

Application of a Small EF Hand Affinity Tag for Expression, Purification and Biophysical Studies of G Protein-Coupled Membrane Receptors

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Abstract

Heptahelical G protein-coupled receptors (GPCR) comprise a large family of integral membrane proteins involved in a wide array of cell signaling pathways. For high resolution structural studies of these receptors, multi-milligram quantities of pure and structurally unperturbed proteins are required. Purification of recombinant GPCRs typically involves their solubilization into detergent micelles followed by chromatographic purification. Because of relatively low expression levels of these recombinant receptors, it is challenging to design an efficient strategy for selective and efficient purification with high yield. Here, we describe a recently introduced purification system employing a high affinity molecular switch based on fragment complementation, with a calcium dependent capture and EDTA mediated chelation elution. This technique was successfully applied to the purification of the recombinant cannabinoid receptor CB₂, a promising target for the development of drugs for inflammation, immunological disorders and pain. It is feasible that similar strategies can be successfully employed for expression and purification of other membrane protein targets.

Introduction

G protein-coupled receptors (GPCR) comprise a large family of integral membrane proteins involved in a wide array of cell signaling pathways. The high resolution structural studies require large quantities of pure, homogenous and stable proteins. Purification of GPCRs typically requires solubilization of these receptors from cell membranes into detergent micelles, followed by one or more steps of affinity purification. This is notoriously challenging, because of the relatively low expression levels of GPCR in membranes and their instability in detergents. In recent years, significant improvements have been made in designing efficient protocols for expression of GPCRs in different cell types, their stabilization, and purification by affinity chromatography.¹⁻⁵

Human cannabinoid receptor CB₂ is primarily expressed in cells of immune origin, and is an attractive target for the development of drugs for management of inflammation, immunological disorders and pain.⁶⁻⁸ Structural studies can provide critical contributions to the rational design of novel, specific drugs targeting this receptor.

Purification of a recombinant GPCR typically involves one or more steps of affinity chromatography, relying on engineered tag(s) fused to the expressed protein. Poly-histidine (immobilized-metal affinity chromatography, IMAC) and FLAG tags have been successfully used.^{3,9-15} However, IMAC is not always efficient for purification of GPCRs, especially for those targets that are expressed at low levels, because of the low affinity of interaction between the His-tag and the resin in detergent-containing buffers. The antibody-epitope interaction exploited by such systems as FLAG-tag/FLAG resin may produce a high purity of the target protein, even in the presence of detergents.^{3,16} However, the application of FLAG resin for preparation of multi-milligram quantities of GPCR is expensive, especially in the case of commercial preparation of affinity resins. Several other affinity tag/matrix pairs have been described in the literature, but very few of them have been applied successfully to preparative-scale production of GPCR.¹⁷

When choosing the tag/affinity matrix pair for purification of membrane proteins for structural studies, the following parameters should be considered:

- High affinity in the presence of detergents (ideally in the picomolar or low nanomolar range)
- Ability to elute the captured protein in mild buffer conditions
- High binding capacity
- Ability to regenerate/re-use the matrix
- Low cost
- Small size of the affinity tag

Currently, there only a few examples of commercial kits that satisfy these requirements. One feasible alternative to the antibody/epitope interaction is a Strep-tag/StrepTactin system that relies on interaction between a small eight amino acid-long epitope (Strep-tag and a modified Streptavidin (StrepTactin). The recently introduced modification of the system StrepTactin XT affords pico- to nano-molar affinities between the Strep-tag and a resin, even in the presence of detergents.¹⁸ This approach has been successfully applied to purification of both soluble and membrane receptors.¹⁹ The limitation of the system is that the protein captured on the resin is eluted with high concentrations of biotin, and the elution kinetics are quite slow. Because of its amphiphilic properties, biotin incorporates readily into detergent micelles, which may interfere with downstream applications of the purified protein.

An alternative strategy utilized in this work relies on a calcium-dependent molecular switch based on the high affinity fragment complementation between the EF1 and EF2 domains of the Ca²⁺-binding protein calbindin D9k.²⁰⁻²³ The high affinity binding of the two EF domains is based on interaction between hydrophobic core residues located in the α helices and loops of these fragments (Figure 1 A). The interaction between these domains can be broken by simply chelating the Ca²⁺ ions resulting in the loss of the helical secondary structure. Therefore, the elution step for EF-based purification of the recombinant protein can be performed at very mild conditions, at physiological pH, with addition of EDTA (Figure 1 B).

We tested the application of such Ca²⁺-dependent complementation in the purification of cannabinoid receptor CB₂.²⁵ The procedure takes advantage of the binding of calcium ions by the EF1 epitope fused to the recombinant receptor, and the EF2 affinity ligand coupled to the agarose matrix. In the presence of calcium, the helix-loop-helix motif on both EF1 and EF2 fragments is restored, facilitating a high-affinity binding of the target receptor to the resin.

Several constructs with the EF1 domain and other expression partners fused in various positions relative to the CB₂ receptor were expressed in *E. coli* cells (Figure 2 A). The sequence of the DNA fragment encoding the 43 amino acid-long EF1 tag was optimized for codon usage of *E. coli* cells. Confirming our earlier observations for expression of CB₂ receptor in bacterial cells,²⁶ we determined that the presence of maltose-binding protein (MBP) of *E. coli* as the N-terminal fusion partner was required for expression of CB₂, and for its accumulation in bacterial cell membranes (Figure 2 B). When expressed with its signal sequence intact, the MBP is transported to the periplasmic space of *E. coli*, and is believed to act as a chaperone directing the downstream part of the fusion (CB₂) to the cytoplasmic membranes.²⁶ The periplasmic location also facilitates the formation of a disulfide bridge in the extracellular loop 2 of CB₂, which is essential for stability of this receptor.²⁷

Consequently, only the fusion constructs that harbor the N-terminal MBP fused to CB₂ were expressed in *E. coli* cells; the construct CB₂-351 (comprising the EF1-CB₂ without an N-terminal MBP) did not appear to express as determined by Western blot with anti-CB₂ antibody (Figure 2 B). Another significant observation is that the location of EF1 tag upstream of CB₂ appears to have a negative effect on the expression levels of this fusion protein (construct 352). The location of EF1 tag at

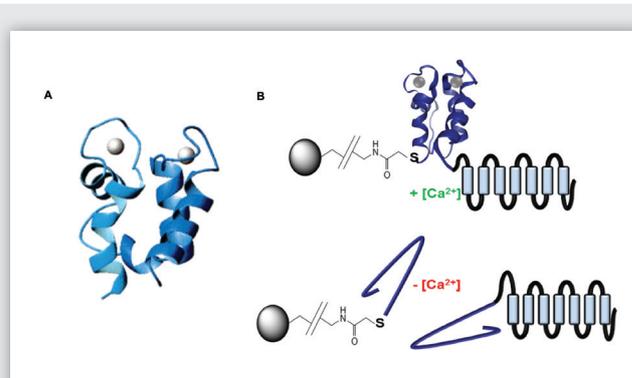


Figure 1. Fragment complementation between EF hand domains of calbindin D9k as a basis for affinity purification (A) Ribbon diagram of calbindin D9k based on X-ray structure coordinates²⁴ (PDB entry 4icb), with calcium ions shown as silver spheres (B) Affinity purification of EF1-tagged protein depends on calcium dependent structural organization of the EF-hands, high-affinity complementation between the EF1 peptide tag and EF2-GGC affinity ligand immobilized on agarose beads (grey) and the dissociation of the bound complex upon chelation of calcium releasing pure protein.

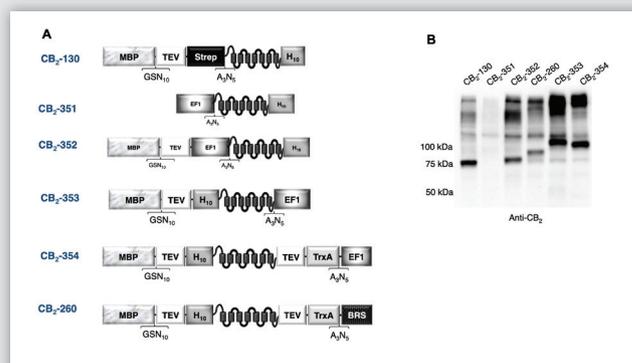


Figure 2 A, Constructs for expression of CB₂ receptor in *E. coli*. 2B. Expression levels of EF1-CB₂ constructs in *E. coli* membranes (determined by Western blot, probed with anti-CB₂ antibody).

the C-terminus of the fusion (constructs 353, 354) did not have any negative effects on expression of these proteins.

We further examined the expression of EF1-CB₂ fusion proteins in other expression hosts besides *E. coli*. Figure 3 depicts the results of the expression of these constructs in suspension culture of Expi293F HEK cells. In these mammalian cells the constructs were expressed without the MBP fusion partner. The sequences of the 43 amino acid- EF1 tag, CB₂, and a small linker were optimized for expression in mammalian cells. The expression levels of both CB₂-352 (EF1 in the N-terminal position) and CB₂-353 (EF1 in the C-terminal position) were approximately equivalent. Both constructs express at levels slightly lower than the control construct CB₂-370 that harbors a twin-Strep-tag (22 amino acids including linker sequence) upstream of CB₂. The expression levels of MBP-CB₂-130 fusion construct in *E. coli* (normalized per milligram of membrane protein) were the highest among the construct and expression hosts tested. These findings underscore the importance of the nature, size and the relative

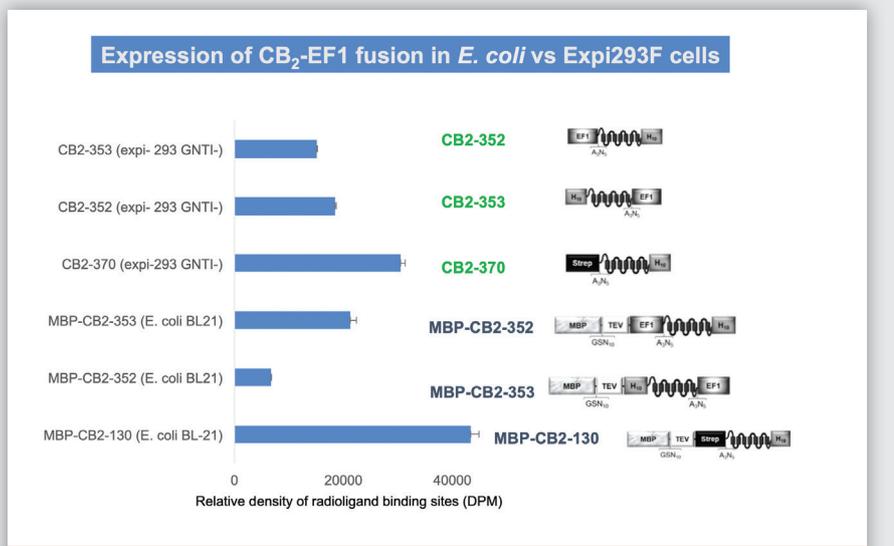


Figure 3. Expression levels of CB₂-EF constructs in Expi293F cells vs *E. coli* cells.

position of the affinity tag on the expression levels of the target protein. Naturally, the effects of the affinity tags on expression levels of the target protein also depend on a host cell. Our data show a potential utility of the EF1 tag for expression of integral membrane GPCR not only in a bacterial but also in a mammalian host.

The functionality of the recombinant receptor can be assessed by a ligand binding assay (typically, using a radioisotope-labeled ligand) and by measuring the activation of G protein on the receptor in response

to ligand binding.^{27,28} Both assays are typically performed on preparations of cell membranes expressing the recombinant receptor. Figure 4A shows the saturation binding of the tritium-labeled high affinity agonist CP-55,940 on the membranes expressing construct CB₂-354. The binding of the ligand is specific, with the K_d~ 1.4 nM, similar to the high affinity binding of this synthetic cannabinoid ligand to other EF1-CB₂ expression constructs.²⁵

Another informative functional test quantifies the rates of activation of G protein on CB₂

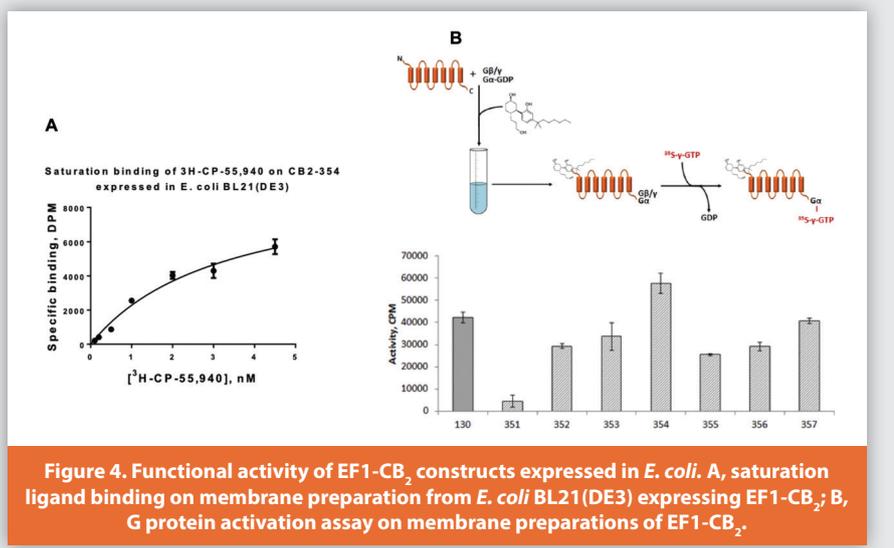


Figure 4. Functional activity of EF1-CB₂ constructs expressed in *E. coli*. A, saturation ligand binding on membrane preparation from *E. coli* BL21(DE3) expressing EF1-CB₂; B, G protein activation assay on membrane preparations of EF1-CB₂.

receptor in response to cannabinoid agonist binding. The assay can be performed with either bacterial membranes expressing CB₂ protein or with purified receptor reconstituted into liposomes. Briefly, the receptor preparation is incubated with saturating concentrations of the activating ligand and is supplemented with purified subunits of G protein (in this case, Gα₁₁ and Gβ_{1γ2}), in the presence of GDP. The reaction is initiated by the addition of radiolabeled analog of GTP, ³⁵S-γ-GTP. This nucleotide analog is poorly hydrolyzible, and upon G protein activation on the receptor it tightly binds to the Gα₁₁ subunit. Thus, the complex of the labeled GTP with G protein accumulates for the duration of the reaction and provides a quantitative assessment of the amount of functional CB₂ receptor. Figure 4B illustrates this concept by comparing membranes expressing different CB₂ constructs. It reveals that the N-terminal (relative to CB₂) location of EF1 tag is detrimental for the expression, while the C-terminal location of the tag results in higher expression levels. Furthermore, the placement of a small thioredoxin epitope between the C-terminus of CB₂ and EF1 tag has a positive effect on expression levels of construct CB₂-354. To summarize, these data confirm the correct folding and full functionality of EF1-CB₂ constructs expressed in *E. coli*.

The purification of CB₂ receptor performed using the EF1-EF2 complementation strategy with affinity resin prepared by thiol coupling of the EF2-GGC peptide to SulfoLink coupling agarose resin as described earlier.²⁵ The recombinant fusion protein is solubilized from cell membranes in detergent micelles comprised of zwitterionic detergent CHAPS and non-ionic n-dodecyl-β-D-maltopyranoside (DDM). For stabilization of the receptor, cholesteryl hemisuccinate (CHS) and a high affinity ligand (CP-55,940) are added, and all purification buffers are supplemented with 15-30% (v/v) glycerol. All steps of purification are performed at 4°C or on ice to preserve the correct fold of the recombinant protein.

The detergent extracts with solubilized receptor are mixed with EF2-agarose, and the binding is typically performed as a batch purification overnight, in the presence of Ca²⁺. The batch mode helps to maximize the

binding capacity of the resin, which can be as high as 0.5 mg of protein per mL of resin. The resin is then washed extensively with Ca^{2+} -containing buffers to ensure that the high affinity interaction between EF1 and EF2 epitopes is maintained. No significant loss of the captured protein is detected during this step. The elution is performed by the addition of EDTA that chelates Ca^{2+} ions. The protein bound to the affinity matrix is released gradually; typically, 4-5 column volumes of EDTA-containing elution buffer are necessary to complete the process (Fig. 5). Both the C-terminal location of the EF1 tag (constructs CB_2 -353, CB_2 -354) and mid-fusion location (construct CB_2 -352) are suitable for purification using this strategy, although in the case of mid-fusion EF1 tag, the binding capacity of the resin appears to decrease, possibly due to steric hindrance effects from the large MBP fusion partner. Also, in the case of construct CB_2 -352 some spontaneous cleavage of the fusion construct can occur during purification that results in the removal of MBP and release of the EF1-CB_2 . Importantly, a significant purity of the protein preparation can be attained after just one chromatography step, with an enrichment factor for the EF1-CB_2 protein of at least 1000-fold.

Both the capture and elution steps are performed at neutral pH values. The mild condition of the purification preserve the functional structure of the receptor, as confirmed by activity tests of the purified products as described earlier.²⁵

We next determined the binding kinetics between EF1-CB_2 proteins and immobilized EF2-GGC in the detergent and glycerol rich solubilization buffer used to purify EF1-CB_2 at 25, 10 and 4 degrees Celsius. EF2-GGC that was thiol coupled to the carboxymethyl dextran surface of a BiaCore CM5 sensor chip and purified EF1-CB_2 -352 and EF1-CB_2 -354 fusions were injected over the surface for 30 minutes followed by buffer flow for 60 minutes. EF1 wild type peptide was injected as a positive control (Figure 6A).²⁵ The equilibrium dissociation constant, K_D , for the CB_2 -354 – EF2 interaction was estimated from the ratio of the fitted rate constants as $K_D = k_{\text{off}} / k_{\text{on}} = 3.10^{-9}$ M (Figure 6B) and 5.10^{-9} M for EF1-CB_2 -352, that had an apparently faster off rate than that of EF1-CB_2 -354, which while not significant, may be due to the N terminal

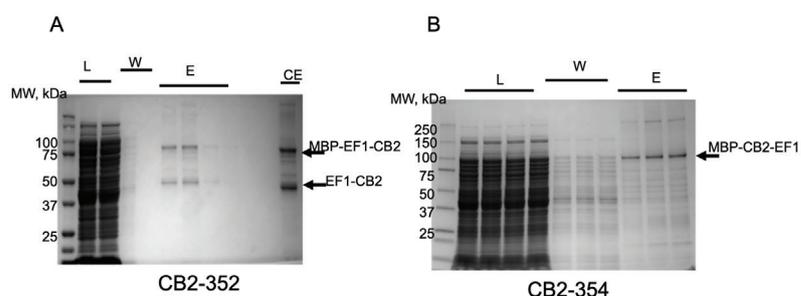


Figure 5. Purification of EF1-CB_2 constructs on EF2 agarose resin. SDS-PAGE stained with Instant Blue. A, construct CB_2 -352. B, Construct CB_2 -354. L, load (crude solubilized protein extract); W, wash fractions; E, elution fractions; CE, combined, concentrated elution fractions. The position of MBP-EF1-CB_2 fusion protein and the cleaved EF1-CB_2 are indicated with arrows.

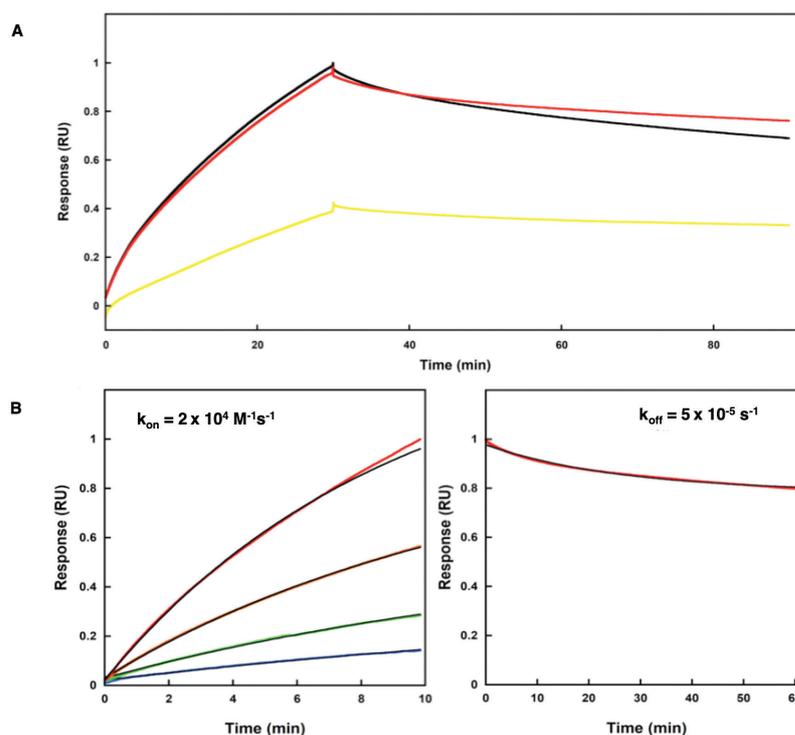


Figure 6. SPR analysis of the interaction between EF1-CB_2 fusion proteins and EF2-GGC functionalised surface in solubilization buffer. (A) Sensorgrams showing the relative response recorded during injection of CB_2 -352 (black), CB_2 -354 (red) and EF1 peptide (yellow) at a concentration of 100 nM, followed by buffer flow, over the EF2-GGC surface. (B) Fitting to (i) the association phase in a concentration series of 100 nM (red), 50 nM (orange), 25 nM (green) and 12.5 nM (blue) CB_2 -354, and to (ii) the dissociation phase after injecting 100 nM CB_2 -354. The equilibrium dissociation constant, K_D , for the CB_2 -354 – EF2 interaction was estimated from the ratio of the fitted rate constants as $K_D = k_{\text{off}} / k_{\text{on}} = 3.10^{-9}$ M.

location of the EF1 -tag in this fusion. The maintenance of the high affinity of interaction between EF1 and EF2 sequences in this buffer at low temperature, with the simple

method of elution, highlights the potential to optimize this system further in the capture, purification and analysis of functional integral membrane proteins.

To summarize, the use of EF1 tag for recombinant production of CB₂ and affinity purification on EF2 matrix facilitates the isolation of the fully functional protein in just one chromatography step. The yield of the recombinant protein may be further improved by optimizing the expression conditions and purification parameters. The technology of preparation of the recombinant CB₂ can be adapted to expression and purification of other GPCR and recombinant receptors and open a path for a meaningful biophysical characterization of these proteins.

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