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Application of HaloTag technology to expression and purification of cannabinoid receptor CB_2

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ABSTRACT

Expression of milligram quantities of functional, stable G protein-coupled receptors (GPCR) for high-resolution structural studies remains a challenging task. The goal of this work was to evaluate the usefulness of the HaloTag system (Promega) for expression and purification of the human cannabinoid receptor CB_2, an important target for development of drugs for treatment of immune disorders, inflammation, and pain.

Here we investigated expression in *Escherichia coli* cells of the integral membrane receptor CB_2 as a fusion with the 34 kDa HaloTag at N- or C-terminal location, either in the presence or in the absence of the N-terminal maltose-binding protein (MBP). The CB_2 was flanked at both ends by the tobacco etch virus (TEV) protease cleavage sites to allow for subsequent removal of expression partners. Expression by induction with either IPTG (in *E. coli* BL21(DE3) cell cultures) or by auto-induction (in *E. coli* KRX cells) were compared.

While the N-terminal location of the HaloTag resulted in high levels of expression of the fusion CB_2, the recombinant receptor was not functional. However, when the HaloTag was placed in the C-terminal location, a fully active receptor was produced irrespective of induction method or bacterial strain used. For purification, the fusion protein was captured onto HaloLink resin in the presence of detergents. Treatment with specific TEV protease released the CB_2 upon washing. To our knowledge, this study represents the first example of expression, surface immobilization and purification of a functional GPCR using HaloTag technology.

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Introduction

The cannabinoid receptors CB_1 and CB_2 belong to the large family of G protein-coupled receptors (GPCRs)[1] and are central to the endocannabinoid system that also includes endocannabinoid ligands as well as enzymes of their respective anabolic and catabolic pathways. CB_2 is predominantly expressed by cells of the immune system and is a primary target for the treatment of immune disorders, inflammatory diseases and pain sensing [1–4]. The primary signaling function of CB_2 appears to be the inhibition of cAMP accumulation [5]. Rational design of specific drugs for controlling function of CB_2 relies on functional and structural information on the receptor that requires highly pure preparations of correctly folded receptor.

Previously, we reported on expression of CB_2 in *Escherichia coli* membranes as a fusion with maltose binding protein (MBP), thioredoxin (TrxA) and two small affinity tags, a Strep-tag III and a polyhistidine tag [6,7]. Purification via the affinity tags placed at opposing ends of the receptor and cleavage of the fusion by tobacco etch virus protease yielded up to 0.4 mg of active CB_2 per liter of culture [7]. However, in the presence of detergents required for solubilization of CB_2, the affinity of the Strep-tag for the StrepTactin resin is relatively low resulting in substantial losses during this chromatographic step. Hence, to achieve a higher yield and purity of the receptor, optimization of purification conditions is desirable.

In order to improve the yield of CB_2 in the equilibrium-based chromatographic step, we take advantage of the HaloTag (Promega), a 34 kDa catalytically inactive derivative of Haloalkane dehalogenase from *Rhodococcus sp.* that rapidly, specifically and covalently binds to synthetic chloroalkane ligands. The HaloTag technology [8,9] relies on the binding of the Halo-tagged target protein to chloroalkane linker attached to the chromatographic resin. Since the binding of the HaloTag to the resin is virtually irreversible, an extensive washing procedure can be employed which improves the purity and yield of the target protein substantially.

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[1] *Abbreviations used:* GPCRs, G protein-coupled receptors; MBP, maltose binding protein; CHS, cholesteryl hemisuccinate Tris salt; DDM, *n*-dodecyl-β-D-maltopyranoside; gor, glutathione reductase.

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Thus, a Halo-tagged protein can be covalently immobilized on a resin, efficiently purified, and eluted from the resin upon cleaving of the fusion with TEV protease at a specific site located between the target protein and the tag.

Recombinant expression of CB_2 in bacterial (*E. coli*) cells has certain advantages over the insect- or mammalian cell-based production, including relatively low cost of fermentation, rapid cell growth, potentially high yield of recombinant protein and the ability of cells to grow in mineral salt media of defined composition. The latter is particularly important for selective labeling of CB_2 with stable isotopes required for NMR studies. While some of these features are also shared by the yeast *Pichia pastoris* expression system, the CB_2 produced in yeast was reported to be non-homogeneously glycosylated and non-functional [10]. One of the major difficulties of expressing mammalian proteins in *E. coli* cells is related to the fact that heterologous protein expression often results in low expression levels and/or poor solubility of the produced protein. These problems are frequently overcome by introducing N-terminal expression tags (i.e. MBP or HaloTag). Integral membrane proteins present a particular challenge since their expression in *E. coli* often results in formation of inclusion bodies that require subsequent refolding. This can be avoided by targeting these membrane proteins for insertion into the plasma membrane of *E. coli* by adding a tag such as MBP. This approach has been shown to yield properly folded and functional membrane proteins [11,12].

Production of recombinant proteins in *E. coli* by induction with IPTG is a widely used methodology. Typically, in a host cell such as BL21(DE3), the target protein is encoded in a plasmid under control of the T7 promoter that is recognized by T7 RNA polymerase. In turn, the expression of the chromosomal copy of T7 RNA polymerase is under control of the IPTG-inducible lacUV promoter [13,14]. However, the basal expression of RNA polymerase may result in expression of substantial levels of recombinant protein that is potentially toxic for the growing cell. Because of this leakage problem, as well as other practical considerations, an alternative method of expression of heterologous proteins in *E. coli* via auto-induction is gaining more attention. In this latter case, the inducer is present in the media from the beginning of fermentation, but the synthesis of recombinant protein is initiated only when the repressor is depleted during the course of cell growth. An important advantage of this method is that the target protein can be produced without the need to closely monitor cell density. Furthermore, while the level of expression per unit of culture density, and the solubility of recombinant proteins were comparable for both IPTG-induced and auto-induced cultures, higher culture densities as well as a higher content of target protein were reached under conditions of auto-induction [15].

To our knowledge, there is only one report on successful purification of *E. coli*-expressed, soluble proteins using the HaloTag technology [9]. In that case, expression was performed in *E. coli* strain KRX by auto-induction. KRX is an *E. coli* K strain that contains a chromosomal copy of T7 RNA polymerase gene that is tightly regulated by a rhamnose promoter ($rhaP_{BAD}$). This promoter is subject to catabolite repression by glucose, and is induced by rhamnose once glucose in the medium is consumed. This allows a precise control of T7 RNA abundance and, thereby, tight control of T7 promoter-driven production of the protein of interest [16]. Protocols for auto-induction of protein expression with rhamnose have been developed [17].

In the present study, we examine conditions for expression of a functional Halo-tagged CB_2 in *E. coli*. Our approach includes testing of constructs with the HaloTag placed at different locations in the fusion, relative to CB_2, either as a sole tag or in combination with MBP, as well as testing expression in a variety of *E. coli* strains using different induction methods. Furthermore, we examine the usefulness of the HaloTag technology for surface immobilization and purification of recombinant CB_2.

Materials and methods

Chemicals and reagents

Oligonucleotides were purchased from Eurofin MWG Operon. Restriction enzymes and DNA-modifying enzymes were obtained from New England Biolabs. HaloLink and HisLink protein purification resins and TEV protease for protein elution were from Promega. The monoclonal antibody against CB_2 (NAA-1) was from Epitomics, anti HaloTag – from Promega and anti rabbit Cy5 – from GE Helathcare. [^{35}S]-γ-GTP was purchased from Perkin-Elmer. Cholesteryl hemisuccinate Tris salt (CHS), the detergents 3[(cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) and *n*-dodecyl-β-D-maltopyranoside (DDM) were obtained from Anatrace.

Expression vectors and strains

E. coli strains DH5α and BL21(DE3) were purchased from Invitrogen, strain KRX from Promega and strain Rosetta-Gami 2(DE3) pLysS from EMD Millipore. Vectors pFC20K, pFN19K, and pFN18K were from Promega. The plasmid for expression of G_α was a kind gift from Dr. J. Northup (NIDCD, NIH). The plasmid for expression of MBP-TEV(S219V)-Arg_5 protease (pRK1043) was a gift from Dr. D.S. Waugh (NCI-Frederick, NIH).

Primers

5MCC: 5′-GGCTGCGATCGCCATGAAAATAAAAACAGGTGCACG-CATC-3′
3MCC: 5′-GATGCTCGAGGCAATCAGAGAGGTCTAGATCTCTGGA ATC-3′
5CC: 5′-GGCTGCGATCGCCATGGAGGAATGCTGGGTGACAGAG-ATA-3′
3CC: 5′-GATGGTTTAAACGCAATCAGAGAGGTCTAGATCTCTGG-3′
5CN: 5′-GGCTGCGATCGCCGAGGAATGCTGGGTGACAGAGATA-3′
3CN: 5′-GATGGTTTAAACGCAATCAGAGAGGTCTAGATCTCTGG-3′

Construction of plasmids for Halo-tagged CB_2 expression

The plasmid pAY 241 encodes human CB_2 receptor, flanked by TEV protease recognition sites and fused to the *E. coli* MBP at the N-terminus, and to haloalkan dehalogenase (HaloTag) at the C-terminus. The plasmid pAY 242 encodes human CB_2 fused to the HaloTag at the C-terminus.

The plasmid pAY 241 was constructed as follows: the 2339 bp DNA fragment encoding MBP-CB_2 was amplified from the plasmid pAY107 [1] using primers 5mCC 3MCC to add the SgfI and XhoI sites, respectively. This fragment was ligated with the 4.4-kb SgfI–XhoI-digested pFC20 K vector. The plasmid pAY242 was constructed as follows: the 1105-bp fragment encoding CB_2 was amplified from pAY107 by PCR using primers 5CC and 3CC to add SgfI and PmeI sites, respectively. The amplified fragment was ligated with 4032-bp pFC20K vector digested with SgfI and EcoICRI.

Plasmids pAY 243 and pAY 244 for expression of human CB_2 fused to the HaloTag at the N-terminus were generated by using the 1102-bp DNA fragment encoding CB_2, obtained by PCR with template pAY107 and primers 5CN and 3CN that add SgfI and PmeI sites, respectively. The fragment was ligated with the 4.4-kb SgfI PmeI- digested pFN19K or pFN18K vectors, to create pAY243 or pAY244, respectively. The resulting plasmids confer resistance to