Validating Antibodies to the Cannabinoid CB2 Receptor: Antibody Sensitivity Is Not Evidence of Antibody Specificity

Yannick Marchalant,[1] Philip W. Brownjohn,[1] Amandine Bonnet, Torsten Kleffmann, and John C. Ashton

Aix-Marseille University, Neurobiologie des Interactions Cellulaires et Neurophysiopathologie, Marseille, France (YM,AB); Department of Pharmacology and Toxicology (PWB,JCA); Centre for Protein Research, Biochemistry Department, University of Otago, Dunedin, New Zealand (TK)

Summary

Antibody-based methods for the detection and quantification of membrane integral proteins, in particular, the G protein-coupled receptors (GPCRs), have been plagued with issues of primary antibody specificity. In this report, we investigate one of the most commonly utilized commercial antibodies for the cannabinoid CB2 receptor, a GPCR, using immunoblotting in combination with mass spectrometry. In this way, we were able to develop powerful negative and novel positive controls. By doing this, we are able to demonstrate that it is possible for an antibody to be sensitive for a protein of interest—in this case CB2—but still cross-react with other proteins and therefore lack specificity. Specifically, we were able to use western blotting combined with mass spectrometry to unequivocally identify CB2 protein in over-expressing cell lines. This shows that a common practice of validating antibodies with positive controls only is insufficient to ensure antibody reliability. In addition, our work is the first to develop a label-free method of protein detection using mass spectrometry that, with further refinement, could provide unequivocal identification of CB2 receptor protein in native tissues. (J Histochem Cytochem 62:395–404, 2014)

Introduction

The problem of antibody reliability in research has now reached the point where many journals reject any work that lacks adequate controls for antibody validation. Despite this, it is still common for researchers to rely on positive controls and only minimal negative controls (such as blocking peptide controls). Using careful cross-comparisons between antibody labeling and other labeling techniques (such as autoradiography or in situ hybridization) it is possible to criticize and even validate antibodies using this approach; for example, that performed for the cannabinoid CB1 receptor in the analysis carried out by Grimsey and colleagues (Grimsey et al. 2008). However, the fundamental distinction between antibody sensitivity and specificity, corresponding to positive and negative controls, respectively, remains insufficiently appreciated. In this study, we developed a very strong positive control for immunoblot labeling of the cannabinoid CB2 receptor. Our work shows clearly that an antibody may pass stringent positive controls, but still fail specificity tests using strong negative controls.

Antibodies for G protein-coupled receptors (GPCRs), of which the cannabinoid CB2 receptor is a member, have been particularly challenging (Ashton 2011; Baek et al.

Corresponding Author:
Philip W. Brownjohn, The School of Physical Education, Sport and Exercise Sciences, University of Otago, PO Box 56, Dunedin 9054, New Zealand.
Email: phil.brownjohn@gmail.com

2013; Brownjohn and Ashton 2012). Despite this, only very recently have commonly used CB2 antibodies been critically scrutinized using CB2 knock-out mice and other controls for their use in western blotting (Cecyre et al. 2013). Despite a dearth of critical testing up to now, western blotting continues to be used to determine apparent CB2 expression, with one antibody in particular used in the majority of these studies (Alvaro-Bartolome and Garcia-Sevilla 2013; Brownjohn and Ashton 2012; Choi et al. 2013; Curto-Reyes et al. 2010; Gong et al. 2006; Walczak et al. 2005; Wotherspoon et al. 2005) despite the validity of this antibody being called into question over a decade ago (Matias et al. 2002). This antibody, No. 101550 from the Cayman Chemical Ltd. (Ann Arbor, MI) is raised in rabbit against amino acids 20-33 (NPMKDYMILSGPQK) of the human CB2 receptor N-terminus. One problem in evaluating this antibody is that it is polyclonal; consequently, it is difficult to ascertain if different lots have been produced and employed in the literature over the considerable period of its manufacture.

Our results may help to explain why CB2 has been called a receptor with an "identity crisis" (Atwood and Mackie 2010). Although expression of CB2 in peripheral immune cells is well established (Galiegue et al. 1995; Munro et al. 1993), CB2 expression in the CNS is controversial (Atwood and Mackie 2010). We argue that—at least with respect to immunoblotting—the situation is not so much one of an identity crisis, but of "identity theft", with cross-reacting protein(s) confounding results obtained for an antibody that appears to be sufficiently sensitive to label CB2, even as it lacks specificity for CB2. In the course of our experiments, we assayed CB2-overexpressing and CB2-negative cell lines, as well as spinal cord, spleen and cortex tissues for possible CB2 content. The spinal cord was used because it has been extensively studied as a possible target for CB2 agonists in neuropathic pain and because of our previous experience with this antibody in spinal cord tissues (Brownjohn and Ashton 2012).

Materials & Methods

Cell Culture

Irradiated Chinese hamster ovary (CHO-K1) cells overexpressing the rat CB2 receptor (EZ Cells™, Applied Cell Sciences Inc.; Rockville, MD) and non-irradiated CHO-K1 cells over-expressing the human neuropeptide FF receptor (as a negative control for CB2 expression) (GenScript USA Inc.; Piscataway, NJ) were grown in Ham's F12 media, supplemented with 10% fetal bovine serum in 95% O_2, 5% CO_2 at 37C. Cells were harvested after 48 hr for western blotting and mass spectrometry analyses.

Rodent Tissue

All experiments at the University of Otago were approved by the University of Otago Animal Ethics Committee, and all experiments at Aix-Marseille University were approved by the Ethics Committee of the Medical Faculty of Marseille and conform to National and European regulations (European Community Council Directive of November 24th 1986 (86-609/EEC). Rats used in this study were naïve male Wistar rats (200–350 g), which were kept in a 12 hr light/dark cycle with access to food and water ad libitum. Rats were decapitated following brief anesthesia with CO_2, and spleens as well as L_4-L_6 lumbar spinal cords were rapidly removed. All tissue samples were immediately frozen and stored at -80C until use. Mice used in this study were wild type and CB2 knock-out mice (Buckley et al. 2000), from a C57BL background, which were also kept in a 12 hr light/dark cycle. Mice were sacrificed before dissection of spleen and cortex, and tissues were snap-frozen with liquid nitrogen.

Western Blot

Sample Preparation. CHO-K1 cells, rat spleen and lumbar spinal cords were homogenized and sonicated in homogenization buffer (50 mM Tris-HCl, pH 7.6) containing complete protease inhibitor cocktail (Roche Applied Science; Indianapolis, IN). For whole preparations of native rat tissues, homogenized samples were centrifuged at 13,000 ×g for 10 min at 4C to remove undisrupted tissue, and the supernatant was stored at -80C until use. The supernatant was then combined with an equal volume of loading buffer (4% SDS, 20% glycerol, 10% 2-mercapto-ethanol, 2 mM EDTA, and 0.025% bromophenol blue in 125 mM Tris, pH 6.8) and incubated at 95C for 5 min. Alternatively, for membrane enrichment of cells and native tissues, homogenized samples were first centrifuged at 1000 ×g at 4C for 10 min to remove undisrupted tissue, and the resulting supernatants further centrifuged at 50,000 ×g for 10 min. The second supernatant, largely containing soluble cytosolic components, was discarded, and the membrane rich pellet was re-suspended in an equal volume of 2× solubilization buffer (10% SDS and 9.3% dithiothreitol (DTT) in 300 mM Tris-HCl, pH 6.8) containing protease inhibitors at a 2-fold concentration (Roche Applied Science). The solubilized pellets were then stored at -80C until use. For SDS-PAGE, samples were combined with an equal volume of loading buffer (10% SDS, 9.3% DTT, 30% glycerol, and 0.025% bromophenol blue in 300 mM Tris-HCl, pH 6.8), and incubated at 95C for 5 min.

Spleen and cortex from wild type and CB2 knock-out male mice were microdissected and homogenized with a 1 ml syringe and 20-gauge needle, at 25% (w/v) in

homogenization buffer (150 mM NaCl, 2mM EDTA, 1% Triton X-100, and 0.05% SDS in 50 mM Tris-HCl, pH 7.5) containing a protease inhibitor mixture (Calbiochem; San Diego, CA). The homogenates were then sonicated and stored at -20C until use. Protein concentrations from homogenates were determined using the Bio-Rad DC protein assay (Bio-Rad; Hercules, CA). For SDS-PAGE, 120 μg of protein of each sample was dissolved in loading buffer (8% SDS, 40% glycerol, 5% beta mercaptoethanol, and 0.04% bromophenol blue in 240 mM Tris-HCl, pH 6.8) and incubated at 95C for 5 min.

Immunoblotting. Samples derived from cells or rat tissue, or human alpha-enolase protein (MBS203689, MyBioSource Inc.; San Diego, CA) were electrophoresed on a 7.5% Tris-Bis gel alongside a molecular weight marker (Bio-Rad), and transferred to a polyvinylidene fluoride membrane, which was blocked for 4 hr with 10% skim milk powder and 0.1% BSA in a solution of 0.1% Triton X-100 in Tris-buffered saline (T-TBS). Membranes were then probed with (1) a rabbit-derived polyclonal antibody raised against amino acids 20-33 of the N terminus of the human CB2 receptor (#101550, Cayman Chemical Company; Ann Arbor, MI), at a concentration of 1:500 in western antibody diluent (0.1% BSA in T-TBS) or (2) a 1:4000 solution of a mouse-derived polyclonal antibody against beta-actin (#sc-47778, Santa Cruz Biotechnology Inc.; Dallas, TX). Membranes were incubated overnight at 4C. Following washing in T-TBS, membranes were incubated with horse radish peroxidase-conjugated sheep anti-rabbit IgG (Chemicon; Temecula, CA) at a concentration of 1:1000 or anti-mouse IgG (Santa Cruz Biotechnology Inc.) at a concentration of 1:1000 in T-TBS for 1 hr at room temperature. Bands were visualized on enhanced chemiluminescence (ECL) film with western blot ECL reagents (GE Healthcare; Buckinghamshire UK).

Samples derived from wild type and CB2 knock-out mice were separated on 10% Tris-glycine gels and proteins then transferred to a nitrocellulose membrane (Amersham Biosciences, GE Healthcare; Piscataway, NJ). After transfer, membranes were blocked for 1 hr with a solution of 0.2% Tween-20 and 5% milk in TBS and then incubated overnight at 4C in a 1:1000 solution of the same rabbit-derived primary antibody raised against the human CB2 receptor (#101550). Membranes were incubated with a horseradish peroxidase-conjugated anti-rabbit IgG at a concentration of 1:2000 (Jackson ImmunoResearch Laboratories; West Grove, PA). Specificity of the primary antibody for the target peptide was probed by pre-incubation of the anti-CB2 antibody (1:1000) with its blocking peptide (1:100) for 60 min at room temperature. Bands were revealed using western blot ECL reagents (GE Healthcare).

Mass Spectrometry

In-gel Digestion. Replicate samples were electrophoresed as described above, with an equal loading of the sample on each half of the gels: one half was processed for western blot analysis as described above, and the other half stained with colloidal Coomassie Blue. The stained gel was then overlaid on to the developed film of the immunoblot detection of CB2, performed with the other half of the gel. To correctly align the gel with the corresponding immunoblot signal, the gel size was adjusted to exactly the size of the gel used for blotting by short incubation steps in 50% methanol in water. Regions of the fixed gel corresponding to bands on the film were excised and subjected to in-gel digestion with trypsin using a robotic workstation for automated protein digestion (DigestPro Msi, Intavis AG; Cologne, Germany). The protocol for automated in-gel digestion is based on the method of Shevchenko et al. (1996). Eluted peptides were concentrated using a centrifugal vacuum concentrator.

Liquid Chromatography-coupled Mass Spectrometry (LC-MS). Samples were re-solubilized in 5% (v/v) acetonitrile, 0.2% (v/v) formic acid in water and injected onto an Ultimate 3000 nano-flow uHPLC-System (Thermo Scientific; Wilmington, DE) that was in-line coupled to the nanospray source of a LTQ-Orbitrap XL hybrid mass spectrometer (Thermo Scientific). Peptides were separated on an in-house-packed emitter-tip column (75 μm ID PicoTip-fused silica tubing (New Objective Inc.; Woburn, MA) packed with C-18 material on a length of 8–9 cm). Samples were analyzed by either an untargeted shotgun approach or, for more sensitive peptide identification, by a targeted analysis using selected ion monitoring (SIM) and selected reaction monitoring (SRM).

Untargeted Protein Identification. Peptides were separated by an increasing gradient of solvent B (0.2% formic acid in acetonitrile) in solvent A (0.2% formic acid in water) from 5% to 45% B over 30 min, followed by an increase to 90% B over 8 min at a flow rate of 400 nl/min. For peptide precursor analysis, the Orbitrap analyzer was operated in a mass range between m/z 300-2000 at a resolution of 60,000 at m/z 400 and an AGC target of 5e5. Preview mode for the FTMS master scan was used to generate precursor mass lists. The strongest five signals were selected for collision-induced dissociation (CID) MS/MS in the LTQ ion trap at a normalized collision energy of 35% using an AGC target of 2e4 and one microscan. Dynamic exclusion was enabled with two repeat counts during 30 sec and an exclusion period of 180 sec.

Targeted Protein Identification. For SIM and SRM analyses, peptides were separated by the same gradient as used for untargeted analyses or a very short gradient from 5% to

20% solvent B over 10 min, followed by an increase to 90% solvent B over 10 min at a flow rate of 800 nl/min. For SIM and SRM analyses, using the same gradient as the untargeted approach, the following precursor ions were selected at a retention time determined by the untargeted analysis: m/z 590.2907 (SIM and SRM); m/z 535.9417 (SIM); m/z 526.2747 (SIM); 752.4036 (SIM and SRM). For the short gradient, the precursor ions m/z 590.2907, 535.9417, and 526.2747 were selected in a mass range between m/z 520-600 during the first part of the gradient (up to 20% acetonitrile) and m/z 752.4036 ± m/z 2 during the following part of the gradient. For SIM analysis, the Orbitrap analyzer was operated in full MS-mode with a narrow mass window of the specified precursor m/z ± 4 at a resolution of 100,000 at m/z 400. Injection waveform for both linear ion trap and Orbitrap analyzers were enabled. Dynamic exclusion was disabled. CID MS/MS in the ion trap was triggered by the detection of one of the selected precursor ions (± m/z 0.01) at a signal intensity of 4e4. SRM scans were performed in the ion trap analyzer using two transitions per precursor ion. Transitions were selected based on the fragmentation pattern of peptides identified in the untargeted analysis of CB2 over-expressing CHO-K1 cells. The following transitions were selected for precursor (i) m/z 590.27: y-6 ion at m/z 676.35; y-8 ion at m/z 906.44; and (ii) m/z 752.40: y-4 ion at m/z 460.25; y-5 ion at 561.30.

Data Analysis. Raw data were processed through the Proteome Discoverer software (version 1.3, Thermo Scientific) and analyzed by both an in-house Mascot server (version 2.3; http://www.matrixscience.com) and the SEQUEST program (Thermo Scientific). Spectra were searched against a subset of the NCBInr amino acid sequence database containing all entries matching the taxon *Rattus* (78244 sequence entries; download April 2012) including corrected sequence entries for the CB2 receptor protein expressed by the rat CB2 cannabinoid receptor cell line (CHO-K1; Chan Test Corp.; Cleveland, OH). All NCBInr amino acid sequence entries for rat CB2 receptor protein are inconsistent with the sequence published by Griffin et al. (2000), which has been used for the CHO-K1 cell line (Genbank accession number AF176350). We found amino acid variations in either position 224 (T or A) or 227 (Q or L) in combination with the variable C-terminus of the two isoforms of CB2. We therefore integrated all nine permutations between position 224, 227 and the variable C-terminus into the database. Searches were set up for full tryptic peptides with a maximum of two missed cleavage sites and carboxyamidomethyl cysteine, deamidated asparagine and oxidized methionine as variable modifications. The mass tolerance thresholds were set to 10 ppm for precursor ions and 0.8 Da for fragment masses. The significance thresholds for high-confidence Mascot ion scores and SEQUEST Xcorr were calculated by the Percolator algorithm (Kall et al. 2007)

allowing a false discovery rate (FDR) of < 1% (q<0.01). Low-confidence identifications were also checked manually for false-negative identification of the CB2 receptor protein. Peak intensities measured in the targeted approach were analyzed manually using the Xcalibur software (Thermo Scientific).

Results

Antibody Sensitivity

When we probed membrane-enriched homogenates of rat CB2-overexpressing CHO-K1 cells with the primary antibody, we detected a protein band at 37 kDa (Fig. 1A). LC-MS/MS analysis of this gel fraction significantly identified seven unique peptides of the rat cannabinoid receptor 2, isoform 1. The identified peptides covered amino acid (aa) positions 224 and 227, which vary between different database entries and confirmed the presence of T224 and Q227 as well as the short C-terminus of isoform 1 (Fig. 1B, 1C). The identified sequence is consistent with the sequence published for rat CB2 receptor protein by Griffin et al. (2000) but varies from all entries in the NCBInr database, including the corresponding Genbank entry, AF176350, which shows a leucine in position 227 instead of a glutamine. We concluded at this step that the band produced by antibody labeling is consistent with detection of the 360 aa short isoform of the rat CB2 receptor.

The spleen contains various immune cells known to express CB2 (Galiegue et al. 1995; Munro et al. 1993). We therefore probed whole rat spleen homogenates with the same antibody (Fig. 2). In these experiments, two additional bands at 44 and 59 kDa were detected. Membrane enrichment resulted in an enhancement of the 37 kDa band and elimination of the 44 and 59 kDa bands in spleen tissue. The spinal cord has also been identified as a possible site for CB2 expression (Beltramo et al. 2006; Hsieh et al. 2011), with western blot analysis commonly used to quantify expression (Brownjohn and Ashton 2012; Curto-Reyes et al. 2011; Curto-Reyes et al. 2010; Ikeda et al. 2013; Walczak et al. 2006, 2005). We therefore carried out the same sequence of experiments on rat lumbar spinal cord homogenates. In the whole cell preparation, only the 44 kDa band was present. After membrane enrichment this 44 kDa band disappeared and was replaced with a band at 37 kDa (Fig. 2). This supports the hypothesis that increased intensity of bands corresponding to CB2 would be seen following membrane enrichment, which is consistent with the hypothesis that CB2 is an exclusively membrane-bound receptor.

Antibody Specificity

When we probed non-CB2-overexpressing CHO-K1 cells alongside CB2-over-expressing CHO-K1 cells at equalized
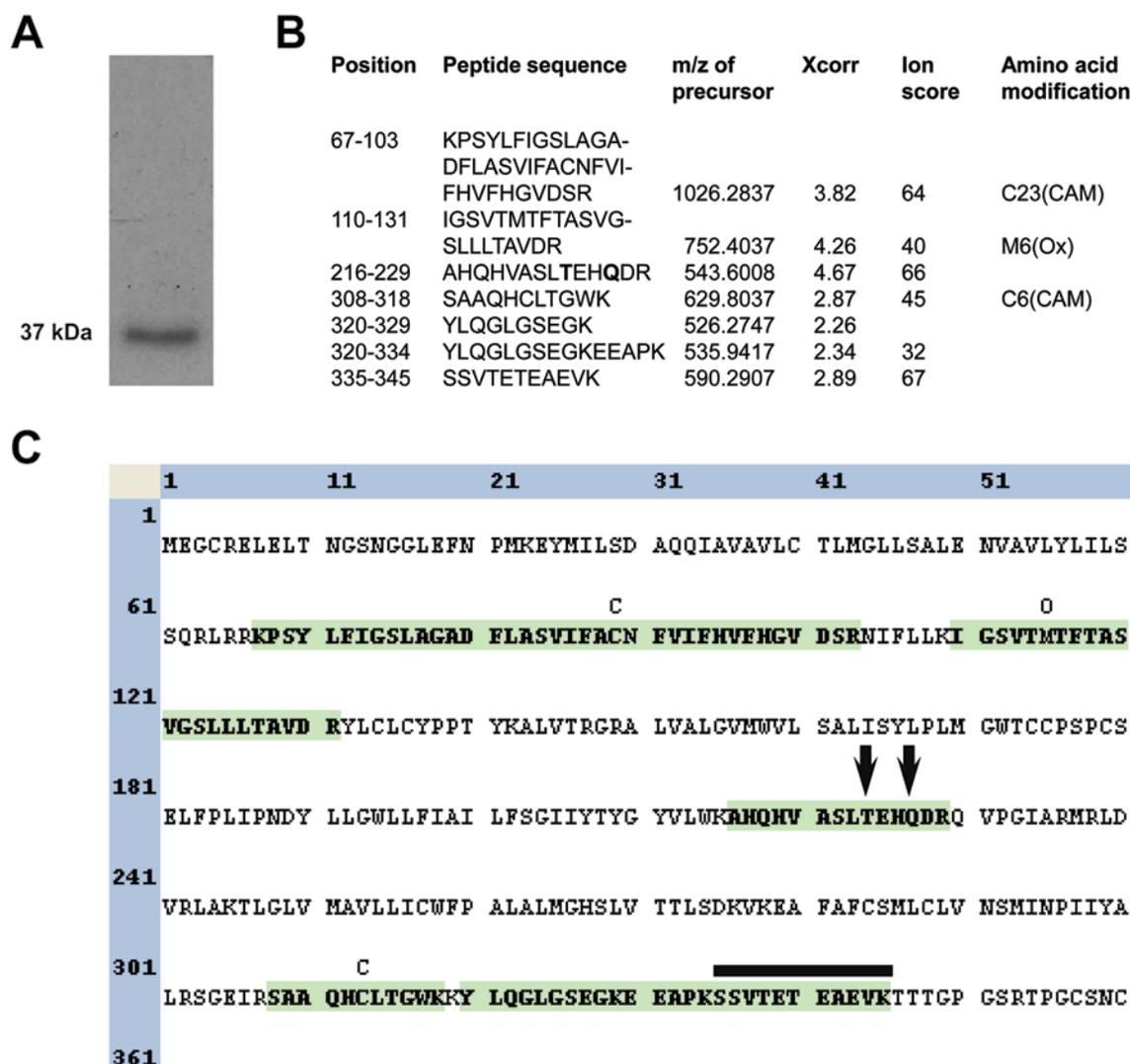
Figure 1. The rat cannabinoid receptor 2 isoform 1 was unambiguously identified by mass spectrometry in the membrane-enriched fraction of lysates from CHO-K1 cells. (A) Western blot of the CB2 receptor in CB2-overexpressing CHO-K1 cells. A single band of 37 kDa is detected. (B) Table view of the identified peptides and the precursor mass-over-charge ratio (m/z), SEQUEST peptide score (Xcorr: cross-correlation coefficient), Mascot ion score and peptide modifications (CAM: carboxyamidomethyl cysteine; Ox: Oxidation of methionine). (C) Sequence view of the significantly identified peptides highlighted in green. Arrows indicate the positions of amino acid sequence variations between the expressed protein and the NCBI accession AF176350 (T224 instead of A224 and Q227 instead of L227). The peptide clearly identifying the presence of the shorter C-terminus of isoform 1 is shown by a horizontal bar.

protein concentration loadings, we found that a band at 37 kDa was present in both cell lines, but was very strongly upregulated in the CB2-overexpressing cells (Fig. 3A). We were unable to determine whether CB2 was indeed absent from the non-overexpressing cells using mass spectrometry for reasons discussed below, and so the cells were not used as a decisive negative control. However, the very strong modulation of the 37 kDa—such that it was very significantly upregulated for the CB2-overexpressing cells—is strongly suggestive that the antibody is sensitive to CB2 protein in cell preparations; i.e., at least some of the staining seen at 37 kDa in the immunoblots of the CB2-overexpressing

cells in particular was due to CB2 labeling by the antibody.

A decisive negative control test was performed using tissue from wild type and CB2-knockout mice (Buckley et al. 2000); the whole lysates of which were then tested by immunoblotting using the same antibody (Figure 3B). As with rat tissue, we again used spleen as a positive control, as well as cortex, which, like spinal cord, is a central nervous system tissue commonly tested for CB2 receptor expression using western blot analysis (Alvaro-Bartolome and Garcia-Sevilla 2013; Benito et al. 2003; den Boon et al. 2012). Using the CB2-directed primary antibody, we observed a
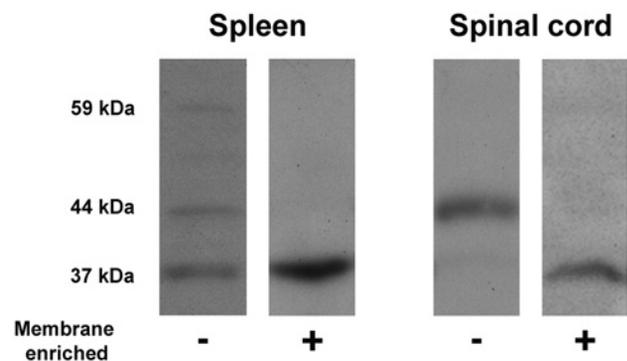
Figure 2. Western blot analysis of rat spleen and lumbar spinal tissue using a CB2-directed antibody. Using a standard western blot sample preparation methods, bands of 37, 44 and 59 kDa were detected in spleen, and a band of 44 kDa was detected in spinal tissue. Membrane enrichment and exclusion of the cytosolic fraction (indicated with +) resulted in abolition of the 44 and 59 kDa bands in spleen, and an enhancement of the 37 kDa band, whereas the same technique in lumbar spinal tissue resulted in abolition of the 44 kDa band and the appearance of a band at 37 kDa.

number of bands between 38 and 55 kDa in spleen and cortical lysates from wild type and CB2-knockout mice. All bands were reduced or abolished when the primary antibody was pre-adsorbed with an excess of the corresponding blocking peptide, indicating primary antibody-dependent binding.

We concluded that, although the antibody is sufficiently sensitive to detect CB2 under some conditions, it is not specific; i.e., in rat, the antibody labels at least one other cytosolic protein of a similar molecular weight to the predicted 410-aa long isoform 2 of the rat CB2 receptor, whereas in mouse, the antibody labels non-specified proteins at a number of weights, including one protein at around 38 kDa which, crucially, has a very similar molecular weight as the predicted unmodified 347-aa mouse CB2 receptor.

Identification of the Cross-reacting Protein Remains Unresolved

With strong circumstantial evidence indicating cross-reactivity of the Cayman Chemical antibody with unknown targets in both rat and mouse, we attempted to identify some likely candidates. We focused on the 44 kDa band in rat tissue, as this species and band weight is commonly reported in studies of the CB2 receptor, and aligns with the predicted weight of the 410-aa isoform 2 of the CB2 receptor. Given that the 44 kDa band we had observed in rat spleen and spinal cord tissue disappeared after membrane enrichment, we reasoned that any potential cross-reactive candidate, at least in rat tissue, would likely be cytosolic. To gain further information on candidate proteins with

which the antibody might cross-react, we performed BLAST searches of the 13 amino acid peptide sequence (NPMKDYMILSGPQ) used to generate the Cayman Chemical CB2 receptor antibody against all protein sequences identified in the different samples. Part of the peptide sequence (MKDYMIL) was matched to a short 7 amino acid sequence stretch (MQEFMIL) from alpha-enolase in all samples corresponding to the immunoblot signal at 44 kDa. Alpha-enolase is a widely expressed glycolytic enzyme and it is highly conserved among mammals. This protein is almost exclusively cytosolic, and has a reported molecular mass of ~47 kDa when separated by SDS-PAGE and detected with immunoblotting (Watanabe et al. 1996). We next attempted to confirm cross-reactivity of the antibody with this protein using western blot analysis. However, any attempts to detect purified human alpha-enolase (MBS203689, MyBioSource Inc., CA, USA) by immunoblotting using the CB2-antibody failed (data not shown) and we therefore excluded alpha-enolase as a potential cross-reactant. Thus, the identity of the cross-reacting protein at this molecular weight remains to be determined.

Sensitivity of Mass Spectrometry

We tested whether the methods that we used to detect CB2 in overexpressing cells could also be used to detect CB2 in native tissues. Utilization of the same shotgun approach with mass spectrometry (as used successfully on the 37 kDa-band in the CB2-overexpressing CHO-K1 cells) did not result in the identification of CB2 in either the rat spleen or spinal cord 44 kDa fractions or the spleen 59 kDa fraction without membrane enrichment. Similarly, analysis of the rat spleen and spinal cord 37 kDa fractions either after membrane enrichment or in the whole cell preparations did not identify CB2.

Given the wealth of corroborating evidence, CB2 receptor protein is almost certainly present in spleen homogenates (Hohmann and Herkenham 1999; Marini et al. 2013; Munro et al. 1993; Schatz et al. 1997), and therefore a lack of positive identification in this case is surprising. It may be that the wider dynamic range of protein abundance, together with the lower abundance of endogenous CB2 receptor protein in the native rat tissue compared to overexpressing CHO-K1 cells, impedes a positive identification of CB2. Using the average MS peak intensity of the three strongest peptide signals per protein as a rough measure of relative protein abundance (Silva et al. 2006), CB2 receptor protein was ranked 41 of 70 identified proteins in the respective gel fraction of overexpressing CHO-K1 cells. This indicates the difficulty in identifying the CB2 receptor protein even in overexpressing systems. The 37 kDa and 44 kDa fractions of the rat spleen and spinal cord tissue samples showed a significantly higher dynamic range of protein abundance, along with the presence of highly abundant cytoskeletal

A

CB2 over-expressing   +   -

CB2

β - Actin   +   -

B

Spleen   Cortex   Spleen   Cortex

WT   CB2 -/-   WT   CB2 -/-   WT   CB2 -/-   WT   CB2 -/-

55 kDa

47 kDa

42 kDa

38 kDa

+ Blocking peptide

Figure 3. Testing of CB2-directed antibody specificity by western blot analysis. (A) With equal protein loading, a band of 37 kDa is detected in membrane-enriched fractions of both CB2-overexpressing and non-overexpressing CHO-K1 cells, with greater band intensity in the overexpressing cell line. (B) Using the same CB2-directed antibody, a number of bands from 38 to 55 kDa were detected in spleen and cortical lysates from wild type and CB2-knockout (-/-) mice. All bands were reduced when the primary antibody was pre-adsorbed with an excess of the corresponding blocking peptide.

proteins such as actin and tubulin, which limits the detection of low-abundance proteins.

Thus, we further employed a targeted, more sensitive approach to detect endogenous CB2. Using the known elution times, mass-to-charge ratios of peptide precursors and their fragmentation pattern detected in the 37 kDa fraction of CB2 over-expressing CHO-K1 cells, SIM and SRM analyses were performed on the 37 kDa and 44 kDa fractions of rat spleen and spinal tissue and on the 37 kDa fraction of non-CB2-overexpressing CHO-K1 cells using the ion trap analyzer of the LTQ Orbitrap system. Positive detection of the CB2 receptor using these targeted approaches was confirmed on protein extracts from CB2-overexpressing CHO-K1 cells. However, we failed to detect the expected CB2 receptor protein fragments in tissue and native cells, despite standard peptide controls indicating sensitivity down to 100 attomol, which would correspond to a total amount of 3-4 pg of digested CB2 receptor protein loaded onto the LC-MS system. The lack of success in detecting CB2 in the spleen left open the questions of the actual amount of receptor protein in the analyzed tissue samples, the efficiency of extraction from cell culture preparations versus tissue samples and the sensitivity of the here-used mass spectrometry application to detect the chosen CB2 target peptides. Since the threshold for detection remained unknown, we cannot conclude that failure to detect CB2 in the tissue samples and the non-CB2-overexpressing CHO-K1 cells was due to the absence of CB2 expression.

Discussion

In this article, we present evidence that the antibody under investigation has the sensitivity to detect CB2 protein when the receptor is expressed in sufficient abundance along with evidence that the antibody also labels other unidentified proteins of a very similar molecular weight to expected CB2 isoforms. Thus, although the antibody appears to have a relatively high affinity and avidity for CB2, it lacks specificity and is therefore unreliable. Reliance on positive controls alone, along with consideration of band number and molecular weight in western blotting, could mislead researchers into thinking that they have strong evidence for the antibody's reliability.

To summarize the key results, the antibody detected a strong band at 37 kDa in membrane-enriched homogenates of rat CB2-overexpressing CHO-K1 cells, in line with the expected weight and distribution of the unmodified short isoform of the rat CB2 receptor. This result was confirmed by LC-MS/MS analysis, which positively identified the short isoform of the rat CB2 receptor at 37 kDa in the membrane preparation of overexpressing CHO-K1 cells. Mass spectrometry has previously been used to identify and characterize the human CB2 receptor (Filppula et al. 2004; Zvonok et al. 2007). Zvonok et al. (2007) detailed a nearly 100% sequence coverage of human CB2 when it was overexpressed in a Baculovirus expression system and purified with a FLAG tag. Interestingly, Zvonok et al. (2007) obtained greater sequence coverage with purification than the 30% reported in this study.

Western blot analysis of commonly prepared whole cell homogenates of rat spleen and central nervous system tissue revealed a band of 44 kDa in spinal and spleen tissue, in line with the expected weight of the long isoform of the rat CB2 receptor. Along with the 44 kDa band, two additional bands of 37 and 59 kDa were detected in spleen tissue. The 44 and 59 kDa bands disappeared in both tissues after

membrane enrichment, indicating that the proteins detected at these higher weights are not membrane associated, and are therefore inconsistent with the membrane-bound CB2 receptor, despite the apparent alignment of the 44 kDa band with the expected weight of the long isoform of this receptor. The identity of the cross-reactive cytosolic protein at 44 kDa in rat spleen and spinal cord was hypothesized to be the widely expressed cytosolic enzyme alpha-enolase, although this was not able to be confirmed. However, our results do clearly show that the unmodified short isoform of the rat CB2 receptor is detected by the antibody in the membrane-enriched fraction of CB2-overexpressing CHO-K1 cells at 37 kDa, as confirmed by mass spectrometry, and that a membrane-associated protein is detected by the same antibody at this weight in rat spleen, of which there is ample corroborating evidence for the expression of CB2 (Hohmann and Herkenham 1999; Marini et al. 2013; Munro et al. 1993; Schatz et al. 1997). In this regard, the results of this study are consistent with those of Matias et al. (2002), who report bands of 39, 47 and 59 kDa in naïve rat spleen whole homogenates using the same CB2-directed primary antibody. Our results indicate that higher weight bands detected in whole spleen homogenates are likely to represent unspecified cytosolic proteins, and not glycosylated variants of the CB2 receptor as previously suggested (Gong et al. 2006; Matias et al. 2002). Spleen tissue is commonly used as a positive control for the CB2 receptor, and western blot analyses of whole homogenates of spleen and other tissues frequently cite a band of approximately 40–50 kDa as the CB2 receptor.

The spinal cord has been extensively studied as a possible site for CB2 expression and function, particularly with respect to the pain pathway (Beltramo et al. 2006; Brownjohn and Ashton 2012; Curto-Reyes et al. 2011; Curto-Reyes et al. 2010; Hsieh et al. 2011; Walczak et al. 2006, 2005; Zhang et al. 2003). However, investigations that have employed western blotting have invariably used a ~45 kDa band as an indicator of CB2 expression (Brownjohn and Ashton 2012; Curto-Reyes et al. 2011; Curto-Reyes et al. 2010; Walczak et al. 2006, 2005). In similar fashion, immunoblotting studies seeking to characterize possible CB2 expression in the brain have also focused on bands over and above 37 kDa, detected either in whole cell homogenates or cytosolic fractions (den Boon et al. 2012; Gong et al. 2006). We have previously shown that a 44 kDa spinal cord band does not change in intensity in the neuropathic spinal cord as compared with healthy control spinal cord, and that the antibody is not specific for CB2 in spinal cord when employed in immunohistochemistry (Brownjohn and Ashton 2012). Membrane enrichment of rat spinal cord lysates did reveal a band in spinal cord tissue at 37 kDa, indicating that CB2 protein may be present in low amounts in the rat spinal cord.

Further analysis with tissue prepared from wild type and CB2-knockout mice revealed strong bands in the spleen and cortical tissues between 38 and 55 kDa from both strains. Given the strength of the evidence that spleen tissue contains CB2, and the unmodified mouse CB2 receptor has a predicted molecular weight of 38 kDa, it is likely the band present at this weight in wild type mouse spleen contains the CB2 receptor protein. The detection of the same band in knockout tissue from the same species, however, indicates that another protein of this weight is also being detected by this antibody, and it lacks sufficient specificity, in line with the findings of a recent study on the same antibody (Cecyre et al. 2013). Unlike Cecyre et al. (2013), however, who employed an N-terminus CB2 receptor knockout, we have used a C-terminus knockout mouse, which, through homologous recombination, lacks a 341-base pair exon fragment, coding for amino acids 217–347 from the C-terminus of the CB2 receptor protein (Buckley et al. 2000). Importantly, we saw no additional bands of a lower molecular weight in spleen tissue preparations from knockout mice, suggesting that a potential truncated protein sequence is not translated in this case. The results from the knockout control therefore strongly suggest that this antibody is not reliable for the detection of CB2, and, in addition to our earlier differential centrifugation experiments, highlights the strong probability that although likely sensitive for the detection of CB2 in native tissues, this antibody also labels at least one, if not several, additional protein targets in mouse.

Rat CB2 receptor protein was positively identified by mass spectrometry in overexpressing CHO-K1 cells, but not in rat spleen tissue; this is likely due to the inherent issues associated with low yield when analyzing in vivo tissue, as well as the hydrophobicity of the CB2 receptor (Filppula et al. 2004; Zvonok et al. 2007) and the detection limit of the here conducted MS approaches. Given the identification of CB2 at 37 kDa in CB2-overexpressing CHO-K1 cells by mass spectrometry, and the presence of the same band in rat spleen tissue both prior to and following membrane enrichment, it is likely that the membrane integral CB2 receptor is present in spleen tissue at 37 kDa, despite a negative result with mass spectrometry. Our results further suggest the possibility that refinements in tissue preparations in conjunction with more sensitive targeted mass spectrometry assays may lead to a method for the detection and quantification of unmodified CB2 receptor protein in native tissues. Although lacking the speed and convenience of immunolabeling and immunoblotting methods, mass spectrometry provides unequivocal identification of protein expression, and is therefore likely to be an increasingly valuable tool in the study of receptor proteins of pharmacological interest, as has been recently advocated (Aebersold et al. 2013). Unfortunately, the here-conducted targeted MS approaches were insufficient to detect CB2 in tissues extracts. The LTQ Orbitrap XL system lacks sensitivity when operated in SRM mode as compared with the contemporary triple quadrupole instruments especially for the detection of low abundance targets in very complex

matrices, such as whole tissue extracts. Current methods for the identification of CB2 ex vivo have been challenged with many issues (Atwood and Mackie 2010). The development of more sensitive SRM assays using triple quadrupole-related instrumentation will provide a promising methodology for the detection and quantification of CB2 receptor protein in crude tissue extracts. Such a method with superior specificity is crucial for the validation of antibodies used in immunoblotting and immunohistochemistry.

In conclusion, prior to carrying out knockout controls, our experiments seemed to provide compelling results in favor of antibody validity: the labeling of bands at weights consistent with proposed isoforms of CB2; the correspondence of one of these bands with labeling in a strong positive control; the identification of CB2 in the positive control at this band weight with mass spectrometry; as well as the disappearance of all identified bands after pre-incubation of the primary antibody with immunizing peptide. Nevertheless, when the antibody was tested with strong negative controls, it became clear that this antibody is not specific for CB2 receptor protein. This highlights the need for stringent negative controls when employing antibodies for the detection and quantification of protein targets. Our results suggest that this antibody detects one or multiple additional and non-specific protein targets, so that any results that have been obtained with this antibody should be interpreted with caution. This may be because of similarity between proteins with respect to the epitope region, or because multiple epitopes exist for the antibody (Bogen et al. 1993). Polyclonal antibodies, in particular, are liable to shifts in epitope binding between batches. More generally, this lesson applies to many antibodies not only in western blotting but also in immunocytochemistry and immunohistochemistry; though, it is difficult to define in advance exactly what controls will be required for a given antibody, multiple coincidences do occur, and just as in courts-of-law, such circumstantial evidence is of heuristic value only, and cannot be used as evidence for antibody specificity.

Acknowledgments

Declaration of Conflicting Interests

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