE2 and STE3, respectively, share the putative 7-helix structural motif and couple to a trimeric G protein with sequence similarities to mammalian G proteins [4,5]. In the present study, we have examined *S. cerevisiae* as a heterologous host for the expression of mammalian G protein-coupled receptors using a cloned gene encoding the M1 subtype of human muscarinic receptor (HM1) [6].

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Abbreviations: NMS, *N*-methyl scopolamine; QNB, quinuclidinyl benzylate; 4-DAMP, 4-diphenylacetoxy-*N*-methylpiperidine methobromide; PMSF, phenylmethylsulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; ADH, alcohol dehydrogenase, mAR, muscarinic acetylcholine receptor

2. MATERIAL AND METHODS

2.1. *Chemicals*

[^{3}H]NMS was purchased from Amersham, atropine, oxotremorine and carbachol were from Sigma and 4-DAMP, pirenzepine, methoctramine and (±)QNB were from Research Biochemical Inc. Zymolase 100T was purchased from ICN Biomedicals, Klenow enzyme, restriction enzymes and *Bam*H1 linkers from Boehringer-Mannheim, PMSF from Gibco/BRL and guanidinium thiocyanate from Fluka.

2.2. *Vector construction*

All recombinant DNA procedures were based on standard protocols [7]. A ca 4 kb human genomic fragment was provided [6] which contained the intronless coding portion of the HM1 gene plus approximately 1.7 and 0.9 kb of 5'- and 3'-non-coding sequences, respectively. Digestion with *Sau*I and SmaI yielded the 1.4 kb coding region flanked by short non-coding sequences. This fragment was blunt-ended using Klenow DNA polymerase I fragment and subcloned into the *Bam*H1 site of the yeast expression vector pVT102-U [8] using synthetic *Bam*HI linkers. The remaining non-coding portions of the

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HM1 gene were removed by oligonucleotide-directed mutagenesis [9] using a kit from BioRad (Mutagene). One oligonucleotide (5'-TACAATCAACTCCAAGCTGGATCCATGAACACTCCAGCC-CCA-3') directed the 'loop-out' removal of the 5'-flanking sequence (73 nucleotides), and a second (5'-TCCCGCCAATGCTGA-TAGGGATCCAGAGGTTTGGTCAAG-3') deleted the 3'-flanking sequence (33 nucleotides). The final sequences at the ends of the HM1 coding segment, as verified by dideoxy sequencing, are shown in Fig. 1B. The resulting construct, termed pVTUHM1 (Fig. 1A), contains the complete HM1 coding sequence with *Bam*H1 sites immediately flanking the translational start and stop codons, and places the insert under control of the promoter and 3'-non-coding regions of the yeast ADH gene. The yeast strain DJ 213-6-3-a (ade2° his4-580^a lys2 trp1^a tyr1° tryl sup 4-3^{ts} leu2 ura3 STE2::leu2) [10] was transformed [11] with either pVTUHM1 or the pVT102-U vector, and colonies selected by growth on solid medium lacking uracil. The HM1 coding segment was also inserted into the *Bam*H1 site of pGEM1 (Promega) for the synthesis of HM1 riboprobes.

2.3. *RNA analysis*

Cloned transformants grown overnight at 30°C in yeast minimal medium lacking uracil (Y MIN-URA) were converted to spheroplasts [12] and total RNA prepared [13]. Following electrophoreses on a 1% agaraose gel in the presence of formaldehyde and transfer to nitrocellulose [7], the Northern blot was hybridized with ^{32}P-labeled HM1 anti-sense RNA, washed at high stringency and exposed to X-ray film.

2.4. *Membrane preparation*

Yeast grown in Y MIN-URA to approximately 1×10^7 cells/ml were harvested by centrifugation, washed twice in ice-cold lysis buffer (50 mM Tris, pH 7.4, 1 mM EDTA, 0.1 mM PMSF, 10% glycerol) and resuspended in the same buffer to yield approximately 5×10^9 cells/ml. The cells were disrupted by two passages through a French press at 20 000 psi, the resulting suspension centrifuged at $500 \times g$ for 5 min and the supernatant centrifuged at $100\,000 \times g$ for 90 min. The pellet of crude membranes was resuspended in a minimal amount of lysis buffer, homogenized in a glass/teflon homogenizer and stored in aliquots at −80°C. The protein concentration of the membrane preparations [14] was between 25 to 35 mg/ml.

2.5. *Binding assays*

Membranes were thawed on ice, homogenized again and diluted as appropriate in the incubation buffer (50 mM Tris, pH 7.4, 0.1 mM PMSF). Assays were conducted in triplicate at a protein concentration of 0.5 mg/ml in a final volume of 1 ml. Incubations were performed for 25°C for 60 min, at which time equilibrium was achieved (not shown). Saturation binding of [^{3}H]NMS was measured at 12–15 concentrations in the range of 0.003 and 20 nM. For competition experiments, 12 concentrations of non-radioactive muscarinic agonist or antagonist were used with a single concentration of [^{3}H]NMS (0.5 nM). Non-specific binding was measured in the presence of 1um atropine or (±)QNB, both of which gave the same result. Incubations were terminated by vacuum filtration on GF/B filters (Whatman) pretreated with 0.3% polyethylenimine for 3 h to reduce non-specific binding of the ligand to the filter. The filters were washed 3 times with 5 ml of ice-cold 50 mM Tris, pH 7.4, placed in vials with 7 ml of liquid scintillation cocktail (ACS, Amersham) and radioactivity counted the next day at 33% efficiency. Saturation and competition binding parameters were calculated by non-linear multiple regression using a computerized curve fitting program (RADLIG, Elsevier-Biosoft).

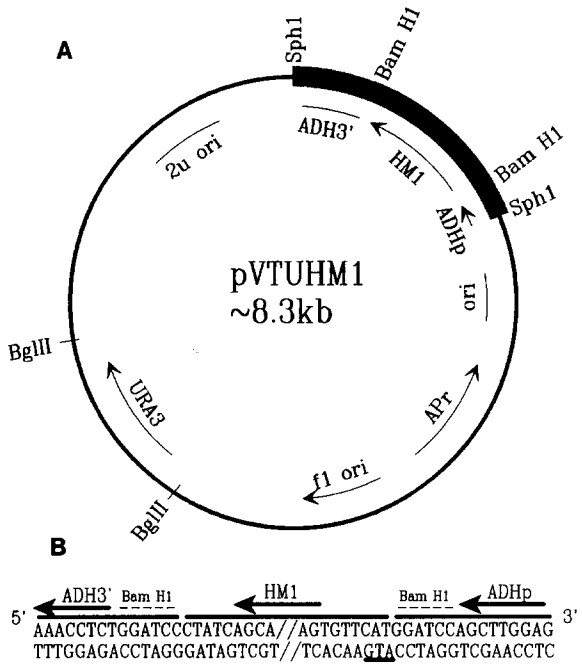

Fig. 1. (a) Partial map of the expression vector pVTUHM1 constructed as described in the text. The HM1 1.4 kb coding sequence (filled arc) is flanked in the vector by the promoter (ADHp) and 3'-non-coding sequence (ADH3') of the yeast ADH gene (stippled arcs). Approximate map position and orientation are also for the *E. coli* of replication (ori), the yeast 2μ origen of replication (2μ ori) and the origin of replication and intergenic region of the phage f1 (f1 ori), as well as the β-lactamase (APr) and URA3 genes used as selectable markers in *E. coli* and yeast, respectively. (b) Nucleotide sequence at 5'- and 3'-ends of the HM1 coding segment in the final pVTUHM1 expression vector. The initiator codon ATG is underlined.

3. RESULTS AND DISCUSSION

Yeast transformants carrying the vector pVT102-U alone or the pVTUHM1 construct were examined for the presence of HM1 RNA transcripts by Northern blotting. As shown in the autoradiogram in Fig. 2, a hybridizing band of approximately 1.6 kb was observed in the total RNA extract from yeast transformed with the recombinant plasmid but was absent from the control. This corresponds in size to that expected for a transcript of the HM1 coding region plus the ADH 5'- and 3'-non-coding segments. The cloned HM1 gene is thus transcribed in yeast from pVTUHM1.

Transformants were tested for the presence of muscarinic binding sites using the antagonist [^{3}H]NMS in saturation experiments. Intact cells did not exhibit specific [^{3}H]NMS binding over background with either plasmid (not shown). Crude membranes prepared from cells transformed with pVTUHM1, but not with vector alone, showed specific, saturable binding of [^{3}H]NMS (Fig. 3a). Data analysis indicated a single class of binding sites with a dissociation constant of 0.179 ± 0.05 nM (mean ± SE, $n = 3$) and densities of between 1 and 20 fmol/mg protein for several different preparations (Fig. 3B). This value represents roughly 2–40 sites/cell. By comparison, the endogenous yeast pheromone receptor STE2 is normally present in *a*-cells at 3000–8000 copies/cell, this value being raised to as high as 100 000/cell in yeast overexpressing the cloned STE2 gene from a high-copy plasmid [15]. The difference could conceivably reflect inefficient translation

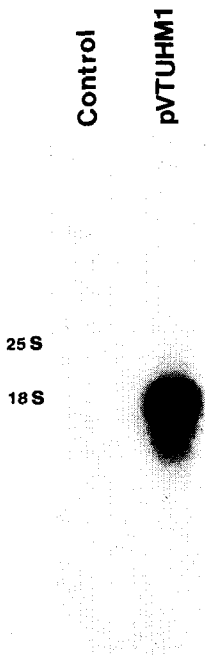

Fig. 2. Autoradiogram of a Northern blot of total RNA (2 μg per lane) from yeast (DJ-213-6-3-a) transformed with pVT102-U alone (control) or the vector harboring the HM1 gene downstream the ADH promoter (pVTUHM1). The position of the yeast 18 S and 25 S ribosomal RNAs are indicated on the left.

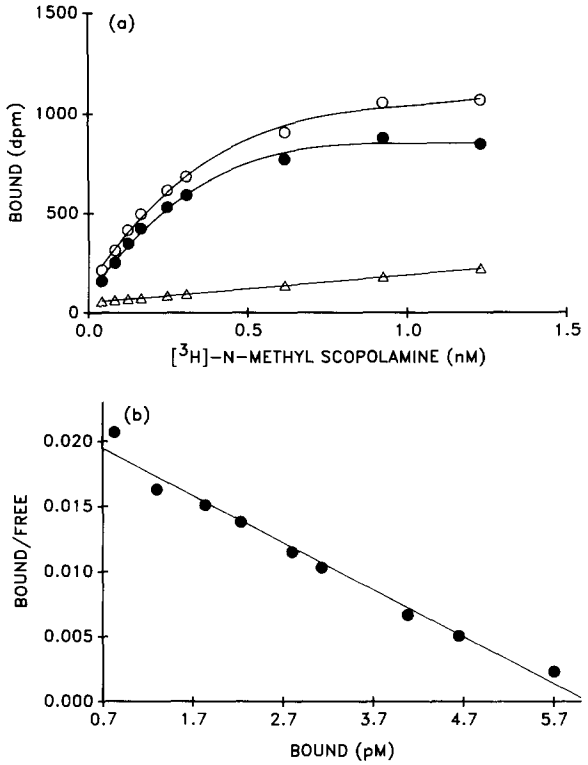

Fig. 3. (a) Representative saturation analysis for [^{3}H]NMS binding to membranes from yeast pVTUHM1 transformants. Shown are total binding (○), non-specific binding in the presence of 1 μM atropine (△), and specific binding (●), (b) Scatchard representation of the specific binding shown in (a).

of HM1 transcripts detected in transformants, or to problems in stability, folding and/or processing (e.g. glycosylation) of human receptor protein in the heterologous yeast host. All of these phenomena have been encountered in studies to express mammalian genes in *S. cerevisiae* (reviewed in [16]). The development of suitable antisera permitting estimates of the total level of HM1 translation products would aid in clarifying this point.

The structural integrity of the muscarinic binding sites detected in transformants was assessed by determining the affinities of a series of muscarinic ligands in competition binding studies with [^{3}H]-NMS. The ligands included two agonists (carbachol and oxotremorine), the non-selective antagonist atropine, and 3 selective antagonists (pirenzepine, 4-DAMP and methoctramine) which distinguish 3 subclasses of muscarinic binding sites in mammalian tissues [17–19]. The competition curves are presented in Fig. 4, and the calculated apparent dissociation constants shown in Table I. All the ligands tested completely inhibited [^{3}H]NMS binding, and the data for each could be fitted to a single class of sites with a Hill coefficient not significantly different from unity ($P>0.05$). The binding sites expressed in yeast membranes from HM1 therefore appear to constitute a homogeneous population.

The apparent dissociation constants determined for all of the antagonists correlate well with the values reported for muscarinic binding sites in rat as well as human cerebral cortical membranes [20,21]. In particular, the 3 subtype-selective antagonists inhibited [^{3}H]NMS binding with the same rank order of potency (4-DAMP $\geqslant$ pirenzepine $>>$ methoctramine) as described for mammalian cerebral cortex [21–24], and for the HM1 gene product expressed in cultured mammalian cells [6,25]. This finding suggests that the recombinant protein detected in binding assays has assumed its 'native' conformation in the yeast membrane.

The dissociation constants observed for the two agonists in the present work are comparable to the low-affinity sites described in brain membranes and in transformed mammalian cells expressing cloned HM1. In these mammalian systems, an additional high affinity component is observed which is abolished by incubation with GTP and its non-hydrolyzable analogues. The high-affinity GTP-sensitive binding of agonists is believed to reflect functional interactions with intracellular G proteins [1]. Based on the present findings, it would appear that recombinant HM1 expressed in *S. cerevisiae* does not couple to the endogenous G protein homologue responsible for signal transduction by receptors for mating pheromones. Consistent with this conclusion, yeast expressing HM1

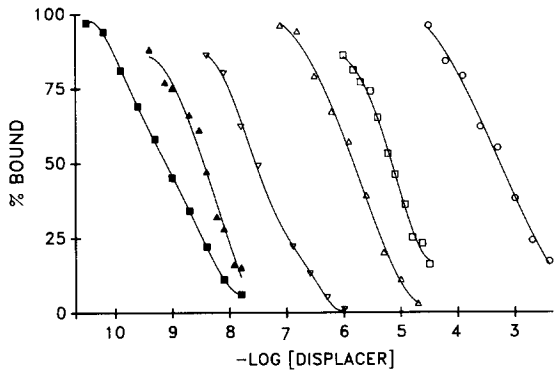

Fig. 4. Inhibition of specific [^{3}H]NMS binding to yeast membranes (from yeast transformed with pVTUHM1). The concentration of [^{3}H]NMS was 0.5 nM and specific binding was defined as that displaced by 1 μM atropine. Shown are values obtained for atropine (■), 4-DAMP (▲), pirenzepine (▽), methoctramine (△), oxotremorine (□), and carbachol (○).

showed no detectable response to muscarinic agonists in a growth inhibition ('halo') assay employed for mating pheromone activity [5] (data not shown). This could reflect the inability of the HM1 gene product to recognize the yeast G protein, a plausible explanation given the limited sequence similarities between the yeast and mammalian homologues [5,26]. It is also possible that the heterologous yeast host may fail to transport the recombinant receptor to the appropriate subcellular compartment, i.e. the plasma membrane. Our inability to detect specific [^{3}H]NMS binding to intact cells is consistent with the latter possibility, though the levels measured in crude membranes were near the detection limit for the assay procedure with intact cells.

The number of receptors expressed per cell in yeast in this work was considerably lower than those reported for a variety of mammalian cell lines stably transformed with the cloned HM1 gene (ca. 20/cell vs 10 000–100 000/cell, respectively) [25,27]. On the other hand, the specific activity of muscarine binding sites in yeast membrane preparations (ca. 20 fmol/mg protein) is within 1–2 orders of magnitude of that observed with transformed mammalian cells (ca. 200 fmol–2 pmol/mg protein). The levels obtained in yeast with the present system are sufficient to perform detailed characterization of antagonist binding properties on recombinant receptor, for example in drug screening programs, and should prove useful in the analysis of antagonist binding to site-directed receptor mutants in structure-function studies. Given the ease and low cost of preparing large quantities of yeast membranes containing recombinant receptor, this system offers significant advantages over mammalian cell expression for such applications. In its present form, however, yeast expression of HM1 does not generate sufficient amounts of receptor protein to undertake purification and structural characterization. Studies are in progress to increase receptor levels using different expression vectors and yeast strains.

Previous reports have described the use of microbial hosts for the expression of neurotransmitter receptors. The 4 subunits of the nicotinic acetylcholine receptor of *Torpedo californica*, a ligand-gated cation channel, have been co-expressed in *S. cerevisiae* [28]. In that study, the four polypeptides represented 0.1–1.0% of the total membrane protein, but no evidence for assembly of functional receptors nor any data on ligand binding were presented. Human β-adrenergic receptors, members of the G protein-coupled receptor family, have been expressed in *E. coli* from β-galactosidase [29] or lamB [30] fusion genes with retention of 'native' ligand binding profiles. The results of these studies indicate that microbial hosts are potentially useful tools for receptor studies. A major drawback to the use of microorganisms for the expression of mammalian G protein-coupled receptors, however, is the absence of biochemical and cellular responses to agonists, due to the lack of apropriate intracellular transduction components. The previous finding that a mammalian G protein α subunit (α-s) can partially compensate for the genetic disruption of the yeast alpha homologue [26] suggests that functional interactions between mammalian receptors and yeast transduction machinery may be possible.

In conclusion, we have shown here for the first time that a human G protein-coupled receptor can be expressed in *S. cerevisiae* with retention of its characteristic ligand binding properties. Studies to establish functional coupling of the recombinant receptor to the mating pathway via genetic engineering of the yeast G protein subunits are in progress.

Table I

Binding parameters of muscarinic ligands to membranes from yeast transformants

	Atropine	4DAMP Methiodide	Pirenzepine	Methoctramine	Oxotremorine	Charbachol
K_{app}	$(2.35\pm0.19)\times10^{-10}$	$(1.09\pm0.22)\times10^{-9}$	$(7.26\pm0.27)\times10^{-9}$	$(2.30\pm0.95)\times10^{-7}$	$(1.19\pm0.12)\times10^{-6}$	$(1.71\pm0.32)\times10^{-4}$
N_H	0.86 ± 0.05	1.19 ± 0.05	0.92 ± 0.04	1.11 ± 0.10	1.04 ± 0.05	0.92 ± 0.09

The data were derived from [^{3}H]NMS inhibition experiments shown in Fig. 4 and represent the mean ± SE ($n=3$) for the values of apparent affinity (K_{app}) and Hill coefficient (n_H) for each drug

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