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Expression, surface immobilization, and characterization of functional recombinant cannabinoid receptor CB_2

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ABSTRACT

Human peripheral cannabinoid receptor CB_2, a G protein-coupled receptor (GPCR) involved in regulation of immune response has become an important target for pharmaceutical drug development. Structural and functional studies on CB_2 may benefit from immobilization of the purified and functional receptor onto a suitable surface at a controlled density and, preferably in a uniform orientation. The goal of this project was to develop a generic strategy for preparation of functional recombinant CB_2 and immobilization at solid interfaces. Expression of CB_2 as a fusion with Rho-tag (peptide composed of the last nine amino acids of rhodopsin) in *E. coli* was evaluated in terms of protein levels, accessibility of the tag, and activity of the receptor. The structural integrity of CB_2 was tested by ligand binding to the receptor solubilized in detergent micelles, captured on tag-specific monoclonal 1D4 antibody-coated resin. Highly pure and functional CB_2 was obtained by sequential chromatography on a 1D4- and Ni-NTA-resin and its affinity to the 1D4 antibody characterized by surface plasmon resonance (SPR). Either the purified receptor or fusion CB_2 from the crude cell extract was captured onto a 1D4-coated CM4 chip (Biacore) in a quantitative fashion at uniform orientation as demonstrated by the SPR signal. Furthermore, the accessibility of the extracellular surface of immobilized CB_2 and the affinity of interaction with a novel monoclonal antibody NAA-1 was studied by SPR. In summary, we present an integral strategy for purification, surface immobilization, ligand- and antibody binding studies of functional cannabinoid receptor CB_2.

1. Introduction

Human peripheral cannabinoid receptor CB_2, a 7-transmembrane domain, G protein-coupled receptor (GPCR) is part of the endocannabinoid system and is primarily found in tissues and cells of the immune system. CB_2 mediates physiological pathways implicated in regulation of the immune response primarily via inhibition of adenylate cyclase [1].

With the goal of performing structural and functional studies on CB_2, procedures for expression of the receptor in *E. coli*, chromatographic affinity purification using His- and Strep-tagged constructs and stabilization in detergent micelles have been developed in our laboratory [2–4]. However, certain challenges inherent to this target protein still need to be overcome. For example, the necessity of using detergents for protein solubilization during purification has known negative influence on the affinity of His- and Strep-tags to their respective resins, thus directly impacting protein yield and purity. Characterization of the functional properties of the recombinant CB_2 by ligand binding is also non-trivial due to the strong hydrophobicity of cannabinoid ligands, endogenous 2-AG and anandamide, but also of the plant derived- and synthetic cannabinoids like Δ^9-tertahydrocannabinol (Δ^9-THC) and the high-affinity agonist CP-55,940. Finally, immobilization of the receptor on a solid support for ligand binding or surface-plasmon-resonance (SPR) assays requires careful preservation of the functional structure of CB_2 at conditions that are potentially harmful for stability of this very labile protein [2]. The objective of this work was to develop procedures for tight, reversible and specific surface immobilization of CB_2 for purification, functional characterization, and the study of molecular interactions including binding affinity and kinetics by SPR.

In a search for a small affinity tag that would be compatible with high level expression of a functional GPCR in *E. coli*, efficient purification in the presence of detergents, and a specific, tight and reversible surface immobilization, we selected a peptide composed of the last nine amino acid residues of bovine rhodopsin (Rho-tag) that selectively binds to 1D4 monoclonal antibody. The 1D4 antibody was first introduced and characterized by Molday et al. [5] and is widely used for affinity purification of rhodopsin [6] and other proteins engineered to contain the epitope [7], as well as in expression of other GPCR like CB_1-Rho-tag [8], the chemokine receptors CXCR4 and CCR5 [9], a series of GPCR expressed in a cell-free system [10], and for production of

Abbreviations: CB_2, peripheral cannabinoid receptor; GPCR, G protein-coupled receptor; MBP, maltose binding protein; NTA, nitrilotriacetic acid; SPR, surface plasmon resonance; TEV, tobacco etch virus; CHS, cholesteryl hemisuccinate Tris salt; CHAPS, 3[(cholamidopropyl)dimethylammonio]-1-propanesulfonate; DDM, *n*-dodecyl-β-D-maltoside; OG, N-octyl-β-D-glucopyranoside; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine; DTPA, diethylenetriamine pentaacetate

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paramagnetic liposomes [11,12]. For SPR, the Rho-tag was first used to capture GPCR from crude cell lysates on a hydrazide-modified L1 sensor chip (Biacore) via 1D4 antibody followed by reconstitution of a lipid environment, and on CM5 (Biacore) sensor chip without reconstitution into bilayers by Stenlund et al. [13]. Later attempts have used a CM4 chip (Biacore) for immobilization of 1D4 and consecutive capturing of GPCR with a Rho-tag to explore solubilization [14,15] and crystallization [16] conditions for chemokine receptors, as well as binding of ligands and small-molecule inhibitor to CXCR4 and CCR5 receptors [17,18].

Although the Rho-tag/1D4 antibody system has been used successfully for purification of several other GPCR expressed in eukaryotic cells, the expression of Rho-tagged proteins in bacterial cells has not been explored so far. Therefore, the first stage of our study was devoted to examining the suitability of the Rho-tag for expression of the fusion construct in *E. coli*. The functional characterization and purification of the Rho-tagged CB_2 from the best performing constructs was carried out taking advantage of Rho-tag interaction with resin-immobilized 1D4 antibody. Finally, we characterized the Rho-tag/1D4 interaction in detergent-containing buffers and used the Rho-tag on CB_2 for surface capturing on CM4-1D4 antibody-covered chips for surface plasmon resonance (SPR) experiments to assess the efficiency of capturing, the feasibility of enzymatic removal of the MBP fusion partner of the immobilized receptor, and its interaction with a monoclonal NAA-1 antibody raised against CB_2.

2. Materials and methods

2.1. Chemicals and reagents

Oligonucleotides were purchased from Operon Biosciences. Restriction- and DNA-modifying enzymes were obtained from New England Biolabs. The Ni-NTA resin was from Qiagen, the CNBr-activated Sepharose from GE Healthcare. The monoclonal antibody against CB_2 (NAA-1) was from Epitomics and 1D4 antibody was from the University of British Columbia, Vancouver, Canada. [^{3}H]-CP-55,940 (specific activity 139.6 Ci/mmol) and [^{35}S]-γ-GTP (specific activity 1250 Ci/mmol) were purchased from Perkin-Elmer. Alexa Fluor488 reactive dye was from Invitrogen. 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. N-Octyl-β-D-glucopyranoside (OG) was purchased from Calbiochem. 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.

2.2. Expression vectors and strains

E. coli strain DH5α was obtained from Invitrogen and *E. coli* strain BL21(DE3) was purchased from Agilent Technologies. Plasmid for expression of G_α was a kind gift from Dr. J. Northup (NIDCD/NIAAA, NIH). The plasmid for expression of MBP-TEV protease (pRK1043) was a gift from Dr. D.S. Waugh (NCI-Frederick, NIH).

2.3. Expression and purification of CB_2

Construction of plasmids, expression of CB_2 fusion proteins[1], preparation of membranes, functional characterization, solubilization of CB_2 into detergent micelles, expression and purification of TEV protease, expression and purification of subunits of G proteins, preparation and regeneration of 1D4-Sepharose resin and chromatographic purification of CB_2 are described in Supplemental materials.

2.4. Ligand binding on CB_2 in detergent micelles

Ligand binding on CB_2 in detergent micelles was performed as follows.

2 mL of wash buffers was prepared by mixing 50 mM Tris, pH 7.5 containing 200 mM NaCl, 30% (v/v) glycerol, 0.5% (w/v) CHAPS, 0.1% (w/v) DDM, and 0.1% (w/v) CHS (buffer A). Buffers were supplemented with a mixture of [^{3}H]-CP-55,940 and unlabeled CP-55,940 (specific activity 50 mCi/mmol), so that the ligand concentration ranged from 0 to 50 μM, and kept on ice until use.

Fusion CB_2-255(Table 1) was purified on a 1D4-Sepharose (as described in Supplemental materials), and the resin with immobilized protein was stored frozen at −80 °C until use. The resin was re-suspended in 2 bed volumes of ice-cold buffer A supplemented with 10 μM of stabilizing ligand CP-55,940, and aliquoted into 0.5 mL Ultrafree centrifugal filters (PVDF 0.45 μm pore diameter, Millipore). Typically, each sample contained 50 μL of resin with ~25 μg of immobilized CB_2-255. Samples were washed 3× with 300 μL of buffer A + 10 μM CP-55,940 at 1500 ×*g*, 1 min each in a refrigerated Eppendorf centrifuge. The stabilizing ligand (unlabeled CP-55,940) was then removed by washing the resin 5× with 200 μL of corresponding [^{3}H]-CP-55,940-containing wash buffers and by centrifugation at 500 ×*g* for 1.5 min. The resin was then re-suspended in 200 μL of a wash buffer and incubated on ice for additional 2 h. Upon incubation, samples were centrifuged at 12,000 ×*g* for 30 s and washed 4× with 300 μL ice-cold buffer A (without ligand). The resin was re-suspended in 250 μL of elution buffer (buffer A supplemented with 4 mM Rho peptide and NaCl concentration raised to 1 M) and incubated for 15 min on ice, and the eluate was collected by centrifugation (500 ×*g*, 2 min). The elution was repeated 3 more times, and the eluate fractions were combined. An aliquot was used to determine the content of [^{3}H]-CP-55,940 on a scintillation counter.

Since the concentration of the radiolabeled CP-55,940 in this assay is in the micromolar range, it is not feasible to determine levels of non-specific binding by adding much higher concentrations of non-labeled ligand. Therefore, to correct for non-specific binding of CP-55,940, we measured the amount of the radiolabeled ligand that remained bound to the receptor immobilized to the resin upon exposure of the sample to 65 °C for 1 h. We have shown earlier that such treatment completely inhibits specific ligand binding to CB_2 [2]. The levels of nonspecific binding to the resin with immobilized receptor did not exceed 25% of the total binding (results not shown).

2.5. Thermoinactivation of CB_2 in micelles

The thermoinactivation of CB_2 in detergent micelles was measured by quantifying the loss of binding of [^{3}H]-CP-55,940 to resin-immobilized fusion CB_2-255. The experiment was performed as follows: 50 μL of 1D4-Sepharose containing 25 μg of purified fusion CB_2-255 was dispensed in Ultrafree centrifugal filters (Millipore) on ice and washed 3× with 250 μL of wash buffer (either buffer A or buffer A without CHS) supplemented with 20 μM of CP-55,940 by centrifugation in a refrigerated centrifuge at 1500 ×*g* for 1 min. The resin was re-suspended in 200 μL of wash buffer and placed into a water bath kept at 4 °C. A set of samples was then exposed to a linear temperature gradient of 1 °C/min from 4 to 84 °C and one sample was withdrawn at temperature intervals of 10 °C and placed on ice. Samples were then centrifuged at 1500 ×*g* for 1 min, and washed 3 times with 250 μL of buffer A supplemented with 20 μM CP-55,940 and 3.6 nM of [^{3}H]-CP-55,940 by centrifugation at 500 ×*g* for 2 min. The resin was re-suspended in 200 μL of the same buffer and incubated

[1] For convenience we define fusion as CB_2 fused at its N-terminus with the *E. coli* maltose binding protein (MBP), while in the purified CB_2 this expression partner is no longer present. Other tags may or may not be present in the purified CB_2, depending on a construct (see Table 1).

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