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Expression and functional purification of a glycosylation deficient version of the human adenosine 2a receptor for structural studies

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Abstract

A glycosylation deficient (dG) version of the human adenosine 2a receptor (hA2aR) was made in *Pichia pastoris* strain SMD1163. Under optimal conditions, expression levels of between 8 and 12 pmol receptor/mg membrane protein were obtained routinely. In a shake flask, this is equivalent to *ca.* 0.2 mg of receptor per litre of culture. The level of functional receptor produced was essentially independent of the pH of the yeast media. In contrast to this, addition of the hA2aR antagonist theophylline to the culture media caused a twofold increase in receptor expression. A similar effect on dG hA2aR production was also observed when the induction temperature was reduced from 29 to 22 °C. In *P. pastoris* membranes, dG hA2aR had native-like pharmacological properties, binding antagonists with rank potency ZM241385 > XAC > theophylline, as well as the agonist NECA. Furthermore, the receptor was made with its large (*ca.* 120 amino acid) C-terminal domain intact. dG hA2aR was purified to homogeneity in three steps, and its identity confirmed by electrospray tandem mass spectrometry following digestion with trypsin. The secondary structure of the entire receptor is largely (*ca.* 81%) α-helical. Purified dG hA2aR bound [^{3}H]ZM241385 in a saturable manner with a B_{max} of 18.1 ± 0.5 nmol/mg protein, close to the theoretical B_{max} value for pure protein (21.3 nmol/mg protein), showing that the receptor had retained its functionality during the purification process. Regular production of pure dG hA2aR in milligram quantities has enabled crystallisation trials to be started.
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Mammalian cells are able to respond to a diverse range of stimuli including light, hormones, neurotransmitters, and odorants via a superfamily of proteins called G-protein coupled receptors (GPCRs)[1] [1]. The function of these proteins at the cell surface is to perceive specific chemical messages in the extracellular environment and then relay this information to the inside of the cell by selectively activating a particular (subset of) G-protein(s). Common to all GPCRs is a 7-transmembrane domain helical motif. Although many GPCRs have now been cloned and their pharmacological and signalling properties characterised, very little is known regarding their structures or the molecular details of how the receptors become activated.

The arrangement of the transmembrane helices in bovine rhodopsin, a light-activated GPCR, was first visualised in an electron microscope study of two-dimensional crystals [2]. Since then, the structure of the inactive conformation of this receptor has been determined to atomic resolution by X-ray crystallography [3–5]. At present, no other GPCR has had its 3-dimensional structure solved. This is in no small part due to the difficulties associated with making milligram quantities of pure receptor for sustained crystallisation trials. A range of heterologous systems (including

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[1] *Abbreviations used:* dG, glycosylation deficient; hA2aR, human adenosine 2a receptor; XAC, xanthine amine congener; NECA, 5′-(*N*-ethylcarboxamido)adenosine; GPCR, G-protein coupled receptor; FH, FLAG-His_{10}; DDM, dodecyl-β-D-maltoside; CHS, cholesteryl hemi-succinate; MSMS, electrospray tandem mass spectrometry; BB, binding buffer; PB, purification buffer.

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re-folding from *Escherichia coli* inclusion bodies [6,7], functional expression in *E. coli* [8–10], yeast [11–19], insect [20–23], and mammalian cells [24]) have been used to make GPCRs for structural studies. At present, there is no clear consensus approach for making large quantities of GPCRs. The methylotropic yeast *Pichia pastoris* is, however, likely to become the preferred organism for recombinant GPCR production in the future. The reasons for this are threefold : as a simple eukaryote, *P. pastoris* has all of the necessary cellular machinery to correctly make, fold, insert and post-translationally modify mammalian membrane proteins. Furthermore, high levels of recombinant protein production are often achieved in *P. pastoris* as expression is driven from a very strong, methanol inducible promoter. Finally, as *P. pastoris* grows well in fermentation culture, it is possible to obtain large (upto 500 g wet weight cells/litre of culture) quantities of cell biomass from which the recombinant protein can be purified. Although several GPCRs can be made in *P. pastoris* with native-like pharmacology, no-one has yet shown how to get from high levels of good quality receptor in *P. pastoris* membranes to active, pure protein in solution useful for structural studies.

The physiological effects of adenosine in the human body are mediated by four different GPCRs. One of these, the adenosine 2a receptor (A2aR), is involved in inhibiting activated immune cells and in protecting tissue from acute inflammatory damage in vivo [25,26]. Consequently, the hA2aR is an important therapeutic target for those illnesses where uncontrolled inflammation is a major contributor to disease pathogenesis. These include cancer, heart disease, atherosclerosis, and sepsis [26].

Previously, it has been shown that the hA2aR can be expressed at high levels in a functional form in *E. coli* [9]. Unfortunately, only a truncated form of the receptor could be made as the receptor's large cytoplasmic domain (*ca.* 120 amino acids) was cleaved by the host cell's proteases. Here, the production of a glycosylation deficient version of the entire hA2aR in *P. pastoris* is described. In the membrane, the expressed receptor has native-like pharmacological properties. dG hA2aR can be purified to homogeneity in a functional state in three steps. The regular production of milligram quantities of receptor has allowed crystallisation trials to be started.

Materials and methods

Strains

Escherichia coli strain DH5α (Invitrogen) was used for transforming and propagating the recombinant expression plasmids. Protein expression was performed in the protease deficient *P. pastoris* strain SMD1163 (*pep4 prb1 his4*).

Plasmid construction

The hA2aR cDNA (Accession Number X68486) was amplified by PCR using the primers GCGCCTGAATTCATGCCCATCATGGGCTCCTCG and GAGTCGGCGGCCGCTCAGGACACTCCTGCTCCATCC that contained the restriction sites *Eco*RI and *Not*I, respectively. The PCR product was purified before it was cloned into the pGEM-T Easy Vector (Promega). The hA2aR coding sequence was excised by digestion with *Eco*RI and *Not*I, and subcloned into the *P. pastoris* expression vector pPICZαA (Invitrogen). The N-linked glycosylation site at Asn154 in hA2aR was changed to a Gln using the primer GGTCAGCCAAAGGAGGGCAAGCAGCACTCCCAGGGCTGCGGGGAG. Following this, a His_{10} tag was introduced into the vector immediately upstream of the *Eco*RI site using the primer CGAGAAAAGAGAGGCTGAAGCTCATCATCATCATCATCATCATCATCATCATGAATTCATGCCCATCATGGGC. Finally, a FLAG-tag was introduced into vector flush to the Kex2 cleavage site, immediately upstream of the His_{10} tag using the primer GAAGAAGGGGTATCTCTCGAGAAAAGAGATTATAAAGATGACGATGACAAGCATCATCATCATCATCATCATCATGAATTCATGC. All mutagenesis reactions were performed using the Quick Change Multi Site-Directed Mutagenesis Kit (Stratagene). The recombinant plasmid was sequenced prior to transformation into the yeast.

Yeast transformation

Twenty micrograms of pPICZαA-FH–dG hA2aR linearised with *Pme*I was introduced into competent SMD1163 cells by electroporation as described in the *Pichia* Easy Select manual (Invitrogen). The yeast were plated on YPDS plates containing either 0.2, 0.5, or 1 mg/ml Zeocin (Invitrogen) and then incubated at 29 °C for 72 h. Single colonies isolated from the 1 mg/ml plate were re-streaked on fresh plates, from which glycerol stocks were prepared. These were stored at −80 °C.

Small-scale receptor expression

Yeast cells were grown up from the −80 °C stocks in 10 ml of BMGY (1% yeast extract, 2% peptone, 100 mM potassium phosphate pH 6, 1.34% yeast nitrogen base, 4×10^{-5} % biotin, and 1% glycerol) at 29 °C until the A_{600} was between 2 and 6. The cells were spun down (1500 g, 5 min) and then re-suspended in 10 ml BMMY (same as BMGY except it contains 0.5% methanol instead of 1% glycerol), and grown for a further 24 h at 29 °C. Expression optimisation experiments were performed by altering the BMMY pH (3, 4, 5, and 7), the induction temperature (22 °C) and adding 10 mM theophylline (a hA2aR antagonist) to the media. For each set of conditions, two yeast cultures were grown and induced separately.

Large-scale production of FH–dG hA2aR

Ten litres of SMD1163-FH–dG hA2aR were routinely grown as 20× 500 ml cultures in 2 L conical flasks. The

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