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Application of Strep-Tactin XT for affinity purification of Twin-Strep-tagged CB₂, a G protein-coupled cannabinoid receptor

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Abstract

Human cannabinoid receptor CB₂ belongs to the class A of G protein-coupled receptor (GPCR). High resolution structural studies of CB₂ require milligram quantities of purified, structurally intact protein. Here we describe an efficient protocol for purification of this protein using the Twin-Strep-tag/Strep-Tactin XT system.

To improve the affinity of interaction of the recombinant CB₂ with the resin, the double repeat of the Strep-tag was attached either to the N- or C-terminus of CB₂ via a short linker. The CB₂ was isolated at high purity from dilute solutions containing high concentrations of detergents, glycerol and salts, by capturing onto the Strep-Tactin XT resin, and was eluted from the resin under mild conditions upon addition of biotin.

Surface plasmon resonance studies performed demonstrate the high affinity of interaction between the Twin-Strep-tag fused to the CB₂ and Strep-Tactin XT with an estimated K_d in the low nanomolar range. The affinity of binding did not vary significantly in response to the position of the tag at either N- or C-termini of the fusion. The variation in the length of the linker between the double repeats of the Strep-tag from 6 to 12 amino acid residues did not significantly affect the binding.

The novel purification protocol reported here enables efficient isolation of a recombinant GPCR expressed at low titers in host cells. This procedure is suitable for preparation of milligram quantities of stable isotope-labelled receptor for high-resolution NMR studies.

Introduction

Human cannabinoid receptor CB₂ is an integral membrane G protein-coupled receptor (GPCR) predominantly expressed in cells of immune origin. It is implicated in regulation of metabolic pathways of inflammation, neurodegenerative disorders, obesity and pain sensing and thus is an important target for pharmaceutical drug development.^(1, 2) Rational design of

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novel drug candidates specifically targeting this receptor relies on studies of its structure, binding with specific ligands, and activation of the interacting partners (G protein, β -arrestin) etc. The biophysical methods that yield high structural resolution require purified, structurally intact receptor. We previously reported on the methodology for expression of CB₂ and its purification and stabilization in a functional state. ⁽³⁻⁷⁾

With the notable exception of rhodopsin, GPCR are present at low levels in native tissues and recombinant expression is required to generate milligrams quantities of protein sample for structural characterization. For studies by NMR the target protein must be labelled by stable isotopes either uniformly or at selected positions ⁽⁶⁾. This latter condition can be met by expressing the protein in a medium of a defined composition supplemented with labelled amino acid(s). However, cultivation in minimal medium typically yields lower titers of the target protein which, in the case of CB₂ does not exceed 0.1% of the total protein in the cell. Thus, a very efficient method of purification is required to recover the recombinant receptor from dilute extracts.

High concentrations of detergents required to solubilize the GPCR from cell membranes impose further restrictions on downstream purification methods. Conventional chromatographic methods such as ion exchange or hydrophobic chromatography are not effective for high-level purification of large quantities of proteins from dilute, detergent-containing solutions. ^(4, 8) A more practical alternative approach is the use of small affinity tag(s), provided that they do not alter the structural stability and function of the target protein. ^(9, 10) A large number of fusion protein tags have been developed in the past two decades to enhance expression ^(11, 12), facilitate proper folding and solubility of the target protein ⁽¹¹⁾ as well as enable detection, surface capture, purification and studies of protein-protein interaction, and these studies were thoroughly analysed in several excellent reviews. ^(10, 12-17) Some of the tags can serve several purposes, such as detection and purification of the target protein. These include c-myc, HA, FLAG, 1D4, polyArg and polyHis, Strep-tag, HaloTag, S-tag, to name a few. ^(12, 13, 17, 18) While the use of affinity tags for protein purification is currently widespread, the application of this technology to preparation of membrane proteins for structural studies has to satisfy a number of stringent requirements:

- (i) The affinity resin should be relatively inexpensive, to allow purification of milligram quantities of the target protein required for structural studies.
- (ii) Resin matrices should be sufficiently robust to withstand multiple cycles of regeneration
- (iii) It is often advantageous not to remove the affinity tag from the target protein after purification, since the tag can be subsequently used for immobilization on a solid support for detergent/ ligand exchange and biophysical studies. Thus, the tag should be relatively small and should not affect the fold of the target protein and interfere with its function.
- (iv) The interaction between the tagged protein and the affinity resin should be sufficiently strong even in the presence of detergents, glycerol, salts and other additives used to solubilize and stabilize membrane receptors. This is especially

important for low-expressing proteins like GPCR in which case the affinity of interaction should be ideally in picomolar- or low nanomolar range.

(v) The tag should dissociate from the affinity resin under mild conditions that ensure preservation of the functional structure of the target protein.

These requirements limit the choice of purification systems for membrane protein targets. For instance, several excellent systems that are based on a small peptide/ antibody interaction are not practical for purification of large quantities of target proteins due to prohibitively high cost of antibody-based resins, with the possible exception of Rho-tag/ 1D4 (13, 19). However, the affinity of interaction for this tag/ antibody pair ($K_d \sim 20$ nM even in the presence of detergents and glycerol) may be too high for purification of the target proteins from very dilute solutions.⁽¹⁹⁾

In addition to a single tag (17), the use of a combination of two or more affinity tags flanking the target protein gains increased popularity (9, 13, 20). We previously described the tandem affinity chromatography for purification of CB₂ from detergent-containing solutions, using a combination of the IMAC and the chromatography on a Strep-Tactin resin. (3, 8, 16, 19). This allows isolation of the full-length CB₂ receptor with high yield and purity. One notable restriction for chromatography on the Strep-Tactin resin is the apparently low affinity of the Strep-tag for the resin. Therefore, the concentration of the target protein in the detergent extract should be quite high, in the high-nanomolar or low-micromolar range.⁽²¹⁾ Thus, in the case of very dilute protein solutions it is difficult to attain satisfactory yield and purity of the target protein (6). Therefore, there is a need for a better performing, high affinity resin for purification of proteins expressed at low titers. In this study we demonstrate that the Twin-Strep-tag/ Strep-Tactin XT system is an appropriate choice for purification of membrane proteins, especially for those expressed at low levels.

The Strep-tag system has been thoroughly described in various articles and reviews (8, 16-18, 22-26). Currently, two different streptavidin binding tags are being used most commonly: the Strep-tag II (WSHPQFEK)⁽²²⁾, and its tandem variant– the Twin-Strep-tag (WSHPQFEKGGGSGGGSGGSAWSHPQFEK) (22). The latter is characterized by higher affinity but still reversible binding, and increasingly gains popularity⁽²²⁾. Through its enhanced binding properties, the Twin-Strep-tag enables a wide spectrum of applications, and is the preferred choice when larger tag size on a target protein is acceptable⁽²²⁾.

Among the streptavidin-based affinity reagents currently available are native streptavidin, the affinity engineered Strep-Tactin (22), as well as its modified version with higher affinity for the Strep-tag.⁽²⁷⁾ These resins cover affinities ranging from micromolar to picomolar thereby enabling the most common purification tasks under native conditions. Competitive elution is performed by biotin⁽²⁷⁾ and biotin analogs, i. e. diaminobiotin for streptavidin (28-30) and desthiobiotin for Strep-Tactin (31-34), resulting in a displacement of Twin-Strep-tag fusion proteins from the column and, simultaneously, allowing for easy regeneration of the streptavidin affinity resin for the next purification cycle.^(5, 8)

The bivalent Twin-Strep-tag comprised of two 8 amino acid-long epitopes separated by a small linker enhances binding to the Strep-Tactin through the avidity effect. Due to the

comparatively fast off-rate of each individual Strep-tag II module, the Twin-Strep-tagged protein can be efficiently displaced from the complex with Strep-Tactin upon addition of desthiobiotin, resulting in sharp elution ⁽¹⁸⁾. To exploit this concept for even more demanding applications, Strep-Tactin was further engineered resulting in the development of Strep-Tactin XT⁽²⁷⁾. The new resin has reportedly double digit-picomolar affinity to the Twin-tag, while preserving reversibility of binding as determined for interaction with soluble proteins containing the Twin-tag⁽²⁷⁾.

In addition to affinity purification, some assays for membrane receptors may be quite challenging, particularly when extensive washing of the surface is required. Examples are ELISA or BiaCore™ experiments where the recombinant Twin-Strep-tag fusion protein is immobilized on a microtiter plate or a sensor chip, coated with Strep-Tactin XT. Low off-rate helps to reduce the protein loss and improve the performance of the assay.

In this presentation we describe the application of the Strep-Tactin XT resin for purification of the integral membrane cannabinoid receptor CB₂ either as one-step or a two-step affinity purification procedure. We also present the application of the strong Twin-Strep-tag/ Strep-Tactin XT interaction for Biacore binding experiments. The effect of the length and the position of the Twin-Strep-tag on the expression and binding properties of the fusion protein will also be discussed.

Materials and Methods

1. Chemicals and reagents

Oligonucleotides were purchased from Operon Biosciences. Restriction enzymes and DNA-modifying enzymes were obtained from New England Biolabs. The Complete His resin was from Roche. The purified Strep-Tactin XT, Strep-Tactin XT Superflow and MagStrep “type3” XT beads were kindly provided by IBA GmbH. Research grade sensor chip CM4, immobilization reagents NHS, EDC, and ethanolamine and HBS-N buffers for SPR experiments were from GE Healthcare.

Cholesteryl hemisuccinate Tris salt (CHS) and detergents 3 [(cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) and n-dodecyl-β-D-maltoside (DDM) were obtained from Anatrace. Lipids 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine sodium salt (POPS) were purchased from Avanti Polar Lipids Inc. Synthetic cannabinoid ligand CP-55,940 was from Tocris. All other chemicals of reagent grade were purchased from Sigma.

2. Expression constructs

Fusion constructs for expression of CB₂ in *E. coli* and subsequent purification (Fig. 1) were constructed as follows. The construction of CB₂-130 was previously described ⁽⁸⁾.

Plasmid CB₂-130 contains a short linker GSGGAS separating the double repeat of the Strep-tag WSHPQFE. For construction of CB₂-290, plasmid CB₂-130 was subjected to three consecutive rounds of site-directed mutagenesis using pairs of primers 290a-For: 5'-CCGCAGTTTGAAAAAGGAGGCGGATCGGGCGCGAGCTGG-3' and 290a-Rev: 5'-

CCAGCTCGCGCCCGATCCGCCTCCTTTTTCAAACCTGCGG-3'; 290b-For: 5'-GGAGGCGGATCGGGCGGAGGATCGTGGTCGCATCCGCAGTTT-3' and 290b-Rev: 5'-AAACTGCGGATGCGACCACGATCCTCCGCCCCGATCCGCCTCC-3'; and 290c-For: 5'-TCGGGCGGGCGGATCGGGAGGCTCGGCTTGGTCGCATCCGCAG-3' and 290c-Rev: 5'-CTGCGGATGCGACCAAGCCGAGCCTCCCGATCCGCCGCCCCGA-3'. The resulting plasmid encodes the following fusion construct: MBP-Asn₁₀-TEV-GS-WSHPQFEK-GGGSGGGSGGSA-WSHPQFEK-GGGS-CB₂-His₁₀, where MBP corresponds to the full sequence of the *E. coli* maltose binding protein, Asn₁₀- the sequence of a linker containing ten asparagine residues, CB₂-sequence of human cannabinoid receptor CB₂, and His₁₀- a decahistidine tag. The sequence of the plasmid was verified.

For construction of plasmid CB₂-293 that encodes the fusion protein MBP-CB₂ with the His₁₀ tag at the N-terminus, and the Twin-Strep-tag - at the C-terminus of CB₂, the previously published plasmid pAY-109⁽³⁵⁾ was modified as follows. The plasmid was digested with *Bam*HI, dephosphorylated by phosphatase, and ligated with the duplex DNA fragment encoding the His₁₀ tag, and also introducing the unique *Nhe*I site using oligonucleotides 5'-P-GATCACACCATCACCATCACCATCACCATCACCATGCTAGCA-3' and 5'-P-GATCTGCTAGCATGGAGATGGAGATGGAGATGGAGATGGAGT-3' forming the plasmid pAY260a. This plasmid was digested with *Not*I, and the singly cut plasmid dephosphorylated with phosphatase to reduce the background. The duplex DNA formed by the oligonucleotides 5'-P-GGCCGGGATGGTCGCATCCGCAGTTTGAAAAAGGAGGCGGATCGGGAGGCGGATCGGGCGGATCCGCTTGGTCGCATCCGCAGTTTGAAAAAGGG-3' and 5'-P-GGCCCCCTTTTTCAAACCTGCGGATGCGACCAAGCGGATCCGCCCGATCCGCCTCCC GATCCGCCTCCTTTTTCAAACCTGCGGATGCGACCATCC-3' was inserted by ligation. The inserted fragment encodes the Twin-Strep-tag in which two repeats WSHPQFEK are separated by a linker GGGSGGGSGGSA. The oligonucleotides were designed so that both *Not*I sites flanking the insert in the newly formed plasmid were inactivated upon ligation. This allowed for selection for the insert-containing plasmid by digesting the ligation mixture by *Not*I enzyme prior to transformation into the *E. coli* DH5α cells.

The sequences of all plasmids were verified.

3. Expression of CB₂ in *E. coli*

CB₂ was expressed as a fusion with the N-terminal MBP and affinity tags in *E. coli* BL21 (DE3) cell cultivated in 2xYT medium supplemented with glucose and ampicillin as described previously⁽⁴⁾. Cells were collected by centrifugation, washed with phosphate-buffered saline, and stored at -80°C. Membranes containing CB₂ protein were prepared as described previously^(3, 8), the expression levels of the protein determined by semi-quantitative Western blot, and the functional activity of CB₂ measured by the G protein activation assay as described previously.⁽⁵⁾

Preparation of crude extract and solubilisation in detergents—Cells were disrupted with Avestin homogenizer, and CB₂ fusion protein was solubilized at 4°C in 50

mM Tris-HCl pH 7.5, 150 mM NaCl, 30% glycerol, 1% dodecyl maltoside (DDM), 0.5% CHAPS, 0.1% cholesteryl hemisuccinate (CHS) with 10 μ M cannabinoid agonist CP-55,940 added for CB₂ stabilization. The crude extract was centrifuged at 170,000x-g for 1h, and the protein purified from the cleared cell extract as described below.

4. Binding to Strep-Tactin resin

Determination of the binding capacity of Strep-Tactin XT resin—The CB₂-130 fusion protein was partially purified on a Ni-NTA resin (Qiagen) and eluted with 250 mM imidazole in buffer A (50 mM Tris pH 7.5, 150 mM NaCl, 30% glycerol, 0.1% DDM, 0.5% CHAPS, 0.1% CHS) as described earlier ⁽⁴⁾. Fractions containing fusion protein were combined (19 mL) and dialyzed against buffer C (50 mM Tris pH 7.5, 100 mM NaCl, 10% glycerol, 0.1% DDM, 0.5% CHAPS, 0.1% CHS) to remove imidazole and reduce the concentration of glycerol and NaCl.

To study the binding of the fusion CB₂ to the Strep-Tactin XT resin, 1 mL of the dialyzed protein solution was applied directly onto the Strep-Tactin XT resin. To study the binding of the CB₂ devoid of the MBP fusion partner, the remaining 8 mL of protein solution were first incubated with 2 mg of TEV protease for 4 h at 4°C and then reacted with the resin. One mL of the protein extract (either treated with TEV protease or untreated as specified in the text) was slowly passed by gravity through the disposable mini-column packed with 500 μ L of the Strep-Tactin XT resin. The columns were then washed successively with 8 mL of buffer A, 8 mL of buffer A supplemented with 500 mM NaCl, and 8 mL of buffer A supplemented with 1M NaCl. All wash fractions were collected and analysed separately. The protein was eluted by gravity flow by applying 1 column volume of buffer A supplemented with 1M NaCl and 50 mM biotin. Upon 15 min incubation the eluate was collected and the elution procedure repeated 4 more times. All samples were processed in duplicates. Further studies showed that the bound protein can be equally effectively eluted by passing the buffer A containing 50 mM biotin through the column without stopping the flow.

For evaluation of the binding capacity of Strep-Tactin XT magnetic beads, 300 μ L of a 5% suspension of the beads were mixed with 1 mL of the protein extract on ice and incubated for 30 min. Because of the small volume of the beads they were not packed into the column but separated from the solution by brief centrifugation, and the supernatant carefully removed. The beads were then washed four times with 1.7 mL of buffer A, four times with buffer A + 500 mM NaCl, and four times with 1.7 mL of buffer A + 1M NaCl. All wash fractions were collected separately and analysed. Protein was eluted by resuspending the beads in 150 μ L of buffer A + 1M NaCl + 50 mM biotin, incubation for 15 min followed by brief centrifugation. The supernatant was collected, and the elution repeated 3 more times. Samples were processed in duplicates.

Protein concentration was determined in the elution fractions by absorbance measurements at 280 nm, and by the Bio Rad Dc detergent-compatible protein assay kit.

Effect of the position of the twin-Strep-tag on binding of CB₂ to Strep-Tactin XT—Detergent-solubilized CB₂-130 and CB₂-290 proteins were purified by immobilized metal-affinity chromatography (IMAC) on a Complete His resin (Roche) as described above,

and 1, 4, or 14 mL extract were mixed with 0.2 mL Strep-Tactin XT resin. In the case of the construct CB₂-293 that lacks the C-terminal His-tag, a biotin blocking solution (BioLockTM, IBA GmbH) was added to the extract according to the manufacturer's protocol, to sequester endogenous biotin present in *E. coli* cells. Then either 4, 40, or 200 mL of solubilized extract were mixed with 0.2 mL resin. Incubation continued for 1 hour at 4°C. We later demonstrated that shorter incubation for 10-15 min were equally effective in capturing the protein (results not shown). The resin was collected by centrifugation, transferred into a disposable column, washed with 10 mL of buffer A + 0.5 M NaCl + CP-55,940, and protein was eluted with 4x column volumes of buffer A + 1M NaCl + CP-55,940 + 50 mM biotin.

Regeneration of the Strep-Tactin XT resin—Regeneration of the Strep-Tactin XT resin was performed according to the manufacturer's instructions.

Purification of CB₂ using conventional Strep-Tactin resin—The purification of CB₂-130 using conventional Strep-Tactin resin and a Twin-Strep-tag was performed as described earlier⁽⁸⁾.

Large-scale purification of CB₂—*E. coli* BL21(DE3) cells expressing CB₂-290 were grown in minimal salt medium in a 7.5-L fermentation vessel and protein production induced as described earlier⁽⁶⁾. The biomass was collected, resuspended in 50 mM Tris-HCl pH 7.5 and 150 mM NaCl supplemented with protease inhibitors and DNase I, and disrupted by passing twice through an Avestin cell homogenizer. Detergents 1% DDM, 0.5% CHAPS, protein stabilizers 0.1% CHS, 30% (v/v) glycerol were added, protein extracted on ice for 1 h, and the solution cleared by centrifugation at 155,000xg for 1 h. The supernatant was filtered through a 0.22 µm pore size filter and applied to a column packed with CompleteHis resin connected to an Akta Purifier 100 chromatography system. The column was washed with buffer A supplemented with 7.5 mM imidazole, and the protein eluted with buffer A + 250 mM imidazole. The fractions containing fusion CB₂ protein were combined, dialyzed against buffer C, and the fusion protein cleaved upon addition of 2 mg of TEV protease.⁽⁸⁾ The resulting extract (50 mL) was mixed with 4 mL of Strep-Tactin XT resin and incubated overnight at 4°C on a shaker. The resin was transferred into a disposable column and washed with buffer A by gravity flow. Buffer A supplemented with 1M NaCl and 50 mM biotin was added, the flow stopped for 15 min, and the eluate collected. The elution cycle was repeated 4 more times, the elution fractions collected and the protein concentration determined by the BioRad DC assay.

5. Surface plasmon resonance (SPR) experiments

CM4 sensor chips were used for SPR experiments performed in a Biacore X-100 biosensor (GE Healthcare) at a temperature 10 °C as indicated in the text.

Immobilization of Strep-Tactin XT onto the sensor chip—The purified Strep-Tactin XT protein was immobilized on the CM4 chip surface at 25 °C by amine coupling using 10 mM HEPES, pH 7.4, and 150 mM NaCl (HBS-N) as a running buffer at a flow rate of 5 µL/min following the procedure recommended by the manufacturer. Upon activation of surfaces

with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) for 8 min, two consecutive injections, 10 min each, of the Strep-Tactin XT protein diluted in 10 mM sodium acetate to 2 µg/mL at a flow rate of 2 µL/min were performed. Excess activated groups were blocked with two consecutive injections (35 and 5 µL) of 1 M ethanolamine-HCl, pH 8.5. A density of Strep-Tactin XT corresponding to 170 response units (RU) was achieved. The surface was stabilized by several consecutive 50-µL injections of 2 M NaCl and running buffer HBS-N at a flow rate 50 µL/min. A reference surface was generated by treatment with the amine coupling reagent without the protein.

Preparation of CB₂ for SPR binding experiments—CB₂-130 and CB₂-290 were expressed in *E. coli* cells and purified by tandem affinity chromatography on Complete His. The MBP tag was removed by TEV protease treatment, and the released CB₂ purified on Strep-Tactin resin as described previously⁽⁸⁾. To remove biotin from the protein solutions eluted from Strep-Tactin, the proteins were then immobilized onto Complete His resin and washed extensively with buffer A supplemented with 10 µM CP-55,940. The proteins were eluted with 250 mM imidazole in buffer A and concentrated on centrifugal micro spin concentrators to 1-2 mg/mL. Prior to the SPR experiments, the protein samples were dialyzed against four changes of the buffer C (50 mM Tris pH 7.5, 150 mM NaCl, 10% (v/v) glycerol, 0.1% DDM, 0.5% CHAPS, 0.1% CHS) supplemented with 10 µM CP-55,940.

CB₂-290 fusion protein was purified on Complete His resin and then on Strep-Tactin XT resin. Treatment with TEV protease was not performed. The protein was then captured again onto Complete His resin and processed the same way as described above for CB₂-130 and CB₂-290.

CB₂-293 was purified as follows. The MBP-CB₂ fusion was captured onto Strep-Tactin XT resin, treated with TEV protease, and then captured onto Complete His resin. The resin was washed extensively to remove impurities, and the eluted protein concentrated and dialyzed against SPR running buffer as described above.

Affinity of CB₂ to Strep-Tactin XT—Binding experiments were performed at 10 °C at a flow rate of 5 µL/min using a surface coated with Strep-Tactin XT and a reference generated by mock coupling without the protein. Running buffer C supplemented with 10 µM cannabinoid ligand CP-55,940 was used in all experiments. The running buffer was kept on ice during the entire experiment, and samples were kept on ice until injection. Solutions of the purified CB₂ (0.05, 0.3, 5, 30, 100 nM and 1 µM) were injected over both the active and reference surfaces; the contact time for each injection was 20 min, the maximal time allowed by the instrument to allow the sensogram to approach a steady reading. Then the running buffer was passed through the cell for 1 h or longer. The CB₂ was eluted by injecting 50 mM biotin in running buffer for 10 minutes or longer. The injections were repeated one or several times until steady readings were achieved. The surfaces were regenerated as described below.

Regeneration of the sensor chip surface—The removal of captured CB₂ was performed by displacement of the protein with biotin at 10 °C. A solution of 50 mM biotin in buffer C was injected at a flow rate of 1 µL/min. Biotin was then removed at a flow rate

of 50 $\mu\text{L}/\text{min}$ injection of the running buffer, 10 mM NaOH twice followed by two more injections of running buffer.

Results

Fusion constructs for expression of CB₂

To study the effects of the length of the linker between the two binding epitopes WSH₂PQFEK in Twin-Strep tag on the affinity of the fusion protein to the resin, we employed two expression constructs CB₂-130 and CB₂-290 (Fig. 1). In the construct CB₂-130, two Strep-tag epitopes are separated by six amino acid-long linker, while in CB₂-290 the length of the linker is 12 residues. Furthermore, to explore the effect of the position of Twin-Strep-tag relative to CB₂, we placed the tag either in the middle of the fusion protein (CB₂-290) or downstream of CB₂ at the C-terminus of the fusion (CB₂-293). In addition, all expression constructs contain the *E. coli* maltose-binding protein (MBP) as an N-terminal fusion partner that directs the newly synthesized protein to the cytoplasmic membranes of *E. coli* and stabilizes the fusion.⁽³⁵⁾ The TEV protease recognition sequence was placed downstream of the MBP in all constructs to allow the removal of the MBP part of the fusion during CB₂ purification.

Expression levels of CB₂ and activity

The expression of CB₂ in *E. coli*, determination of the levels of protein accumulation in *E. coli* membranes, and determination of functional activity of CB₂ were performed as described in Materials and Methods. The accumulation of CB₂-290 in membranes was almost identical to that of CB₂-130, while CB₂-293 expressed at somewhat lower levels (Fig. 2 A). These results are confirmed by the activity tests measuring the rates of G protein activation by CB₂, with the activity levels of CB₂-293 being about 2.5-times lower than those of CB₂-130. Lower expression levels of the fusion CB₂-293 protein may be attributed to lower rates of translation and/or folding of the nascent polypeptide due to an unfavourable effect of the decahistidine tag located in the middle of the fusion protein.

Determination of the binding capacity of resin variants

To compare the binding of the Strep-Tactin Superflow resin and the magnetic beads the following experiment was performed: The CB₂-130 construct was pre-purified by chromatography on Ni-NTA as described in Materials and Methods. The Strep-Tactin Superflow resin as well as the Strep-Tactin XT magnetic beads were tested. The protein solution in detergent-containing buffer (Buffer A as described in Materials and Methods) was applied to the resin in a batch mode.

To study the effect of the position of the Twin-Strep tag in the fusion protein (Fig.1) on binding of CB₂ to the resin, we compared the binding of the fusion MBP-CB₂-130 protein to the resin with that of the CB₂ protein pre-treated with TEV protease. TEV protease cleaves the fusion protein downstream of MBP, leaving only three amino acid residues (SGS) upstream of the Twin-Strep-tag in case of CB₂-130. In the uncleaved MBP-CB₂ fusion protein, the Twin-Strep tag is located approximately in the middle of the fusion. The results of these experiments are summarized in Table 1.

For the Strep-Tactin XT Superflow, the binding capacity for the N-terminally located Twin-Strep-tag in CB₂-130 was in the range of 37-38 nmol per mL of resin, corresponding to 1.65-1.75 mg of the CB₂ protein (44,065 Da) per 1 mL of resin. For the fusion protein MBP-CB₂ (86,336 Da) the binding capacity decreased by about 3-fold, most likely because of steric hindrance to interaction of the Strep-tag with binding sites on the resin from the MBP fusion partner. The binding capacity of the Strep-Tactin XT magnetic beads for the CB₂ protein was almost two-fold higher than for regular Strep-Tactin XT chromatography resin which can be attributed to a higher density of the Strep-tag binding sites on the functionalized beads⁽²⁷⁾.

The resin samples containing bound protein were extensively washed with a total of 48 column volumes of wash buffer (see Materials and Methods). In all cases we detected only traces of CB₂ in wash fractions, indicating tight binding of the protein to the resin. The elution was achieved by five sequential cycles of treatment of the resin with 1 column volume of the elution buffer containing 50 mM biotin. Over 95% of the protein was eluted in the first 4 fractions.

Position of the Strep-tag and the length of the linker

The position of the Strep-tag in the fusion protein and the length of the linker between the binding epitopes may affect the exposure of the tag for interaction with binding sites on the Strep-Tactin XT resin which, in turn, may influence the binding capacity of the resin and the affinity of interaction. We compared the binding of several CB₂-expressing fusion constructs to the Strep-Tactin XT resin.

E. coli cells expressing CB₂-130, CB₂-290 or CB₂-293 were grown in 2xYT medium, and the recombinant CB₂ protein production induced as described in Materials and Methods. The expression levels of these proteins were determined by Western blot and the activity tests (Fig. 2). Cells were collected, and the fusion proteins solubilized in buffers containing detergents DDM, CHAPS and CB₂ stabilizers CHS and high affinity cannabinoid ligand CP-55,940.

The experiments were designed such that for each sample the extract applied onto the resin contained a large (3-4 fold) excess of CB₂ protein relative to the amount of resin. In order to accomplish this, CB₂-130 and CB₂-290 fusion proteins were purified from the respective crude extracts by IMAC using the C-terminal decahistidine tag and eluted in buffer A supplemented with 250 mM imidazole as described in the Materials and Methods. Imidazole was removed from the eluates by dialysis, and part of the sample was incubated with TEV protease to remove the MBP fusion partner while the other portion of the extract was left intact. The resulting concentration of CB₂ in these eluates was approximately ten-fold higher (0.5-1 μ M) than in the crude extracts prior to the Ni-NTA affinity purification.

For CB₂-293 that lacks the C-terminal polyhistidine tag, the pre-purification by IMAC could not be performed. The crude extract cleared by ultracentrifugation and filtration was treated with BioLock (biotin blocking solution) to remove the biotin that may be present in cell extracts, and mixed with the Strep-Tactin XT resin as described in the Materials and Methods. After the protein binding step was completed, the resin was extensively washed

with buffers with progressively increasing concentrations of NaCl, and the CB₂ protein eluted with biotin. The results are summarized in Table 3 and in Fig. 3.

The results suggest that steric hindrance by MBP negatively affects the binding of the fusion proteins. The location of the Strep-tag in the middle of the fusion is not optimal for binding since the apparent capacity of the resin is significantly lower for the fusion protein. In the case of CB₂-130, the removal of MBP increased the binding of CB₂ by about 4-fold. A slightly lower increase by about a factor of 2 was observed for the CB₂-290 construct. Interestingly, the increase in length of the linker from 6 to 12 amino acids slightly lowers binding of CB₂ protein but appears to be beneficial for binding of the larger fusion protein.

The C-terminal location of the tag (CB₂-293) appears to be less favourable for binding as opposed to the N-terminal location (CB₂-130 and CB₂-290). However, in the case of CB₂-293 binding occurred from dilute crude extracts, and other proteins may have obstructed interaction of the tag with the resin.

Importantly, the purity of CB₂ in all cases is high (Fig. 3), and leaching of CB₂ from the resin during washes is minimal.

Regeneration of the Strep-Tactin XT resin

Regeneration of the Strep-Tactin XT resin was performed by repeated treatments with NaOH according to the manufacturer's instructions. To estimate the performance of the regenerated resin, it was reacted with IMAC-pre-purified CB₂-130, washed, and the eluted protein quantified. The resin packed in a column was subjected to three consecutive cycles of regeneration/-binding; the binding capacity slightly decreased with each cycle. For CB₂-130 the binding capacity decreased from about 60 nmol/mL to ~45 nmol/mL after 3 cycles of regeneration (Fig. 4)

Large-scale purification of CB₂ from dilute solutions

For NMR studies, the CB₂ protein must be labelled with stable isotopes. This was achieved by expression in *E. coli* cultivated in minimal salt medium supplemented with labelled nutrients.⁽⁶⁾ The titer of the recombinant protein expressed in minimal medium typically decreases several-fold compared to rich medium. To compensate for this decrease and to achieve high protein yield, cells are cultivated to a high density in a fermenter under controlled conditions of aeration, pH, temperature and nutrient supply. ⁽⁶⁾ The large amount of biomass generated in the fermentation process provides additional challenges for downstream purification. Our typical protocols call for processing of as much as 300-400-g of wet cell paste for preparation of 3-4 mg of pure CB₂. The procedure for the purification is described in Materials and Methods.

The biomass obtained from fermentation in minimal medium typically contains lower titers of CB₂ compared to the rich medium, and solubilization of the recombinant receptor requires large volume of detergent-containing buffers. Typically, proteins from 300-400 g of wet biomass are solubilized in 2-2.5 L of buffer, which corresponds to an effective concentration of CB₂ of 50 nM or lower, depending on expression levels of the target protein.

The construct CB2-293 with the C-terminal Twin-Strep tag was successfully purified directly from the cell extracts on a Strep-Tactin XT resin (Fig. 3). Because the level of expression of CB2-293 is lower than for CB2-130 or CB2-290 (see Fig. 2), the latter two constructs are preferred for large-scale production of CB₂. Because in these two fusion proteins the Twin-Strep tag is located in the middle of the construct, binding capacity of the resin for those constructs is low. Furthermore, it would be challenging to pass a large volume of extract through the column because of the very long interaction time required to achieve efficient binding.

Therefore, we purified the CB₂ protein by tandem affinity chromatography, using successively the C-terminal His₁₀ tag and the N-terminal twin-Strep-tag. The first stage of purification (IMAC) is performed on a large excess of relatively inexpensive Ni-resin. The eluates containing CB₂ protein captured from the crude extracts are then dialyzed, treated with TEV protease, and applied onto the Strep-Tactin XT column. While the purity of the CB₂ protein in the IMAC eluate is low, this step increases the concentration of CB₂ by about 20 fold which makes the final affinity purification on the Strep-Tactin XT resin much more efficient (Fig. 5). We estimated that this strategy recovers over 70% of CB₂ present in the original crude extract allowing purification of large quantities of CB₂. About 4 mg of CB₂ protein was obtained from 400 g of wet biomass using this protocol. The purified CB₂ was reconstituted in proteoliposomes ⁽⁷⁾ and was shown to be fully functional by G protein-activation tests (results not shown).

Surface Plasmon Resonance studies of CB₂ binding to Strep-Tactin

Binding affinity of the interaction of CB₂ with Strep-Tactin was further studied by surface plasmon resonance (SPR). The CM-4 sensor chip was functionalized with Strep-Tactin XT, and the CB₂ protein prepared as described in the Materials and Methods.

A typical binding curve of the construct CB₂-130 to the functionalized chip is shown in Fig. 6 A. The protein sample was applied at a flow rate of 5 μ L/ min for 20 min followed by the injection of the running buffer. The concentrations of the protein in the injected samples ranged from 0.05 nM to 1 μ M. Depending on the concentration of CB₂, an increase in the signal from 1 response units (RU) up to ~350 RU was observed (Supplemental Fig. 1). This surface coverage corresponds to 80-90% of the binding capacity of the functionalized sensor chip assuming 1:1 interaction between binding site and CB₂ and a $k_{on} = 3.1 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$. ⁽¹⁹⁾ During the dissociation phase initiated by the application of running buffer over the sensor chip surface that lasted for 1 h or longer, only a slight decrease in the signal was observed with an estimated $k_{off} = 2.3 \times 10^{-4} \text{ s}^{-1}$. The dissociation of the receptor from the chip surface was slow, as expected based on the previous studies with soluble proteins. ⁽³¹⁾ The regeneration of the surface was performed for more than 10 times according to the protocol described in Materials and Methods, with only a small decline of binding capacity.

Because of the very slow dissociation of the receptor from the surface, an accurate determination of the binding affinity of the protein to the functionalized chip at the experimental conditions was not possible. We estimated that the K_d of interaction between the CB₂ and Strep-Tactin XT in the presence of 10% glycerol and various detergents is at least in the low nanomolar to high picomolar range, consistent with experiments performed

with the Strep-Tactin XT resin. Our attempts to more accurately quantify the binding of various constructs of CB₂ to the surface by equilibrium titration at 10 °C (Supplemental figures 1-4) were not successful because a steady signal at each concentration of analyte was not reached on a reasonable time scale.

Among the CB₂ constructs tested, we observed highest binding affinity for CB₂-130 and only slightly lower binding – for CB₂-290 (Supplemental figures S1A-S2A). Binding of the MBP-CB₂-290 construct (figure S3A) corresponded to about 50% of binding density of CB₂-290, probably reflecting steric hindrance effects due to the presence of maltose binding protein in the fusion. Binding capacity of CB₂-293 (Supplemental figure S4A) was about 3 times lower than that of CB₂-290 and CB₂-130 suggesting weaker interactions with the surface due to steric hindrance of the tag located at the C terminus of CB₂.

The injection of biotin resulted in elution of all four constructs tested (S1,B-S4,B). However, the decrease in the signal corresponded to release of no more than 50% of chip-bound receptor. Subsequent injections of biotin-containing buffer over longer periods of time resulted in further release of CB₂. For complete regeneration of the sensor surface repeated cycles of injection NaOH and running buffer (without biotin) as described in Materials and Methods are effective.

Discussion

GPCR are very flexible proteins that respond to binding of specific ligands by changing conformation that ensures transmittal of the signal from the extracellular milieu to intracellular responding elements. To study conformation and dynamics of GPCR, an array of spectroscopic methods is used including nuclear magnetic resonance spectroscopy. Application of this method, however, benefits enormously from the ability to introduce stable isotopes (¹³C, ¹⁵N, ²H) either uniformly or in selected positions of the protein. This is achieved by expressing the protein in *E. coli* cells cultivated in a medium of defined composition (minimal salt medium, MSM) supplemented with stable isotope-labelled nutrients. While this approach enables the incorporation of stable isotope(s) into the target protein, cultivation in MSM typically results in lower levels of target protein expression per unit of biomass. Even at carefully optimized conditions, the titers of CB₂ in MSM reach no more than 40% of that of rich medium. Since purification of the protein requires solubilization of the biomass in detergent-containing buffers, the concentration of CB₂ extracted from biomass obtained from minimal medium is 20 nM or less. This requires a very efficient purification protocol with an affinity to the resin used for purification in the lower nanomolar- or better picomolar range. The resin has to possess a sufficiently high binding capacity, allow elution of the captured protein at mild conditions, and should be priced to permit purification of milligrams of target protein at reasonable cost. We are not aware of other commercially available resins that can fully satisfy these stringent requirements.

We have previously demonstrated a superior performance of the Twin-Strep-tag (formerly known as Strep-Tag III) over the Strep-tag II for purification of CB₂ on a Strep-Tactin resin, likely due to the avidity effect.⁽⁸⁾ While the binding affinity of the Twin-Strep-tag to the

resin was not quantified, we routinely observed 40-50% higher recovery of the constructs of CB₂ containing the Twin-Strep-tag relative to the single tag. However, even for the constructs with Twin-Strep-tag, the previously available version of the Strep-Tactin resin was not optimal due to the requirement of relatively high concentrations (about 0.3-1 μ M) of CB₂ protein applied to the resin, and a larger number of column volumes to elute the protein. Thus, a further increase in affinity of interaction between the tag and the resin was highly desirable.

Here we demonstrate that the Strep-Tactin XT resin that recently became commercially available satisfies stringent requirements for affinity and binding capacity, enabling mild purification of CB₂ receptor at high purity and yield. For the N-terminally located, well exposed tag, we estimated a recovery of 1.5 mg receptor from 1 mL of resin. The elution of the protein is efficient in the presence of 50 mM biotin, and the bulk of the protein (over 80-90%) can be recovered in the first 3 column volumes of eluate. The resin can be efficiently regenerated with only a slight decline in binding capacity over 3 regeneration cycles.

The binding affinity of the Strep-Tactin XT resin is very high. The resin with bound CB₂ was washed extensively, as much as 48 column volumes of detergent-, glycerol- and salt-containing buffers, yet we did not detect any significant CB₂ in the wash fractions.

The significantly higher binding of CB₂ onto magnetic beads relative to the Strep-Tactin XT Superflow resin can be explained by a much denser coating of the beads with Strep-Tactin XT in comparison to the Superflow resin, and also by the smaller size of the beads (25-30 μ m vs. 60-150 μ m). However, the application of the magnetic beads for the purification of large quantities of CB₂ is not practical due to the relatively high price of the beads.

While we demonstrated the feasibility of a one-step purification of CB₂ with Twin-Strep-tag at the N-terminus from crude cell extracts using Strep-Tactin XT resin, this approach is not practical due to relatively low binding capacity of the resin for CB₂ with C-terminal Twin-Strep-tag. A purification in column mode can be performed instead, but it harbours yet another problem: a large volume of extract has to be processed in order to capture the target protein which will lengthen the purification time unreasonably. A similar problem was observed with purification of CB₂ harbouring the C-terminal Rho-tag⁽¹⁹⁾. The purity of the CB₂ fusion protein captured on the 1D4 resin is high. However, the affinity of Rho-tag binding to 1D4 antibody in the presence of detergents was not very high (K_d approximately 20 nM⁽¹⁹⁾) and it can be used C-terminally only. Therefore, this tag is not as practical for recovery of CB₂ from dilute solutions as compared to the Twin-Strep-tag that can be used at the N-terminus as well.

Therefore, our current purification protocol relies on two successive chromatography steps that, in turn, require two affinity tags on the protein. The first step is purification via IMAC that allows recovery of most of the protein from the solution albeit at low purity only, using a large excess (relative to protein) of the inexpensive chromatography resin. The enriched material is further purified by chromatography using Strep-Tactin XT. Due to the slow off-rate, the protein bound to the Strep-Tactin XT resin is not lost during extensive washing,

resulting in much higher purity and overall yield of the target protein. This is a significant improvement over the previous version of the Strep-Tactin resin that was characterized by much lower affinity for the tag and hence much greater losses of the protein during the wash step.

A similar two-step purification protocol has been employed in this laboratory with the previously available Strep-Tactin resins. The recovery of CB₂ from cells expressed in rich 2xYT medium was on average 0.25-0.3 mg/L cell culture. However, the superior binding properties of the recently released Strep-Tactin XT allows for much more stringent washes of the protein bound to the resin, yielding higher purity. Furthermore, the recovery of CB₂ is about 30-40% higher, typically in 0.45-0.5 mg/L range.

Our SPR results confirm the high affinity of interaction between the Twin-Strep tag attached to CB₂ and the Strep-Tactin XT. The interaction is efficient in the presence of high concentrations of detergents, glycerol and salts. It appears that the N-terminal-, – rather than the C-terminal – position of the Twin-Strep tag is preferred for higher density of binding. As expected, the location of the Twin-Strep tag in the middle of the fusion protein results in lower density of surface coverage, most likely due to unfavourable steric effects of the large MBP tag. All surface-captured constructs of CB₂ were stable, and very little of the surface-captured protein was lost during prolonged washing with the running buffer. The protein was eluted under mild conditions by addition of biotin.

To summarize, we demonstrated significant advantages of the Strep-Tactin XT for purification of the difficult-to-express cannabinoid CB₂ membrane receptor. The use of the twin-Strep-tag is preferred over the single Strep-tag, since the higher affinity of interaction with the resin allows efficient capture and recovery of the target protein. The location of the tag at the N-terminus of CB₂ leads to higher density of binding compared to the C-terminal location. However, this effect is likely to be specific to CB₂ protein due to the steric hindrance of the tag. The length of the spacer between the two epitopes of the twin-Strep-tag does not significantly affect the affinity of binding.

The twin-Strep-tag/ Strep-Tactin XT system can be especially useful for purification of recombinant proteins expressed at low titers, and for purification of membrane proteins in the presence of high concentrations of detergents, salts and glycerol.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- A novel high affinity resin Strep-Tactin[®] XT was tested for purification of integral membrane receptor CB2
- We developed an efficient protocol for capture and purification of recombinant cannabinoid receptor CB2 from dilute solutions
- The N-terminal position of the Twin-Strep-tag is preferred for efficient binding to the resin
- Surface plasmon resonance studies with the purified protein confirm the high affinity of interaction between the Twin-Strep-tag fused to the CB₂ and Strep-Tactin XT.

Fig. 1.
Expression constructs for CB₂ protein

Fig. 2.

Expression and activity of CB₂.

A, Western blot performed on *E. coli* membranes expressing CB₂. Proteins were probed with rabbit polyclonal antibody against His₅ tag.

B, activation rates of G protein on *E. coli* membrane expressing CB₂. The receptor was activated by addition of 10 μM agonist CP-55,940 and the rates of activation of G protein analysed by measuring the accumulation of ³⁵S-γ-GTP-Gα_{i1} complex as described previously.⁽⁵⁾

Fig. 3.

Constructs of CB₂ purified on Strep-Tactin XT resin.

A, Western blot detection of CB₂: Lane 1, extract applied onto Complete His resin; 2, eluate from CompleteHis treated with TEV protease; 3, flowthrough from Strep-Tactin XT; 4-wash fractions from Strep-Tactin XT; 5-eluate from Strep-Tactin XT.

B, Eluates from Strep-Tactin XT analysed by SDS-PAGE, gel stained with Instant Blue:

Lane 1, CB₂-130-MBP fusion protein; lane 2, CB₂-130; lane 3, CB₂-290-MBP fusion protein; lane 4, CB₂-290; lane 5, CB₂-293 fusion protein.

Fig. 4. Regeneration of Strep-Tactin XT resin. Western blot probed with anti-His-tag antibody. Lane 1, eluate from IMAC; 2-eluate from IMAC treated with TEV protease; 3-5, flow-through from the Strep-Tactin resin regenerated 0, 1 and 3 times; 6-8 eluate from Strep-Tactin XT resin regenerated 0, 1 and 3 times.

Fig. 5. Purification of CB₂ from dilute solutions. A, SDS-PAGE stained with Instant Blue. B, Western blot probed with anti-His antibody. M, molecular weight marker; 1, crude extract; 2, flowthrough from IMAC; 3-combined wash fractions from IMAC; 4, 5, elution fractions from IMAC; 6, flowthrough from 5 mL Strep-Tactin XT resin; 7-wash fractions from Strep-Tactin XT resin; 8-eluate from Strep-Tactin XT.

Fig. 6.

Binding of CB₂ to Strep-Tactin XT studied by surface plasmon resonance.

A, CB₂-130 at a concentration 100 nM was injected over 20 min over both the functionalized and the reference surfaces of the sensor chip. The dissociation stage was performed in running buffer C.

B, elution of CB₂ from the chip by injection of 50 mM biotin in running buffer. All experiments performed at 10 °C.

Table 1

Determination of the binding capacity of Streptactin-XT resin.

Variant of the Strep-Tactin XT resin	Binder	Resin binding capacity, nmol protein/ mL resin *
Strep-Tactin XT	CB ₂ -130	37.5+/- 1.1
Strep-Tactin XT	CB ₂ -130-MBP fusion	11.4 +/-1.8
Strep-Tactin XT magnetic beads	CB ₂ -130	72.7 +/-4.3

* The results represent an average of two samples.

Table 2Binding of various constructs of CB₂ to Strep-Tactin XT resin

CB ₂ expression construct	Binding capacity of the resin *	
	mg/mL	nmol/mL
CB ₂ -130-MBP fusion	1.3 mg/mL	15
CB ₂ -130	2.8 mg/mL	63.5
CB ₂ -290-MBP fusion	2.2 mg/mL	25.3
CB ₂ -290	1.9 mg/mL	42.9
CB ₂ -293-MBP fusion	1.7 mg/mL	19.6

* The results are the average of two samples