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Methods for recombinant expression and functional characterization of human cannabinoid receptor CB_2

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Abstract: Cannabinoid receptor CB_2 is a seven transmembrane-domain integral membrane protein that belongs to a large superfamily of G protein-coupled receptors (GPCR). CB_2 is a part of the endocannabinoid system that plays vital role in regulation of immune response, inflammation, pain sensitivity, obesity and other physiological responses. Information about the structure and mechanisms of functioning of this receptor in cell membranes is essential for the rational development of specific pharmaceuticals. Here we review the methodology for recombinant expression, purification, stabilization and biochemical characterization of CB_2 suitable for preparation of multi-milligram quantities of functionally active receptor. The biotechnological protocols include expression of the recombinant CB_2 in *E. coli* cells as a fusion with the maltose binding protein, stabilization with a high affinity ligand and a derivative of cholesterol in detergent micelles, efficient purification by tandem affinity chromatography, and reconstitution of the receptor into lipid bilayers. The purified recombinant CB_2 receptor is amenable to functional and structural studies including nuclear magnetic resonance spectroscopy and a wide range of biochemical and biophysical techniques.

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Introduction

Heptahelical G protein-coupled receptors (GPCR) are integral membrane proteins involved in a wide array of cell signaling pathways. The cannabinoid receptor CB_2 that belongs to the rhodopsin-like (class A) GPCR is an attractive target for the development of drugs for management of pain, inflammation and immunological disorders [1-3]. Structural studies will provide critical insights into the molecular mechanisms of ligand binding and signal transduction, and can contribute to the rational design of novel specific drugs targeting this receptor.

The progress in structural studies of GPCR has been relatively slow until recently, primarily due to (i) difficulties in obtaining large quantities of sufficiently pure, homogenous and functional receptors, (ii) conformational flexibility of GPCR that hinder their stabilization in detergent micelles, and (iii) high hydrophobicity of these integral membrane proteins that complicates preparation of well-diffracting crystals for X-ray crystallography. In spite of these obstacles, significant improvements in expression techniques, methods of stabilization and crystal preparation resulted in several high resolution structures of GPCR solved during the past few years [4-12].

With the notable exception of rhodopsin, most GPCR are present in native tissues at relatively low levels, and recombinant expression in a heterologous host is currently the only practical way to obtain these proteins in milligram quantities necessary for structural studies. The commonly used expression systems for GPCR include baculovirus-infected insect cells, yeast, bacterial or mammalian cells as well as cell-free systems [6]. Expression in insect cells has been particularly useful for production of receptors in milligram quantities for crystallization trials [5, 9, 10, 13, 14]. However, the adaptation of insect- or mammalian cells for preparation of stable-isotope labeled proteins for nuclear magnetic resonance (NMR) spectroscopy studies currently is prohibitively expensive due to the complexity of the medium and high cost of labeled nutrients [15]. While the expression in yeast cells has also been used for production of several GPCR [16, 17], this host may not be suitable for some receptors (including CB_2) because it is often prone to non-homogenous glycosylation or partial proteolysis of target proteins [18-20].

These considerations stimulated the development of methods of production of the recombinant CB_2 in *E. coli* cells. In a series of publications we reported efficient expression of CB_2 receptor in bacterial cell membranes in a fully functional form, although in the absence of posttranslational modifications [21], and its purification to over 90% homogeneity by tandem affinity chromatography [22]. Furthermore, the protein can be labeled with stable isotopes by fermentation of *E. coli* in a defined-composition medium supplemented with labeled nutrients [23]. This robust methodology for expression and labeling of CB_2 opens up exciting opportunities to study this receptor by NMR spectroscopy.

In addition to the availability of milligram quantities of purified receptor, structural methods require sufficient stability of the protein over extended periods of time. While solubilization in detergents is needed for isolation of GPCR from cell membranes, preventing irreversible denaturation of these proteins in detergent micelles is a notoriously difficult task [6-8, 13, 24-26]. Here we review the methodological approaches for stabilization and reconstitution of the purified receptor in lipid bilayers and preparation of milligram quantities of functional CB_2 suitable for studies by a broad array of biophysical techniques.

Experimental design

Several laboratories reported expression of either full-length or truncated human cannabinoid receptors in a heterologous host including bacteria, yeast, baculovirus-infected insect cells and cell-free

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system [18-20, 27-32]. While production of a ligand binding-competent receptor was demonstrated, no successful attempt was reported to produce and purify the expressed receptor in large quantities, to label it with stable isotopes and to stabilize it in a functional form suitable for biophysical studies.

A comprehensive program initiated in our laboratory has an objective to develop an extensive set of methods for recombinant expression of human cannabinoid receptor CB_2 in large quantities in *E. coli*, efficient purification, stabilization in detergent micelles, and functional characterization. Various elements of this methodology were reported in several earlier publications [21-24, 33, 34], and the general outline of the experimental strategy is given in Figure 1. The initial stage of the study focused on establishing conditions for production of the functional CB_2 receptor in *E. coli* cells cultivated in a rich 2xYT medium while subsequent work dealt with the adaptation of expression protocols to preparation of stable-isotope labeled receptor by fermentation in minimal salt media of defined composition. Particular attention was devoted to maximizing the recombinant protein yield, reducing the cost of fermentation, stabilization of the functional CB_2 receptor in detergent micelles and achieving high purity and homogeneity of protein preparations. In parallel, methods for functional analysis of the purified receptor by ligand binding and G protein activation (either in detergent micelles or reconstituted in lipid bilayers) were developed. Furthermore, the purified CB_2 was characterized by several biophysical techniques including NMR spectroscopy, surface plasmon resonance, CD-, IR- and fluorescent spectroscopy, and differential scanning calorimetry.

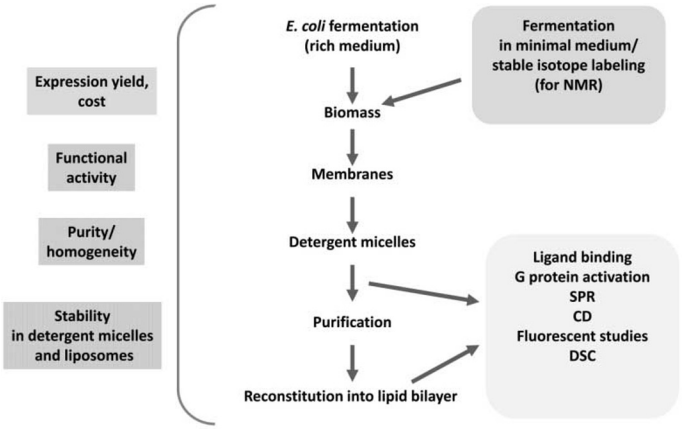

Figure 1. Experimental strategy for preparation of functional CB_2 (adapted from [24]).

Expression of CB_2 receptor in *E. coli*

The choice of the bacterial host, copy number of the expression vector, strength of the promoter, composition of the culture media, concentration of the inducer as well as temperature, method and duration of induction play critical roles in determining both the total yield of the fusion protein and the recovery of functional receptor. Several *E. coli* strains including BL21 (DE3), DH5a, KRX, RosettaGami, C41 and C43 were compared for their effectiveness in production of fusion CB_2, and BL21 (DE3) was selected based on higher yield of the recombinant protein and high levels of functional activity of the receptor [21, 22, 35].

Limited availability of tRNAs for rarely used codons may play a role in controlling the rate of translation of the recombinant polypeptide [36, 37]. We optimized the codon usage of the human CB_2 gene for bacterial expression by designing a synthetic gene enriched with synonymous codons reported to be frequently used in *E. coli* [38]. However, the use of the synthetic gene did not increase the yield of CB_2 when introduced into the expression vector under the control of the *lac* promoter; it even resulted in slightly lower levels of the recombinant receptor compared to the original mammalian gene sequence (Yeliseev et al, unpublished). This suggests that the rate of folding and insertion of CB_2 in the bacterial membrane rather than the rate of translation has a critical influence over the yield and correct fold of the recombinant receptor. Therefore, in all subsequent experiments the native sequence of the human CB_2 gene was used.

The nature and relative position of expression partners fused to the target protein can change dramatically the expression level and activity of the recombinant GPCR [6]. Therefore, a variety of plasmid constructs containing different combinations of several expression- and solubility tags were tested for their efficiency in production of functional CB_2. Figure 2 depicts selected expression constructs currently utilized in our laboratory.

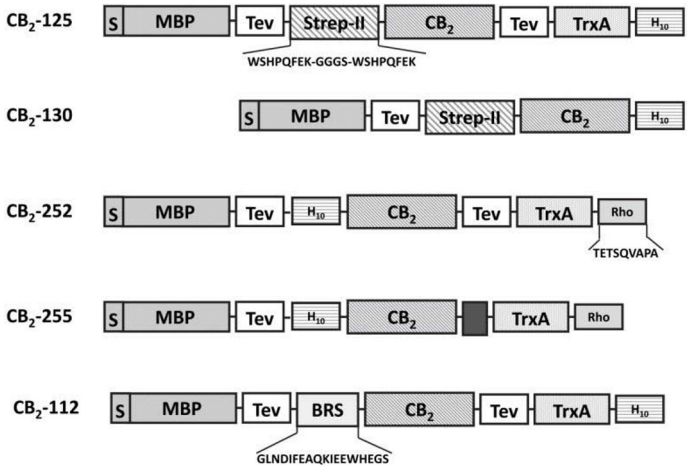

Figure 2. Schematic representation of constructs for expression of CB_2 fusion protein in *E. coli* (adapted from [22, 33, 39]).

To avoid misfolding and aggregation of the recombinant CB_2, and to ensure its expression in a functional form in *E. coli*, an appropriate N-terminal fusion partner is required [22]. For example, the N-terminal Haloalkane dehalogenase (Halotag) significantly increased the levels of the fusion CB_2 protein but a large fraction of the receptor was not functional (likely due to misfolding and aggregation) [35]. On the other hand, the use of the maltose-binding protein of *E. coli* (MBP) fused at the N-terminus of CB_2 was highly beneficial for the high-level functional expression of this receptor [21, 22, 28]. The MBP is normally localized in the periplasm of *E. coli*, and the transport of the nascent polypeptide across the cytoplasmic membrane is facilitated by the Sec translocon system that recognizes the leader sequence of MBP [40]. Importantly, the full length sequence of MBP is required for the maximal beneficial effect. Expression of CB_2 fused to only the 26 amino acid-long leader sequence resulted in a significantly lower (~50-100-fold) expression and activity suggesting that the whole-length MBP is highly beneficial to ensure the correct fold of CB_2 in *E. coli* membranes [22]. A recent study reported the use of N-terminal Mistic (from *Bacillus subtilis*) and C-terminal TarCF (fragment of bacterial aspartate chemosensory transducer) for functional expression of CB_2 in *E. coli* [31]. However, the density of ligand-binding sites in cell membranes was an order of magnitude lower than that of MBP-containing expression constructs [21].