Published in final edited form as:

Protein Expr Purif. 2013 May ; 89(1): 62–72. doi:10.1016/j.pep.2013.02.011.

Application of HaloTag Technology to Expression and Purification of Cannabinoid Receptor CB₂

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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 CB₂, 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 CB₂ 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 CB₂ 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 CB₂, 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 CB₂ upon washing. To our knowledge, this study represents the first example of expression, surface immobilization and purification of a functional GPCR using HaloTag technology.

Introduction

The cannabinoid receptors CB₁ and CB₂ belong to the large family of G protein-coupled receptors (GPCRs) and are central to the endocannabinoid system that also includes endocannabinoid ligands as well as enzymes of their respective anabolic and catabolic pathways. CB₂ 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 CB₂ appears to be the inhibition of cAMP accumulation [5]. Rational design of specific drugs for controlling function of CB₂ relies on functional

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and structural information on the receptor that requires highly pure preparations of correctly folded receptor.

Previously, we reported on expression of CB₂ in *E. 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 CB₂ per liter of culture [7]. However, in the presence of detergents required for solubilization of CB₂, 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 CB₂ 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. 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 CB₂ 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 CB₂ with stable isotopes required for NMR studies. While some of these features are also shared by the yeast *Pichia pastoris* expression system, the CB₂ 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 CB₂ in *E. coli*. Our approach includes testing of constructs with the HaloTag placed at different locations in the fusion, relative to CB₂, 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 CB₂.

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 Protein Purification System (Promega). The monoclonal antibody against CB₂ (NAA-1) was from Epitomics, anti HaloTag - from Promega and anti rabbit Cy5 - from GE Healthcare. [³⁵S]-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)-Arg5 protease (pRK1043) was a gift from Dr. D.S. Waugh (NCI-Frederick, NIH).

Primers

5MCC: 5'-GGCTGCGATCGCCATGAAAATAAAAACAGGTGCACGCATC-3'

3MCC: 5'-GATGCTCGAGGCAATCAGAGAGGTCTAGATCTCTGGAATC-3'

5CC: 5'-GGCTGCGATCGCCATGGAGGAATGCTGGGTGACAGAGATA-3'

3CC: 5'-GATGGTTTAAACGCAATCAGAGAGGTCTAGATCTCTGG-3'

5CN: 5'-GGCTGCGATCGCCGAGGAATGCTGGGTGACAGAGATA-3'

3CN: 5'-GATGGTTTAAACGCAATCAGAGAGGTCTAGATCTCTGG-3'

Construction of plasmids for Halo-tagged CB₂ expression

The plasmid pAY 241 encodes human CB₂ receptor, flanked by TEV protease recognition sites and fused to the *E. coli* MBP at the N-terminus, and to haloalkane dehalogenase (HaloTag) at the C-terminus. The plasmid pAY 242 encodes human CB₂ fused to the HaloTag at the C-terminus.

The plasmid pAY 241 was constructed as follows: the 2339 bp DNA fragment encoding MBP-CB₂ 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 pFC20K vector. The plasmid pAY242 was constructed as follows: the 1105-bp fragment encoding CB₂ 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 CB₂ fused to the HaloTag at the N-terminus were generated by using the 1102-bp DNA fragment encoding CB₂, 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 kanamycin (pAY 243) or ampicillin (pAY 244). The sequence of all plasmids described above was verified.

Expression of CB₂-fusion proteins

Expression of recombinant CB₂-fusion proteins in *E. coli* BL21(DE3) and Rosetta-Gami 2(DE3) pLysS cells was performed in double-strength YT medium supplemented with ampicillin (50 µg/ml) or kanamycin (25 µg/mL) and glucose (0.2% w/v) as described previously [6]. To aid production of functional CB₂, high affinity cannabinoid ligand CP-55,940 (2.5 µM) was added to the medium at the time of induction.

Expression of fusion proteins in the *E. coli* strain KRX was performed by auto-induction with rhamnose. After overnight incubation of cells at 37°C in LB medium containing kanamycin (25 µg/mL) or ampicillin (50 µg/mL), the cell suspension was diluted 100 times into fresh LB medium supplemented with antibiotics, glucose (0.2% w/v) and rhamnose (0.2% w/v) and incubated in shaker flasks at 20°C for 48 hours. Cell density at λ=600 nm was measured, and cells were collected by centrifugation, washed in phosphate-buffered saline, and stored at -80°C.

Preparation of membranes

Membranes from the *E. coli* cells expressing recombinant CB₂ were prepared according to our previously published protocol [18] with the following modifications: cells were collected by centrifugation at 4,500×g for 20 min, washed once with a cold PBS buffer, centrifuged again, and re-suspended in a small volume (3–5 mL) of cold TBS buffer supplemented with protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), MgCl₂ (5 mM) and DNase I ((10 µg/mL), Sigma, St. Louis, MO). Cells were disrupted by passing the cell paste through a French Press (Thermo Fisher, Pittsburgh, PA). Unbroken cells and cell debris were removed by centrifugation at 15,000 rpm (Beckman 90Ti) for 30 min, and the supernatant was subjected to high-speed centrifugation (70,000 rpm, Optima TLA 100.4, 1 h). The resulting membrane pellet was washed with PBS and re-suspended in a small volume of PBS buffer supplemented with protease inhibitor cocktail and sucrose (20% w/v). All procedures were performed on ice or at 4°C. Aliquots were flash-frozen in liquid nitrogen and stored at -80 °C until use.

Protein analysis

Recombinant CB₂ fusion proteins were detected by Western blot with rabbit anti-CB₂ monoclonal antibody (1:2000) or rabbit anti-HaloTag antibody (1:4000, Promega). The Western blot was developed with anti-rabbit Cy5 antibody (1:2500) and visualized on a Typhoon 8600 fluorescent scanner (GE Healthcare). Protein concentration in the presence of detergents was determined using the DC Protein Assay Reagent (BioRad), using BSA as protein standard.

Functional characterization of CB₂ receptor

Activation of G proteins by recombinant CB₂ in *E. coli* membranes was tested as reported previously [6]. The amount of membrane suspension used per assay corresponds to a total protein content of 2 µg. When two different types of membranes were tested jointly, each of them contributed 2 µg of total protein. For tests with HaloTag protein, 40 ng of the purified HaloTag protein per assay were added to the reaction mix. Membranes containing temperature-inactivated CB₂-130 were obtained by incubating CB₂-130-containing membranes at 75 °C for 1 h.

The G protein components of the assay, Gα_i and Gβ₁γ₂ were expressed in *E. coli* cells and in Sf9 cells, respectively, and purified according to procedures previously published [19] and [20].

Purification of TEV protease

MBP-TEV(S219V)-Arg₅ was expressed in BL21(DE3) cells and purified by amylose affinity chromatography followed by cation exchange chromatography as described [21-23].

TEV protease cleavage of fusion CB₂ in *E. coli* membranes

Membranes were solubilized to a final concentration of 5 mg/mL of total protein in a buffer containing Tris (50 mM, pH 8), EDTA (5 mM) and DTT (1 mM). Aliquots were kept on ice or incubated either in the absence or in the presence of 1 µg TEV protease per 50 µg of membrane protein. In another format of the experiment 1 µg of TEV protease was used per 5 µg of total protein for 1 h at 25 °C. Samples containing 50 µg of total membrane protein were loaded on a gel for Western blot analysis.

Solubilization of CB₂ fusion protein

The procedure for solubilization of CB₂-fusion proteins from *E. coli* cells is based on a previously described protocol [7] with some modifications. All steps were performed on ice or at 4°C. Frozen cells were homogenized in TBS buffer supplemented with EDTA-free protease-inhibitor cocktail (Roche) in a blender. After addition of MgCl₂ (5 mM), DNase I (10 µg/mL) and anti-foam (Sigma), the mixture was passed two times through a cell homogenizer (Avestin) and supplemented with the buffer to a final concentration of 50 mM Tris-HCl pH 7.5, 200 mM NaCl, 30% glycerol, 0.5% (w/v) 3[(cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 0.1% (w/v) cholesteryl hemisuccinate Tris salt (CHS), and 1% (w/v) n-dodecyl-β-d-maltopyranoside (DDM) (buffer A), and a stabilizing ligand CP-55,940 (10 µM). After stirring the extract for 40-60 min at 4°C, cell debris was removed by centrifugation (Beckman 70Ti rotor, 45,000 rpm, 1 h, 4°C), supernatants were combined and the resulting solution was passed through a 0.2-µm filter.

Purification of Halo-tagged CB₂

Biomass of *E. coli* BL21 cells expressing CB₂-241 from 2.5 L of culture was harvested, cells disrupted, and the receptor extracted at a final volume of 400 mL as described in the previous section. HaloLink resin was equilibrated in buffer A supplemented with 10 µM

CP-55,940. The resin (2.5 mL) was incubated with 200 mL of the extract at 4 °C either for 2 hours (protocol 1) or overnight (protocol 2), and collected by centrifugation (1000× g, 4 °C, 10 min). Washes were performed with 10 column volumes of buffer A+ 10 μM CP-55,940. Afterwards, the resin was incubated with TEV protease (Promega, 150 μl in 2.5 ml of buffer A+ 10 μM CP-55,940) overnight at 4 °C (protocol 1) or for 1 h at room temperature (protocol 2). The supernatant was separated by centrifugation (5000× g, 5 min) and the resin washed with buffer A+CP-55,940. Both supernatants were combined (Elu 1). In order to remove TEV protease, 125 μL of HisLink resin were added and incubated for 1 h at 4 °C (protocol 1) or for 30 min at room temperature (protocol 2). Eluted protein was separated from the resin by centrifugation (Elu 2).

Results

In order to examine the usefulness of the HaloTag technology for expression and purification of CB₂, several expression conditions including two types of *E. coli* cells, two different induction methods and four fusion constructs with or without MBP and with alternating locations of the HaloTag with respect to CB₂ were tested. Performance was evaluated in terms of cell density of cultures, expression levels of fusion proteins, accessibility of HaloTag, and ability of the produced CB₂ to activate G proteins. Further, the efficiency of TEV protease cleavage was tested and CB₂ purification performed.

The constructs for expression of CB₂ are schematically presented in Fig. 1. Four constructs were prepared in which CB₂ was fused to HaloTag either at the C-terminus (CB₂-241 and CB₂-242) or at the N-terminus (CB₂-243 and CB₂-244). Furthermore, CB₂-241 contains an MBP tag at the N-terminal end. TEV protease recognition sites flanking the CB₂ sequence were also introduced. As positive controls for CB₂ expression levels and activity we used constructs CB₂-130 and CB₂-125 [7].

The *E. coli* strains BL21(DE3) and KRX were used as hosts for expression. Expression of Halo-tagged CB₂ in BL21(DE3) cells was performed by induction with 0.5 mM IPTG at 20 °C as previously described [6]. Expression in KRX cells required optimization which is described below.

Optimization of expression of Halo-tagged CB₂ in KRX cells by auto-induction

Optimization of expression was performed by varying the concentration of glucose as well as time and temperature of cultivation (Fig. 2). The different expression conditions were evaluated for final cell density (OD₆₀₀) and CB₂ expression levels (by Western blot). Cultivation was performed either at 25 °C (optimal temperature for auto-induction in KRX cells) or at 20 °C (previously determined to yield the largest amount of functional CB₂ [18]).

When a protocol for early-stage auto-induction (0.05% glucose) was used, relatively low culture densities were reached (OD₆₀₀=1-1.5) independent of the temperature of cultivation (Fig. 2A). Maximal protein expression after induction was reached after 48 hours of incubation at 20 °C, while at 25 °C the maximal levels were reached already after 24 hours (Fig. 2C). The late auto-induction protocol (0.15 % glucose) led to higher optical densities of culture (OD₆₀₀= 2.5-3). A decrease in temperature from 25 °C to 20 °C resulted in a slight delay in biomass accumulation and in a lower final OD₆₀₀ value (Fig. 2B). Comparable expression levels of CB₂ were reached after 48 hours of fermentation at both temperatures (Fig.2D). Increasing the glucose concentration (0.15, 0.20 and 0.25%) resulted in maximal readings after 48 hours of cultivation in all cases. The density of cultures increased progressively with increasing glucose concentration (Fig. 2E). Therefore, subsequent experiments on CB₂ expression in KRX cells were performed at 20 °C for 48 hours using a late auto-induction protocol with 0.2% glucose in the medium.

Expression of Halo-tagged CB₂ in *E. coli* cells

Expression of Halo-tagged CB₂ was performed by induction with IPTG in BL21(DE3) and by auto-induction with rhamnose in KRX cells under similar aeration conditions. After harvesting, cell culture density was measured as OD₆₀₀ (Table 1). An increase in biomass was observed for CB₂-241 under auto-induction (average OD₆₀₀= 3.7) compared to IPTG induction (average OD₆₀₀= 2.2). For constructs CB₂-242 and CB₂-243, no significant difference in culture density between IPTG- and auto-induction protocols were observed, whereas for CB₂-244, the auto-induction method appeared to be detrimental for biomass production (average OD₆₀₀= 5.3 for IPTG and 3.1 for auto-induction).

Expression levels of Halo-tagged CB₂ were evaluated by Western blotting analysis of membrane preparations with monoclonal anti-CB₂ antibody (Fig. 3A) and results quantified by densitometry (Table 1). Among the four expression constructs tested, the CB₂-242 had the lowest levels of expression in both BL21(DE3) and KRX cells. The fusion with MBP at the N-terminus of CB₂ (CB₂-241) resulted in a dramatic increase of expression levels in both types of cells. Likewise, switching the HaloTag from the C- to the N-terminal location resulted in higher expression levels with the following distinctions: CB₂ expressed with construct 244 showed a similar level of expression as CB₂-241 in both types of cells, whereas construct CB₂-243 yielded a moderate expression level in KRX cells and a remarkably high levels (the best of all constructs tested) in BL21(DE3) cells. This intriguing difference in expression levels of construct CB₂-243 between the IPTG-induced and auto-induction systems, and in the expression pattern of CB₂-243 in comparison to CB₂-244 is probably the result of differences in construction of expression plasmids 243 and 244; they differ in antibiotic resistance genes they encode (Km vs Amp) as well as in the arrangement of promoters driving the expression of the target protein.

In addition, expected yields of CB₂ per liter of culture were calculated as presented in Table 1. These results are in reasonable agreement with the results presented above for expression levels.

In order to test the accessibility of the HaloTag for subsequent purification of the fusion protein, the Western blotting analysis with an anti-HaloTag antibody was performed (Fig. 3B). While this antibody appears to be less sensitive than anti-CB₂ antibody, the overall pattern of yield of fusion proteins reproduced results for detection with anti-CB₂ antibody, independent of the location of the tag.

Taken together these results suggest that while the HaloTag is accessible from both ends of the fusion protein, high expression levels of CB₂ receptor can only be achieved when a solubility tag (either MBP or Halo) is placed at the N-terminal end of the receptor.

Functional activity of CB₂

To evaluate the structural integrity of Halo-tagged CB₂ in *E. coli* membranes, a test of G protein activation was performed (Fig. 4). The assay involves measurement of the rates of activation of heterotrimeric G protein (Gα_{i1}β₁γ₂) on CB₂-containing membranes pre-treated with high affinity, synthetic cannabinoid agonist CP-55,940. The assay measures the binding of the non-hydrolyzable analogue of GTP, [³⁵S] GTPγS to the activated Gα_{i1} subunit. Based on the activity values, as well as on expression levels of CB₂ fusion proteins determined by the semi-quantitative Western blotting on membrane preparations, specific activities were calculated for each expression construct. They are presented as a fraction of specific activity of CB₂-130 (that was set as 100%, see Table 1).

We observed that the activity pattern of membranes containing C-terminal HaloTag (CB₂-241 and CB₂-242) fusion protein (Fig. 4) closely reflected the differences of

expression levels of CB₂ (Fig. 3A.). This observation also holds for expression in either BL21 or KRX where each C-terminal Halo-tagged construct had similar levels of specific activity, ~30% for CB₂-241 and ~20% for CB₂-242 (Table 1). In contrast, the activity pattern of N-terminal Halo-tagged CB₂ proteins significantly diverged from the corresponding expression pattern: the well-expressed CB₂-243 and CB₂-244 exhibited only low levels of specific activity of less than 2% compared to the positive control. The biggest differences between the expression yield and G protein activation capacity was found for the construct CB₂-243 expressed in BL21(DE3) membranes: it had the highest level of expression of all four fusion constructs tested, but its specific activity was only ~3.5% of that of the positive control.

Taken together, these results suggest that the N-terminal location of the HaloTag is disadvantageous for production of active receptor. On the other hand, a C-terminal location of the Halo-tag results only in a moderate reduction in activity (perhaps due to more limited access of G proteins to the C-terminal part of the CB₂-HaloTag fusion) but the expression levels of this CB₂ fusion are relatively low. Both expression levels and activity are improved significantly with the addition of an MBP tag to the N-terminus of the CB₂-HaloTag fusion protein.

Effect of N-terminal HaloTag on activity of CB₂

To ascertain whether the low activity of N-terminal Halo-tagged CB₂ is caused by the incorrect folding of fusion protein rather than by the interference of the HaloTag fusion partner (34 kDa) with activation of G proteins by the recombinant receptor, a series of experiments were performed.

While the N-terminal HaloTag is not likely to directly interfere with interaction of the receptor with G proteins, it still may affect the ability of the receptor to undergo a conformational change upon binding of the agonist. To test for this possibility, we measured the G protein activation by CB₂ in membranes after removing the HaloTag, by treatment with specific TEV protease. Removal of the HaloTag from CB₂ in constructs 243 and 244 did not result in an increase of activation rates (Fig. S1), indicating that the presence of this tag at the N-terminus yields nonfunctional receptor. This is probably the result of incorrect folding of the receptor.

Formation of disulfide bonds is considered to be an important factor for stabilization of GPCRs in a functionally relevant conformation [24]. While N-terminally MBP-tagged CB₂ is inserted into cytoplasmic membranes of *E. coli* BL21(DE3) with regions requiring disulfide bond formation exposed to the periplasm [25], the N-terminal-Halo-tagged CB₂ lacks the signal sequence and may not get properly folded in membranes, undergoing folding in the cytoplasm. However, the experimental procedures used in this study cannot separate the correctly folded receptor from misfolded receptor associated with membrane fractions. The oxidized redox environment of the periplasmic space of *E. coli* favors formation of disulfide bonds, while reductive conditions in the cytoplasm of *E. coli* prevent their formation. Thus, the lack of a proper redox environment may contribute to accumulation of non-functional N-terminally, Halo-tagged CB₂ in both BL21(DE3) and KRX cells.

To aid formation of disulfide bridges, expression of CB₂-243 and CB₂-244 was attempted in *E. coli* Rosetta-Gami 2(DE3) pLysS. A combination of mutations in both thioredoxin reductase (*trxR*) and glutathione reductase (*gor*) in this strain enhances disulfide bond formation in the cytoplasm. However, no improvement in activity of CB₂ compared to membranes prepared from BL21(DE3) or KRX cells was observed, suggesting that

(presumed) incorrect folding of the receptor may be the result of factors different from an inadequate redox environment in the host cell (Fig. S2).

To examine whether the presence of the HaloTag and/or of misfolded CB₂ affects the activation of G protein on CB₂, we tested the effect of the purified HaloTag protein, membranes containing thermally denatured fusion CB₂-130 or fusion protein CB₂-243 on activation rates by CB₂-130 (Fig. 5). The addition of purified HaloTag protein (Promega) to CB₂-130 membranes caused a very slight decrease in activation rates (by ~10%). However, an addition of membranes containing thermally inactivated CB₂-130 decreased the activation rates by 24 %. The addition of CB₂-243 membranes resulted in a significant drop in activation rates (by 43 %).

Altering the content of membranes in a reaction mixture may affect the partitioning of amphiphilic G proteins between aqueous and lipidic phases, which in turn may change the rates of nucleotide exchange on a G_{αi} [26]. To account for that, empty BL21(DE3) membranes were added to the control assay resulting in a reduction of activation rates by only 7%. On the other hand, purified HaloTag protein (in the absence of any CB₂) did not cause any measurable increase in nucleotide exchange on G protein.

These results suggest that the addition of misfolded CB₂ to the reaction may have a negative impact on the rates of activation of G proteins by the correctly folded receptor, irrespective of the presence of a HaloTag¹.

In summary, the low levels of specific activity of N-terminal Halo-tagged CB₂ are probably the result of the presence of misfolded receptor in membrane preparations. While the underlying cause for misfolding is not yet clear, our data indicate that the (likely) misfolded HaloTag-CB₂ reduces the rates of G-protein activation, similarly to the effect of thermally inactivated CB₂-130. It is possible that such non-functional receptor retains the ability of binding G proteins while not stimulating nucleotide exchange on a G_α subunit, thus depleting the pool of G proteins available for activation by the functional receptor.

Cleavage of the HaloTag-fusion protein in membranes by TEV protease

CB₂ is released from the fusion by treatment with specific TEV protease at recognition sites located between CB₂ and the fusion partners (Fig. 6). Cleaving of CB₂-241 (MBP-CB₂-HaloTag, 113 kDa) can generate the following fragments detectable with anti-CB₂ antibody: 79 kDa (MBP-CB₂), 71 kDa (CB₂-HaloTag), and 37 kDa (corresponding to untagged CB₂). The presence of the 37 kDa band confirms that at least part of the fusion protein (estimated 50%) is cleaved at both sites. Irrespective of the amounts of TEV protease used in the reaction, bands of 113 kDa and 71 kDa were completely absent from the reaction products indicating that cleavage between CB₂ and the HaloTag was complete. On the other hand, the TEV protease site located between the MBP and CB₂ appears to be more resistant to cleavage since the 79 kDa fragment was still present among the reaction products even after treatment with high amounts of protease. Please note that the control construct CB₂-130 containing one TEV recognition site undergoes only partial (~50%) cleavage probably due to random orientation of the protein in the membranes of vesicles and the inability of TEV protease to penetrate vesicles.

¹This observation suggests the following interpretation of the data presented in figures S1 and S2. The rates of nucleotide exchange on G protein measured in reactions in which no membranes were used (background) are higher than the rates measured in preparations containing CB₂-243 and CB₂-244 membranes. This, most likely, is caused by the tight binding of G-protein to misfolded CB₂ resulting in a net decrease of G_{αi1} subunit bound to ³⁵S-γ-GTP.

Use of HaloTag for purification of CB₂

We evaluated the feasibility of HaloTag technology for purification of CB₂. The purification protocol suggested by the manufacturer consists of the following steps: binding the Halo-tagged protein to the HaloLink resin, elution of the target protein by treatment with TEV protease, and subsequent removal of His-tagged TEV protease by HisLink resin. BL21 cells expressing CB₂-241 were cultured and treated as described in Materials and Methods. Two purification protocols were compared (Fig. 7) that differ in the time of exposure of cell lysate to HaloLink resin, the temperature and duration of treatment with TEV protease, as well as the duration of binding to the HisLink resin. In protocol 1, the fusion CB₂ from the cell extract was captured on a resin during a 2-hour incubation and all purification steps performed at 4°C. In protocol 2, capture of CB₂ to the HaloLink resin was performed during an overnight incubation and treatment with TEV protease and subsequent removal of protease by HisLink resin performed at room temperature as recommended by the manufacturer.

Protocol 1 appeared to be more efficient in capturing the recombinant protein, as less CB₂ fusion was detected in the corresponding flow-through (estimated 83% from load in protocol 1 vs. 88% in protocol 2). Overnight exposure to the resin (protocol 2) was detrimental for binding, probably due to the presence of detergents and/or glycerol in the buffer. However, protocol 2 appears to be more effective in yielding large quantities of purified CB₂ because of the more efficient cleavage by TEV protease at room temperature, as shown by Western blot (Fig. 7). However, even in that case, only ~20 % of the fusion protein was cleaved, and the estimated yield of the purified receptor was quite low (~6.5 µg/L of culture), corresponding to ~2.5 % overall recovery of the recombinant receptor.

In summary, higher yields of purified receptor can be achieved by combining features of both protocols. Fusion CB₂ should be captured from the crude extract at 4°C by incubation with the HaloLink resin for ~2 hours, while the release of CB₂ from the resin can be performed by treatment with TEV protease at room temperature to increase the efficiency of cleavage. It is clear that certain further improvement of the purification procedure can be attempted, in particular, optimizing cleavage of the resin-immobilized fusion CB₂ with TEV protease. Our results also indicate that capturing of the fusion protein from crude cell extracts onto the HaloLink resin is currently not very efficient. Estimates show that only ~17% of the CB₂ fusion protein from the crude extract was captured onto HaloLink resin while less than 1% of the expected binding capacity of the resin was utilized. This severely limits the application of the HaloLink resin as a first chromatography step for a large-scale purification of CB₂. However, the use of the HaloTag technology for surface immobilization of CB₂ still appears attractive since the haloalkane-functionalized surface can be used for rapid purification of the recombinant protein as well as for subsequent biochemical and biophysical studies that require moderate (microgram) quantities of pure, functional receptor.

Discussion

In this work we examined the application of the HaloTag technology for expression and purification of cannabinoid receptor CB₂, a class A GPCR. Evaluation of expression parameters focused on conditions favoring accumulation of functional receptor in cytoplasmic membranes of host *E. coli* cells. Several plasmid constructs encoding fusion of CB₂ with HaloTag and MBP, several *E. coli* strains as well as induction conditions were tested. Furthermore, we attempted purification of CB₂ using HaloLink resin and evaluated conditions yielding highly pure and functional receptor.

In particular, we compared the effect of the relative position of the HaloTag and the presence of the N-terminal MBP tag on expression levels and activity of the CB₂-fusion protein. N-terminal Halo-tagged constructs (CB₂-243 and CB₂-244) showed significant expression levels but disproportionally low specific activity. While the expressed fusion protein was associated with membranes of *E. coli*, subsequent removal of the HaloTag by treatment with TEV protease did not increase the activation rates of G proteins indicating possible misfolding of the recombinant receptor. Furthermore, the attempted expression of the fusion CB₂ in *E. coli* strain engineered to enhance formation of disulfide bond failed to improve the yield of functional receptor, suggesting that the N-terminal location of the HaloTag may be detrimental for function. Thus, the low levels of specific activity of N-terminal Halo-tagged CB₂ are probably a consequence of the presence of misfolded receptor, which reduces the rates of G-protein activation by depleting the pool of G proteins available for activation by the functional receptor.

On the other hand, positioning of the HaloTag at the C-terminus of CB₂ (constructs 241 and 242) significantly improved specific G protein activation rates. Out of four constructs tested in this work, construct CB₂-241 encoding the fusion protein MBP-CB₂-HaloTag combined high expression levels with high specific activity of the receptor while the construct CB₂-HaloTag also had high specific activity but expressed at a much lower level. In summary, while the HaloTag may result in high expression levels of CB₂, the N-terminal location of this tag is detrimental for functional activity of the recombinant receptor, presumably by producing misfolded receptor. Although the C-terminal position of the HaloTag resulted in low expression levels of the fusion protein, it did not adversely affect functional activity of CB₂. Therefore, a combination of two tags: N-terminal MBP and the C-terminal HaloTag emerge as optimal for high-level expression of functional CB₂ for subsequent surface immobilization.

Our data in part support previously published reports of expression of 19 recombinant proteins in *E. coli*, where the HaloTag was placed as the N-terminal fusion partner [9] and demonstrated to be superior to other tags in enhancing levels and solubility of recombinant proteins. In terms of activity, only 2 of the expressed proteins were tested and both were functionally active, but in the same report, additional 6 proteins (among them integral membrane receptors) failed to express as a soluble fraction. Motejaded et al. [27] demonstrated successful expression of an N-terminal Halo-tagged lipase that retained activity after immobilization on magnetic beads. However, in that case MBP was present as an additional N-terminal fusion partner to the Halo-lipase protein. Full-length methyl transferase CARM1 was obtained in active form from mammalian HEK293 cells as both N- and C-terminal HaloTag fusion [28]. However, expression of the N-terminal Halo-tagged CARM1 in *E. coli* yielded mostly insoluble protein. The small portion of protein that remained in the soluble fraction was truncated at the C-terminal end but retained activity. In agreement with the report of Ohana et al. [9], in our studies N-terminal location of the HaloTag was beneficial for high expression levels of CB₂ in *E. coli* but not for functional activity.

In addition to the study of the effect of C-terminally located HaloTag on expression levels and function of methyl transferase CARM1, Ohana et al. [29] reported successful expression and purification in active form of five Halo-tagged kinases in mammalian HEK293 cells. At the same time, the only successful example of C-terminal Halo-tagged proteins expressed in *E. coli* was published by Erkelenz et al. [30]. In that work, two subdomains of the soluble cytochrome P450 BM3 enzyme fused to HaloTag were purified by anion exchange chromatography and bioconjugated with chloroalkane-modified oligonucleotide.

Here we demonstrated that while the expression levels of C-terminal Halo-tagged CB₂-242 were low, the fusion protein activated G proteins in response to the agonist binding suggesting that the C-terminal HaloTag is compatible with a functional fold of the receptor. It has to be taken into account that the interpretation of activity measurements on CB₂ by G protein activation assay depends on an accessibility of the C-terminal region of the receptor for interaction with G proteins. The positive control used in our study (CB₂-130) carries only a small C-terminal affinity tag that was shown not to interfere with interaction of CB₂ with G proteins [26]. On the other hand, the HaloTag fused to the C-terminus of CB₂ is a much larger fusion partner that can partially block the site of interaction of receptor with G proteins. Somewhat reduced rates of activation of G proteins on CB₂-241 may be indicative of such partial blockage rather than unfavorable effect of the C-terminal HaloTag on the fold of the receptor.

Part of our work was devoted to optimizing expression conditions for fusion CB₂ proteins in *E. coli* strains BL21 (DE3) and KRX. Accumulation of biomass, expression levels of different constructs, CB₂ yield per liter of culture, and specific activity of proteins produced by auto-induction in KRX cells [17] or by IPTG-induction in BL21(DE3) cells were compared (Table 1). For constructs without the N-terminal MBP tag the auto-induction method did not improve biomass production compared to IPTG induction. While in the case of MBP-CB₂-HaloTag (CB₂-241) the accumulation of biomass was higher for auto-induced KRX compared to the IPTG induced cells, this did not result in a higher amount of target protein per volume of culture. Thus, both types of cells/ methods of induction appear to be similarly suitable for expression of Halo-tagged CB₂.

Towards purification of CB₂ by HaloTag technology

A prerequisite for successful purification of the expressed fusion protein is the efficient cleavage by the TEV protease to elute purified CB₂ from the HaloLink resin. We evaluated this by using membrane preparations of BL21(DE3) or KRX cells expressing fusion CB₂. TEV protease cleavage of CB₂-241 in *E. coli* membranes generated two fragments, corresponding in size to MBP-CB₂ and CB₂, respectively, demonstrating that CB₂ is efficiently separated from HaloTag upon cleavage. However, in membranes cleavage between the MBP and CB₂ occurs only partially, probably due to a limited access of the TEV protease to this cleavage site. However, this limitation is unlikely to matter for purified MBP-CB₂ fusion protein that was shown to be efficiently cleaved by TEV protease in detergent-containing buffers [7].

Our experiments demonstrate that the construct CB₂-241 was expressed at moderately high levels, the accumulated fusion protein was correctly folded as shown by G protein-activation, the HaloTag was accessible for interaction with the affinity resin, and the CB₂ was released from the fusion upon cleavage with specific TEV protease. However, the binding of Halo-tagged-CB₂ to the HaloLink resin was found to be not very efficient in detergent-containing crude cell extracts, although an excess of resin and sufficient time for binding were given. One likely cause for low binding is the presence of detergents that interfere with interaction of HaloTag protein with the chloroalkane in the resin. Indeed, according to the manufacturer's manual, CHAPS at the concentration used in this study may promote non-specific binding of contaminants to the resin, thereby reducing the effective capacity for Halo-tagged proteins. However, our system is optimized for the use of a mixture of DDM, CHS and CHAPS to assure solubility and stability of CB₂, and these detergents cannot be easily replaced. Furthermore, it is possible that the use of the HaloLink resin as the first step of purification is not optimal since the relative content of the CB₂ fusion in the extract is quite low and other components of the extract may non-specifically interact with the resin and reduce its binding capacity. Thus, a pre-purification step (perhaps

using yet another affinity tag) may be desirable prior to capturing the fusion CB₂ onto the HaloLink resin.

As expected, detergents did not affect the strength of the HaloTag-chloroalkane interaction since extensive washing did not remove captured CB₂ fusion protein from the resin. The treatment with TEV protease was found to be more efficient at room temperature than at 4°C but even in that case the estimated cleavage efficiency was only ~20 %. There might be certain properties of the HaloLink resin that adversely affect the TEV protease cleavage site on fusion CB₂, perhaps by physically limiting access of the protease to the cleavage site on the fusion. It remains to be seen whether new modifications of the resin would be better compatible with the proteolytic cleavage of the captured fusion protein.

In summary, the application of the current protocol to a large-scale purification of CB₂ is not very practical due to relatively low yield of the target protein. However, further optimization of the purification strategy including pre-purification of the fusion protein prior to its capture onto the HisLink resin, and development of resins better compatible with efficient TEV proteolytic cleavage may increase the yield substantially.

Since its introduction, HaloTag technology has been used predominantly for applications in imaging [8, 31-35] and for applications that require protein immobilization on a solid support [36-38]. To a lesser extent, HaloTag has been used for protein purification [9, 28, 29]. Here we have established conditions for expression of the Halo-tagged G protein coupled CB₂ receptor in *E. coli* and demonstrated feasibility of its immobilization and purification.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the Intramural Research Program of the NIAAA, NIH. Authors wish to thank Dr. R. Ohana (Promega Corporation) for gift of materials and helpful suggestions.

References

- [1]. Munro S, Thomas KL, Abu-Shaar M. Molecular characterization of a peripheral receptor for cannabinoids. *Nature*. 1993; 365:61–65. [PubMed: 7689702]
- [2]. Buckley NE. The peripheral cannabinoid receptor knockout mice: an update. *Br J Pharmacol*. 2008; 153:309–318. [PubMed: 17965741]
- [3]. Basu S, Abhyankar A, Kand P, Kumar R, Asopa R, Rajan MG, Nayak U, Shimpi H, Das T, Venkatesh M, Chakrabarty S, Banerjee S. ‘Reverse discordance’ between 68Ga-DOTA-NOC PET/CT and 177Lu-DOTA-TATE posttherapy scan: the plausible explanations and its implications for high-dose therapy with radiolabeled somatostatin receptor analogs. *Nucl Med Commun*. 2011; 32:654–658. [PubMed: 21654355]
- [4]. Console-Bram L, Marcu J, Abood ME. Cannabinoid receptors: nomenclature and pharmacological principles. *Prog Neuropsychopharmacol Biol Psychiatry*. 2012; 38:4–15. [PubMed: 22421596]
- [5]. Felder CC, Joyce KE, Briley EM, Mansouri J, Mackie K, Blond O, Lai Y, Ma AL, Mitchell RL. Comparison of the pharmacology and signal transduction of the human cannabinoid CB1 and CB2 receptors. *Mol Pharmacol*. 1995; 48:443–450. [PubMed: 7565624]
- [6]. Yeliseev AA, Wong KK, Soubias O, Gawrisch K. Expression of human peripheral cannabinoid receptor for structural studies. *Protein Sci*. 2005; 14:2638–2653. [PubMed: 16195551]

- [7]. Yeliseev A, Zoubak L, Gawrisch K. Use of dual affinity tags for expression and purification of functional peripheral cannabinoid receptor. *Protein Expr Purif.* 2007; 53:153–163. [PubMed: 17223358]
- [8]. Los GV, Encell LP, McDougall MG, Hartzell DD, Karassina N, Zimprich C, Wood MG, Learish R, Ohana RF, Urh M, Simpson D, Mendez J, Zimmerman K, Otto P, Vidugiris G, Zhu J, Darzins A, Klaubert DH, Bulleit RF, Wood KV. HaloTag: a novel protein labeling technology for cell imaging and protein analysis. *ACS Chem Biol.* 2008; 3:373–382. [PubMed: 18533659]
- [9]. Ohana RF, Encell LP, Zhao K, Simpson D, Slater MR, Urh M, Wood KV. HaloTag7: a genetically engineered tag that enhances bacterial expression of soluble proteins and improves protein purification. *Protein Expr Purif.* 2009; 68:110–120. [PubMed: 19464373]
- [10]. Feng W, Cai J, Pierce WM Jr, Song ZH. Expression of CB2 cannabinoid receptor in *Pichia pastoris*. *Protein Expr Purif.* 2002; 26:496–505. [PubMed: 12460775]
- [11]. Szmecman S, Clement JM, Jehanno M, Schwartz O, Montagnier L, Hofnung M. Export and one-step purification from *Escherichia coli* of a MalE-CD4 hybrid protein that neutralizes HIV in vitro. *J Acquir Immune Defic Syndr.* 1990; 3:859–872. [PubMed: 1696629]
- [12]. Bertin B, Freissmuth M, Breyer RM, Schutz W, Strosberg AD, Marullo S. Functional expression of the human serotonin 5-HT_{1A} receptor in *Escherichia coli*. Ligand binding properties and interaction with recombinant G protein alpha-subunits. *J Biol Chem.* 1992; 267:8200–8206. [PubMed: 1533220]
- [13]. Studier FW, Moffatt BA. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J Mol Biol.* 1986; 189:113–130. [PubMed: 3537305]
- [14]. Studier FW, Rosenberg AH, Dunn JJ, Dubendorff JW. Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.* 1990; 185:60–89. [PubMed: 2199796]
- [15]. Studier FW. Protein production by auto-induction in high density shaking cultures. *Protein Expr Purif.* 2005; 41:207–234. [PubMed: 15915565]
- [16]. Hartnett J, Gracyalny J, Slater MR. The single stem (KRX) competent cells: efficient cloning and high protein yields. *Promega Note.* 2006; 94 <http://www.promega.com/~media/files/resources/promega%20notes/94/the%20single%20step%20competent%20cells-%20efficient%20cloning%20and%20high%20protein%20yields.pdf?la=en>.
- [17]. Schagat T, Ohana RF, Otto P, Hartnett J, Slater M. KRX autoinduction protocol: a convenient method for protein expression. *Promega Note.* 2008; 98 <http://www.promega.com/~media/files/resources/promega%20notes/98/krx%20autoinduction%20protocol-%20a%20convenient%20method%20for%20protein%20expression.pdf?la=en>.
- [18]. Berger C, Ho JT, Kimura T, Hess S, Gawrisch K, Yeliseev A. Preparation of stable isotope-labeled peripheral cannabinoid receptor CB2 by bacterial fermentation. *Protein Expr Purif.* 2010; 70:236–247. [PubMed: 20044006]
- [19]. Mumby SM, Linder ME. Myristoylation of G-protein alpha subunits. *Methods Enzymol.* 1994; 237:254–268. [PubMed: 7935001]
- [20]. Glass M, Northup JK. Agonist selective regulation of G proteins by cannabinoid CB(1) and CB(2) receptors. *Mol Pharmacol.* 1999; 56:1362–1369. [PubMed: 10570066]
- [21]. Cherry, S.; Tropea, JE.; Waugh, DS. Expression and purification of His-TEV(S219V)-Arg. http://mc11.ncifcrf.gov/qaugh_tech/protocols/pur_histev.pdf
- [22]. Kapust RB, Waugh DS. *Escherichia coli* maltose-binding protein is uncommonly effective at promoting the solubility of polypeptides to which it is fused. *Protein Sci.* 1999; 8:1668–1674. [PubMed: 10452611]
- [23]. Kapust RB, Tozser J, Fox JD, Anderson DE, Cherry S, Copeland TD, Waugh DS. Tobacco etch virus protease: mechanism of autolysis and rational design of stable mutants with wild-type catalytic proficiency. *Protein Eng.* 2001; 14:993–1000. [PubMed: 11809930]
- [24]. Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Le Trong I, Teller DC, Okada T, Stenkamp RE, Yamamoto M, Miyano M. Crystal structure of rhodopsin: A G protein-coupled receptor. *Science.* 2000; 289:739–745. [PubMed: 10926528]
- [25]. Krepiy D, Wong K, Gawrisch K, Yeliseev A. Bacterial expression of functional, biotinylated peripheral cannabinoid receptor CB2. *Protein Expr Purif.* 2006; 49:60–70. [PubMed: 16621595]

- [26]. Vukoti K, Kimura T, Macke L, Gawrisch K, Yeliseev A. Stabilization of functional recombinant cannabinoid receptor CB(2) in detergent micelles and lipid bilayers. *PLoS One*. 2012; 7:e46290. [PubMed: 23056277]
- [27]. Motejadded H, Kranz B, Berensmeier S, Franzreb M, Altenbuchner J. Expression, one-step purification, and immobilization of HaloTag(TM) fusion proteins on chloroalkane-functionalized magnetic beads. *Appl Biochem Biotechnol*. 2010; 162:2098–2110. [PubMed: 20473582]
- [28]. Chumanov RS, Kuhn PA, Xu W, Burgess RR. Expression and purification of full-length mouse CARM1 from transiently transfected HEK293T cells using HaloTag technology. *Protein Expr Purif*. 2011; 76:145–153. [PubMed: 21126607]
- [29]. Ohana RF, Hurst R, Vidugiriene J, Slater MR, Wood KV, Urh M. HaloTag-based purification of functional human kinases from mammalian cells. *Protein Expr Purif*. 2011; 76:154–164. [PubMed: 21129486]
- [30]. Erkelenz M, Kuo CH, Niemeyer CM. DNA-mediated assembly of cytochrome P450 BM3 subdomains. *J Am Chem Soc*. 2011; 133:16111–16118. [PubMed: 21919448]
- [31]. So MK, Yao H, Rao J. HaloTag protein-mediated specific labeling of living cells with quantum dots. *Biochem Biophys Res Commun*. 2008; 374:419–423. [PubMed: 18621022]
- [32]. Strauch RC, Mastarone DJ, Sukerkar PA, Song Y, Ipsaro JJ, Meade TJ. Reporter protein-targeted probes for magnetic resonance imaging. *J Am Chem Soc*. 2011; 133:16346–16349. [PubMed: 21942425]
- [33]. Kovalenko EI, Ranjbar S, Jasenosky LD, Goldfeld AE, Vorobjev IA, Barteneva NS. The use of HaloTag-based technology in flow and laser scanning cytometry analysis of live and fixed cells. *BMC Res Notes*. 2011; 4:340. [PubMed: 21906296]
- [34]. Hong H, Benink HA, Zhang Y, Yang Y, Uyeda HT, Engle JW, Severin GW, McDougall MG, Barnhart TE, Klaubert DH, Nickles RJ, Fan F, Cai W. HaloTag: a novel reporter gene for positron emission tomography. *Am J Transl Res*. 2011; 3:392–403. [PubMed: 21904659]
- [35]. Kosaka N, Ogawa M, Choyke PL, Karassina N, Corona C, McDougall M, Lynch DT, Hoyt CC, Levenson RM, Los GV, Kobayashi H. In vivo stable tumor-specific painting in various colors using dehalogenase-based protein-tag fluorescent ligands. *Bioconjug Chem*. 2009; 20:1367–1374. [PubMed: 19514716]
- [36]. Taniguchi Y, Kawakami M. Application of HaloTag protein to covalent immobilization of recombinant proteins for single molecule force spectroscopy. *Langmuir*. 2010; 26:10433–10436. [PubMed: 20527958]
- [37]. Urh M, Hartzell D, Mendez J, Klaubert DH, Wood K. Methods for detection of protein-protein and protein-DNA interactions using HaloTag. *Methods Mol Biol*. 2008; 421:191–209. [PubMed: 18826056]
- [38]. Nagaki K, Shibata F, Kanatani A, Kashiara K, Murata M. Isolation of centromeric-tandem repetitive DNA sequences by chromatin affinity purification using a HaloTag7-fused centromere-specific histone H3 in tobacco. *Plant Cell Rep*. 2012; 31:771–779. [PubMed: 22147136]

- Cannabinoid CB₂ receptor is expressed in *E. coli* as a fusion with HaloTag
- CB₂ expressed as a fusion with C-terminal HaloTag retains functional activity
- Fusion CB₂-HaloTag can be immobilized and purified on a HaloLink resin
- N-terminal HaloTag results in misfolding of the receptor in *E. coli*

Figure 1.

Constructs for expression of Halo-tagged CB₂ and CB₂ fusion proteins used as controls. Plasmids for expression of CB₂-243 and 244 differ in antibiotic resistance and type of promoter.

Figure 2.

Optimization of conditions for expression of Halo-tagged CB₂ in KRX cells by auto-induction with rhamnose. The course of expression of CB₂-241 by early and late induction were followed at 20 °C and 25 °C by measuring optical density (OD) at 600 nm (A and B, respectively) and by Western blot using anti-MBP antibody (C and D, respectively). Samples for Western blots were prepared by collecting cells from 1 mL of culture by centrifugation and solubilizing proteins in Laemmli loading buffer by sonication. The influence of glucose concentration on cell growth for the late induction process at 20 °C was followed over time by measuring OD₆₀₀ (E). Arrows indicate bands corresponding to CB₂-241.

Figure 3. Expression levels and accessibility of HaloTag in *E. coli* membranes. Solubilized membrane preparations containing equal amounts of total protein were resolved by SDS-PAGE and electroblotted onto a nitrocellulose membrane. Results of Western blots performed with anti-CB₂ (A) or anti-HaloTag (B) antibodies are shown. Membranes expressing CB₂-130 and 125 were used here as controls. The expected locations of CB₂ fusion proteins are indicated by arrows.

Figure 4.

Relative activity of Halo-tagged CB₂ in membranes.

Membrane preparations containing 2 µg of total protein were tested in a G protein activation assay (in duplicates). Relative activity with respect to the activity of a control sample (membranes containing CB₂-130) is presented.

Figure 5.

Effect of N-terminal Halo-tagged CB₂ on activation rates of G proteins.

Rate of activation of G proteins by CB₂-130 membranes (2 µg total protein per assay) upon addition of CP 55,940 was measured in the absence (CB₂-130) or presence of equal amounts of membranes prepared from empty BL21 cells (CB₂-130+BL21), BL21-CB₂-243 (CB₂-130+CB₂243) or membranes BL21-CB₂-130 that were pre-incubated at 75 °C for 1 h (CB₂130+CB₂-130T). As a control, either purified HaloTag protein (40 ng per assay) was added to CB₂-130 membranes (CB₂-130+HaloTag) or tested alone (HaloTag). The relative activity compared to activity of CB₂-130 membrane preparations set to 100% is presented.

Figure 6.

Cleavage of CB₂241 in *E. coli* membranes by TEV protease.

A. Expected pattern of cleavage of fusion proteins with TEV protease when probed with anti-CB₂ antibody.

B. Results of Western blot analysis of the TEV protease cleavage experiment. Membrane preparations containing either CB₂-130 or CB₂-241 fusion proteins were diluted in reaction buffer to a final concentration of 5 mg/mL of total protein. Aliquots were left as such (O= original), or incubated in the absence (I= incubated), or in the presence of 8 μg (T1= treated), or 40 μg (T2= treated) of TEV protease for 1h at 25 °C. Samples (50 μg of membrane protein) were separated with SDS-PAGE and analyzed by Western blot. The position of relevant bands is indicated by arrows.

Figure 7.**Purification of Halo-tagged CB₂**

Comparison of two purification protocols for CB₂-241 by HaloTag technology using Western blot analysis of purified fractions is presented. L= load, FT= flow-through, W= wash, Elu 1=elution from HaloLink resin after treatment with TEV protease, Elu2= elution after removal of TEV protease by HisLink resin. The arrows indicate the expected migration position of fusion and purified CB₂ protein.

Table 1

Comparison of expression systems for Halo-tagged CB₂ constructs

Calculations of expression levels are based on semi-quantitative analysis of Western blots results. Thus, this table is intended to provide a qualitative comparison of expression systems used in this work.

The Halo-tagged CB₂ was expressed in either BL21 or KRX cells cultivated in 250 mL flasks as described in Materials and Methods and OD₆₀₀ was measured at harvesting. The mean (OD₆₀₀ at harvesting) of two independent experiments is presented

Membranes were prepared from BL21 and KRX cells, in which expression of CB₂ was induced by IPTG or rhamnose, respectively. Membranes containing equal amounts of total protein were studied by Western blot and activity tested by a G protein activation assay. Results of a representative Western blots were quantified by densitometry and are presented relative to expression of CB₂-130 (set to 100%).

Densitometry values were compared to dose curves and calculations performed for bands within the linear range of the assay. The expected yield of CB₂ per 1L of culture was calculated based on estimated 3 ng of CB₂-130 present in 1 µg of total protein in membranes as reported previously [26]. The amount of CB₂ per µg of total protein in each membrane suspension was calculated by comparing the intensity of bands corresponding to Halo-tagged CB₂ with the corresponding band of CB₂-130. The expected yield of CB₂ was estimated from the total protein concentration of each membrane preparation, the original volume of suspended membranes, and the volume of the culture from which they were prepared.

Specific activity was calculated by dividing activity/µg of total protein (from the G protein activation assay) by the amount of CB₂/ µg of total protein (from Western blot). Two sets of specific activities were obtained using two independently prepared batches of membranes. Averages were compared with the specific activity of CB₂-130 set to 100 %. Specific activity data (in % with respect to CB₂-130 expressed in BL21 cells) are shown.

Construct	OD ₆₀₀ at harvesting		CB2 expression (%)		Expected yield (µg CB ₂ /L culture)		Specific activity (%)	
	BL21(DE3)	KRX	BL21(DE3)	KRX	BL21(DE3)	KRX	BL21(DE3)	KRX
130	3	NA	100	NA	350-400	NA	100	NA
241	2.2	3.7	70	50	230	150	30	30
242	4.3	3.9	30	10	80	20	20	20
243	3.4	2.9	120	30	400	30	2	2
244	5.3	3.1	70	80	210	140	0 [/]	0

NA: not available

[/] One batch of CB₂-244 BL21-membranes gave activity very close to background levels, the other batch could not be subjected to specific activity calculation because of a very low signal in Western blots.