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Signal Peptide and Denaturing Temperature are Critical Factors for Efficient Mammalian Expression and Immunoblotting of Cannabinoid Receptors*

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Summary

Many researchers employed mammalian expression system to artificially express cannabinoid receptors, but immunoblot data that directly prove efficient protein expression can hardly be seen in related research reports. In present study, we demonstrated cannabinoid receptor protein was not able to be properly expressed with routine mammalian expression system. This inefficient expression was rescued by endowing an exogenous signal peptide ahead of cannabinoid receptor peptide. In addition, the artificially synthesized cannabinoid receptor was found to aggregate under routine sample denaturing temperatures (i.e., 95°C), forming a large molecular weight band when analyzed by immunoblotting. Only denaturing temperatures 75°C yielded a clear band at the predicted molecular weight. Collectively, we showed that efficient mammalian expression of cannabinoid receptors need a signal peptide sequence, and described the requirement for a low sample denaturing temperature in immunoblot analysis. These findings provide very useful information for efficient mammalian expression and immunoblotting of membrane receptors.

Keywords

cannabinoid receptor 1; cannabinoid receptor 2; denaturing temperature; signal peptide; mammalian expression

Cannabinoid receptors (CNRs) are believed to play a critical role in mediating a great number of the biological activities ascribed to plant-derived, synthetic and endogenous cannabinoids. To date, two CNRs, including cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2), have been identified and cloned^[1, 2], though there is mounting evidence suggesting the existence of other receptors through which cannabinoids signal^[3, 4]. Both

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CB1 and CB2 receptors belong to the rhodopsin superfamily of G protein-coupled receptors (GPCRs) and are coupled with Gi or Go proteins^[5]. They share a common membrane topology, possessing an intracellular C-terminus, the signature seven hydrophobic transmembrane (TM) domains and an extracellular N-terminus, with other members of GPCRs.

Membrane proteins typically have a signal sequence in their N-terminus to ensure proper trafficking to the cell surface and avoidance of degradation by proteasomes during the process of protein maturation. However, the majority (about 90%) of GPCRs, including CB1 and CB2, do not contain a signal peptide. This contradiction seems to be resolved in the cellular environment by some yet to be elucidated mechanism.

Strategy of artificial expression of CNRs has been employed for different research purposes, such as for the study of ligand binding affinity, signal transduction, receptor trafficking and metabolism, or for generating large amounts of CNR protein^[6–9]. Overall, the expression systems used in these investigations can be generally divided into two groups. One group uses prokaryotic or fungal expression systems to produce milligram amounts of CNR protein for purification and structural studies, which in most cases included an exogenous signal peptide in the N-terminus of protein to facilitate expression^[9, 10]. Conversely, others have applied mammalian expression systems for certain functional study, where no signal peptide is used^[7]. In the latter scenario, the efficient expression of receptor was evidenced indirectly by functional experiments, and not by direct immuno-analysis. However, direct confirmation of efficient protein expression is normally an obligation for artificial expression research. We speculated there must be some unknown obstacles that prevented researchers from directly confirming efficient expression of CNR proteins by immunoblotting.

In present study, we reproduced mammalian expression of CNR (rat CB1 or human CB2) protein engineered with N- or C-terminal Flag peptide epitope. However, we found neither could be efficiently expressed with routine expression vectors until a plasmid vector with a preset signal peptide sequence, named preprotrypsin, was applied. Furthermore, when performing Western immunoblot analysis, we found that under routine sample denaturing temperatures (i.e., 95°C) CNR fusion proteins aggregated and formed a large molecular weight band characteristic of protein aggregates in immunoblots. Only denaturing temperatures 75°C resulted in band(s) of the predicted molecular weight.

1 MATERIALS AND METHODS

1.1 Construction of Plasmids

Primers for the full length of CNR (rat CB1 and human CB2) coding region were designed from the cDNA retrieved from GenBank (accession No. NM_012784 and NM_001841). Four new plasmids were constructed based on two plasmid vectors, pCMV-Tag1 (without signal sequence) (Stratagene, USA) and pFlag-CMV-3 (with signal sequence) (Sigma-Aldrich, USA). Among them, pFlag-hCB2 and phCB2-Flag were derived from pCMV-Tag1 with N- or C-terminal Flag-epitoped hCB2, respectively. pSig-Flag-hCB2 and pSig-Flag-rCB1 were derived from pFlag-CMV-3 with a preprotrypsin signal peptide and N-terminal Flag-epitoped hCB2 or rCB1. Total RNA was extracted from either human leukemia cell line HPB-ALL or Sprague-Dawley rat brain and then reversely transcribed into first-strand cDNA. PCR reaction was performed with HF-2 DNA polymerase (Clontech, USA). All PCR products were purified and sub-cloned into pCMV-Tag1 or pFlag-CMV-3 vector. All new constructs were submitted to sequencing.

1.2 Mammalian Expression of CNRs

HEK293, CHO-K1 and RINm5F cell lines were obtained from the American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium (Invitrogen Life Technologies, USA) supplemented with 10% fetal bovine serum (Invitrogen Life Technologies, USA) in a humidified 5% CO₂ atmosphere at 37°C. The transfection was performed with Lipofectamine2000™ (Invitrogen Life Technologies, USA) according to the manufacturer's instructions. Transfected cells were harvested for mRNA and protein isolation 24 h after transfection.

1.3 Analysis of CNRs Protein by Western Blotting

Whole cell lysate was prepared with RIPA buffer (20 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mmol/L EDTA, 0.1% SDS) including Protease Inhibitor Cocktail (Roche, USA). Cell membranes were prepared by pipetting cells up and down in hypotonic lysis buffer (7.5 mmol/L sodium phosphate, 1 mmol/L EDTA, pH 7.5) with Protease Inhibitor Cocktail. The nuclei were removed by discarding the pellet after centrifugation at 500 r/min and 4°C for 10 min. The supernatant was centrifuged again at 200 000 r/min and 4°C for 1 h. The membrane pellet was collected and solubilized in 2% SDS, normalized by amount of total protein following the BCA protein quantification (Pierce, USA). Protein samples (10 µg/lane to 100 µg/lane according to different detection purposes) were fractionated by SDS-PAGE and transferred to Hybond ECL nitrocellulose membrane (Amersham Biosciences, USA). The blots were probed with various antibodies, respectively, including anti-Flag antibody (M2 clone, Sigma-Aldrich, USA), anti-CB2 antibody (Kindly provided by Dr. Ken Mackie, University of Washington, USA) and anti-CB1 antibody (Cayman Chemical, USA). Enhanced chemiluminescence detection system was applied to visualize antibody-bound protein.

1.4 Analysis of hCB2 mRNA Level with Real-time PCR

Total RNA was reversely transcribed by using Taqman Transcription Reagent Kit (Applied Biosystems, USA). Real time PCR was performed with SYBR Core Kit in an ABI Prism 7700 system (Applied Biosystems, USA). The upstream primer for hCB2 was 5'-CCGCCATTGACCGATACCT-3' and the downstream primer was 5'-CTAGTGCTGAGAGGACCCACATG-3'.

2 RESULTS

2.1 Inefficient Expression of CB2 Fusion Protein in the Absence of an Exogenous Signal Peptide in a Competent Mammalian Expression System

The DNA sequences of all four constructs were carefully compared with those obtained from Genbank using MacVector software. The results showed the constructs were all verified for expression experiments. Construct pFlag-hCB2, derived from pCMV-tag1, which did not contain a signal sequence, was transfected into HEK293 cells. CB2 mRNA levels were confirmed to be greatly increased (table 1) after transfection by real time PCR. Surprisingly, no Flag-CB2 fusion protein was detected by immunoblot analysis, either in whole cell lysates or cell membrane preparations from transfected cells (fig. 1). To demonstrate the functionality of the mammalian expression system, a positive control plasmid pCMV-Luc-Flag (Stratagene) was transfected into HEK293 cells using identical conditions. Luc-Flag fusion protein was found to be efficiently expressed and readily detected (fig. 1). These results suggested that the expression system was functional.

Further, to exclude the possibility that the N-terminal Flag epitope (8aa peptide, DYKDDDDK) interferes with the first TM or other potential signal sequences, Flag was substituted to the C-terminal of CB2 sequence, i.e. phCB2-Flag, but as before, no CB2-Flag

fusion protein was detected in transfected cells (fig. 1). To verify this inefficient expression was not unique to HEK293 cells, two additional mammalian cell lines including RINm5F (rat) and CHO-K1 (hamster) were applied in transfection experiments. No CB2 fusion protein was detected while Luc-Flag fusion protein always could be efficiently expressed (data not shown).

2.2 Efficient Expression of CNR Fusion Proteins Containing a Preprotrypsin Signal Peptide Sequence

Considering that the inefficient expression of CB2 might be due to the lack of a proper leading sequence, we used a plasmid vector pFlag-CMV-3 containing a preprotrypsin signal peptide sequence. Any fusion protein expressed using this vector contains a preprotrypsin signal peptide during the translation process.

The derivative pSig-Flag-hCB2 was transfected into HEK293 cells. As expected, Flag-hCB2 was efficiently expressed and detected in immunoblots by either anti-Flag antibody or anti-CB2 antibody (fig. 2A). Similar results were obtained from transfected CHO-K1 or RINm5F cells (data not shown). To further confirm that the exogenous signal peptide sequence is necessary for efficient expression, the rat CB1 coding sequence was cloned into the same vector and Flag-rCB1 fusion protein was also efficiently expressed in transfection experiments (fig. 2B).

2.3 Aggregation of CNR Fusion Proteins under High Denaturing Temperature

Interestingly, when performing Western immunoblot analysis, we found that routine sample denaturing temperatures, i.e. 95°C, would cause CNR fusion proteins to aggregate and form a clear band at the top of the gel (fig. 3). Initially, the identity of this band was unknown. To diminish its presence several approaches were attempted including using different reducing agents (DTT or 2-Mercaptoethanol) and SDS concentrations (2% or 4%) in the sample loading buffer, decreasing the PAGE-gel percentage from 10% to 7.5%, and decreasing the amount of protein that was loaded per lane. None of these factors eliminated the unknown protein band (data not shown) until serial sample denaturing temperatures were applied. Clear CNR fusion protein bands of the predicted size appeared in immunoblots with a proportional diminution of the unknown band when denaturing temperatures below 75°C were employed, suggesting that the large unknown molecular weight band was most likely an aggregated form of CNR fusion proteins (fig. 3). This conclusion was further supported by the fact that protein samples from mock and positive (Luc-Flag fusion protein) transfection control never showed this large molecular weight band at any denaturing temperatures tested.

3 DISCUSSION

Both CB1 and CB2 appear to lack a signal peptide in the N-terminus as is typical of most GPCRs. In the case of CNRs, the first TM domain was thought to function as a targeting sequence (reverse signal anchor sequence) for the protein to initiate the maturation process, such as translocation, modification and assembly. However, Andersson and colleagues^[11] discovered in an artificial expression system that the long N-terminal tail of CB1 (about 116 amino acids), severely hampered the translocation of the receptor into endoplasmic reticulum (ER) and thus resulted in more than 90% receptor degradation within an hour. They also found that shortening the N-terminus or the addition of a signal peptide sequence at the N-terminus could greatly enhance the stability and cell surface expression of CB1. Based on these experiments, they predicted that GPCRs with a short N-terminal region, such as CB2 (33 amino acids), could be efficiently expressed in an artificial expression system.

However in the present study, our results suggest that the short N-terminus of CB2 also hindered the translocation of the protein into the ER, as was observed with the native CB1 N-terminus, likely leading to rapid degradation of the protein by proteasomes. Based on these results, we speculated the length of N-terminus played a role in the protein maturation process of CNRs, but not a determinant role. At least in artificial expression systems, an exogenous signal peptide sequence is necessary for efficient CNRs protein expression. It was reported that in a physiological environment some unknown cellular factors were required to facilitate the protein maturation process of CNR and other GPCRs lacking a spontaneous signal peptide^[12–14]. However, in artificial expression systems, where large amounts of proteins are synthesized, these factors may be absent or insufficient, thus leading to the degradation of immature proteins.

An interesting finding in present study is the aggregation of expressed CNR fusion protein under routine denaturing temperature. Normally in Western immunoblot analyses the mature protein structure (i.e., tertiary or quaternary structure) is disrupted using detergent (SDS), a reducing agent (DTT or 2-ME) and high denaturing temperatures. Likewise, mature proteins are returned to primary peptides and peptide side-chains of amino acid residues are bound by SDS, which results in rich negatively charged peptide-SDS complexes to keep proteins isolated, linear and soluble. In light of this, it is highly unusual for CNR fusion proteins to aggregate under such rigorous conditions. One possible explanation may involve the highly hydrophobic domains within CNR peptides, such as TM domains. These hydrophobic structures may not interact under mild temperature conditions but at higher temperatures may gather together to form peptide aggregates across hydrophobic regions.

In summary, although mechanisms remain obscure, two novel observations made in the present study provide very useful information for efficient mammalian expression of CNRs and which are likely applicable to other GPCRs or membrane proteins lacking a signal peptide. First, employment of an exogenous signal peptide can facilitate efficient expression of CNR proteins. Second, low sample denaturing temperatures will prevent protein aggregation of CNRs for immunoblot analysis.

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Fig. 1.

CB2 fusion protein with N- or C-terminal Flag could not be properly expressed, in contrast to the positive control, Luc-Flag fusion protein. Immunoblot analysis was performed with anti-Flag antibody.

Trans: HEK293 cells were transfected with pFlag-hCB2, phCB2-Flag or pCMV-Luc-Flag.

Mock: mock transfection control.

Data were representative of 3 independent experiments.

Fig. 2.

CNRs fusion protein was properly expressed when endowed with the signal peptide, preprotrypsin. Immunoblot analysis was performed with anti-Flag or anti-CB2 antibody (A) and anti-Flag or anti-CB1 antibody (B).

Trans: HEK293 cells were transfected with pSig-Flag- hCB2 or pSig-Flag-rCB1. Mock: mock transfection control.

Data was representative of 3 independent experiments.

Fig. 3.

Aggregation of Flag-CB2 fusion protein was diminished in the presence of decreasing sample denaturing temperatures. Immunoblot analysis was performed with anti-Flag antibody.

Transfect: HEK293 cells were transfected with pSig- Flag-hCB2. Mock: mock transfection control.

Temp: sample denaturing temperatures. RT: room temperature (i.e. 25°C), i.e. not heated.

Data were representative of at least 3 independent experiments.