Biochemical Pharmacology, Vol. 55, pp. 1893–1905, 1998.
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ISSN 0006-2952/98/$19.00 + 0.00
PII S0006-2952(98)00081-1

High-Level Expression of the Human CB2 Cannabinoid Receptor Using a Baculovirus System

Katharine W. Nowell, Denise A. Dove Pettit,† Wayne A. Cabral,* H. William Zimmerman, Jr.,† Mary E. Abood† and Guy A. Cabral*‡*

DEPARTMENTS OF *MICROBIOLOGY/IMMUNOLOGY AND †PHARMACOLOGY/TOXICOLOGY, MEDICAL COLLEGE OF VIRGINIA OF VIRGINIA COMMONWEALTH UNIVERSITY, RICHMOND, VA 23298-0678, U.S.A.

ABSTRACT. A human CB2 recombinant baculovirus (AcNPV-hCB2) was generated by site-specific transposition and employed to express the human CB2 cannabinoid receptor. Northern analysis of total RNA from *Spodoptera frugiperda* (Sf9) insect cells infected with AcNPV-hCB2 revealed novel expression of a unique 2.3 kb transcript when probed with hCB2 cDNA. This transcript corresponded to the size expected for hCB2 generated from the recombinant virus construct. Western immunoblot analysis of whole cell homogenates of recombinant baculovirus-infected Sf9 cells, using affinity-purified antibody to a human CB2 carboxy terminal domain (anti-hCB2.CV), revealed the presence of novel immunoreactive protein. In addition, when anti-hCB2.CV was employed in immunofluorescence staining, an intense signal was observed within AcNPV-hCB2-infected cells but not within uninfected cells or cells infected with a control β-galactosidase recombinant baculovirus. The pattern of immunofluorescence at early periods post-infection was in a perinuclear arrangement with a "signet-ring" appearance, suggestive of glycosylation of the expressed recombinant protein. Transmission electron microscopy revealed regions of intranuclear recombinant virus assembly and the presence of numerous intracytoplasmic proteinaceous vesicular inclusions consistent with hyperproduction of hCB2. Scatchard–Rosenthal analysis of [^{3}H]-(-)3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-[3-hydroxypropyl]cyclohexan-1-ol ([^{3}H]CP 55,940) receptor binding indicated a K_d of 2.24 nM and a B_{max} equal to 5.24 pmol/mg of protein. The lack of [^{3}H]CP 55,940 displacement with *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamidehydrochloride (SR 141716A), the CB1-selective antagonist, confirmed the identity of the receptor as CB2. These data indicate that AcNPV-hCB2 expresses high levels of the human CB2, which retains properties of the native receptor. Thus, this recombinant virus may prove suitable for hyperproduction of receptor for basic biochemical and biophysical characterization studies. BIOCHEM PHARMACOL **55**;11:1893–1905, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. radioligand binding; human peripheral cannabinoid receptor; human CB2 receptor; G protein-coupled receptor; baculovirus expression; delta-9-tetrahydrocannabinol; anti-cannabinoid receptor antibody

It is now recognized that many of the effects attributed to both exogenous and putatively endogenous cannabinoids are elicited by cannabinoid receptors. At least two cannabinoid receptors have been identified. The first of these, designated CB1§, is limited primarily to neural tissue [1–4], although it has been reported to be present also in unfractionated mouse spleen [5], testis [6], and human leukocytes [7]. The second receptor, designated CB2, has been compartmentalized within cells of the immune system [8, 9]. An alternatively spliced variant of CB1, designated CB1a, also has been reported [10]. Whether biologically relevant levels of this variant protein are expressed within neural tissue remains to be determined.

The observation that cannabinoid receptor expression was linked to cellular functional activities was made by Howlett [11], who demonstrated cannabinoid-induced inhibition of cyclic AMP accumulation in the mouse N18TG2 neuroblastoma cell line. Radioligand binding

‡ Corresponding author: Dr. Guy A Cabral, Medical College of Virginia of Virginia Commonwealth University, 1101 E. Marshall St., Box 980678 MCV Station, Richmond, VA 23298-0678. Tel. (804) 828-2306; FAX (804) 828-8220; E-mail: gacabral@gems.vcu.edu.

Received 26 August 1997; accepted 2 February 1998.

§ *Abbreviations:* AcNPV-β-gal, β-galactosidase recombinant baculovirus; AcNPV-hCB2, *Autographa californica* multiply enveloped nuclear polyhedrosis virus human CB2 recombinant; AcNPV-rCB1, rat CB1 recombinant baculovirus; CB1, central or neural cannabinoid receptor; CB2, peripheral cannabinoid receptor; CP 55,940, (−)3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-[3-hydroxypropyl]cyclohexan-1-ol; KLH, keyhole limpet hemocyanin; LB, Luria Broth; MBS, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester; MOI, multiplicity of infection; PCR, polymerase chain reaction; pi, post-infection; PMSF, phenylmethylsulfonyl fluoride; SFM, Serum Free Medium; Sf9, *Spodoptera frugiperda* insect cells; SR 141716A, [*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamidehydrochloride]; SSC, 3.0 M NaCl, 0.3 M $Na_3C_6H_5O_4$, pH 7.0; TBS, Tris-buffered saline; TTBS, Tween-20 supplemented Tris-buffered saline; pfu, plaque-forming units; and WIN 55,212-2, (*R*)-(+)-[2,3-dihydro-5-methyl-3-](4-morpholinyl)-methyl]pyrrolo[1,2,3-*de*]-1,4-benzoxazin-6-yl](1-naphthalenyl)methanone.

studies utilizing a potent tritiated cannabinoid analog (CP 55,940) [12], and anatomical receptor distribution analyses using [^{3}H]CP 55,940 in autoradiography, have confirmed the existence of CB1 in brain and have demonstrated its preponderance in cerebellum, hippocampus, and basal ganglia [1, 2]. The discovery of cDNA clones, isolated from rat cerebral cortex [13] and human brain stem [6] cDNA libraries, shown to bind and functionally interact with cannabinoids, has allowed the molecular characterization of this receptor. The subsequent isolation of a second cannabinoid receptor from a human promyelocytic HL-60 cell line [14] has allowed for its initial characterization and for studies to determine its functional relevance in relation to the immune system. Sequence analyses of CB1 and CB2 clones have revealed a high level of homology with G protein-coupled receptors in that they contain an external domain that possesses glycosylation sites, seven conserved transmembrane domains, and an internal or cytoplasmic domain that is coupled to a G protein (inhibitory) complex.

Although much information has been obtained concerning the molecular characteristics of CB1 and CB2 cDNAs and the expression and compartmentation of their mRNA, little data are available concerning the expression and cellular localization of cannabinoid receptor proteins. Definition of the basic biochemical and biophysical characteristics of cannabinoid receptor protein is important since it is the molecular entity that interacts directly with cannabinoid ligand. Characterization of this interaction will provide essential information concerning the nature of the ligand binding site and may provide insight concerning the potential association of the receptor with other cellular proteins. To address these issues, it is necessary to acquire preparative levels of purified receptor protein that exhibits native properties. However, in contrast to CB1, which is expressed at high levels within the brain, CB2 is expressed at relatively low levels within immunocytes [7, 8]. Thus, a strategy that allows for high level recombinant CB2 protein expression under conditions that approximate those achieved in native tissues is advantageous. We have demonstrated previously that the baculovirus system is an effective means for the relatively high level expression of the rat CB1 [15]. In the present study, we report on the expression of the human CB2 cannabinoid receptor in baculovirus-infected insect cells. The data demonstrate that the baculovirus expression system is a viable means of expressing high levels of human CB2 receptor recombinant protein, which exhibits ligand binding characteristics of this receptor.

MATERIALS AND METHODS

Reagents

All reagents, unless otherwise noted, were obtained from the Sigma Chemical Co. All restriction enzymes were purchased from New England BioLaboratories. Bluo-gal was obtained from Life Technologies. Kanamycin was purchased from Research Organics.

Drugs

[^{3}H]CP 55,940 was purchased from DuPont/NEN. CP 55,940 and SR 141716A were synthesized by Dr. Larry Melvin (Pfizer Inc.) and Dr. John Lowe (Pfizer Inc.), respectively. WIN 55,212-2 was provided by Dr. Susan Ward (Sterling–Winthrop Research Institute).

Cell Culture

Sf9 cells (Invitrogen Corp.) were cultured in Sf-900 II SFM (Life Technologies Inc.) in disposable sterile 125-mL Erlenmeyer flasks with loosened caps to allow for ample aeration. Cells were grown at 28° at 150 rpm in a New Brunswick Scientific Series 25 Incubator Shaker. Cells were subcultured three times weekly.

Construction and Cloning of the Human CB2 (hCB2) Donor Plasmid (pFastBac-hCB2)

The plasmid MC36F1, containing the human peripheral cannabinoid receptor cDNA inserted into the pBluescript II KS-plasmid, was provided by Dr. Sean Munro (Medical Research Council). The plasmid was replicated in XL-1 Blue MRF′ cells (Stratagene) and purified using the Qiagen Spin Miniprep kit (Qiagen). To optimize protein expression, the 5′ noncoding sequence of the hCB2 cDNA was removed using a PCR subcloning strategy (see Fig. 1). The following oligonucleotide primers (P1 and P2) were synthesized and used to amplify a 310 bp fragment containing the initiation codon of the cannabinoid receptor and the start of the cannabinoid receptor coding sequence:

P1: CGG CCT GCA GAT GGA GGA ATG CTG

GGT G

P2: GCC TTG GAA TCC ACA CCA

P1 is homologous to the cDNA sequence at the CB2 start site and was engineered to contain a unique *Pst*I site (underlined) for subcloning into the parent plasmid following *Pst*I digestion. The P2 sequence was selected to allow for the amplification of a unique *Sph*I site within the coding sequence of hCB2. The PCR reaction was carried out as previously described [16], using a DNA Thermal Cycler (Perkin–Elmer Corp.) that was programmed for 25 cycles in the following manner: 1-min denaturation at 95°, 1-min annealing step at 45°, and 1-min extension period at 72°. Digestion of MC36F1 with *Pst*I and *Sph*I allowed for the removal of the 5′ noncoding sequence and 184 bp of coding sequence. Digestion of the amplified 310-bp fragment with the same restriction enzymes followed by ligation into digested MC36F1 allowed for the replacement of the removed coding sequence. This cloning procedure resulted in the generation of the recombinant plasmid, pBS-CB2, containing the hCB2 cDNA lacking the 5′ noncoding sequence. Recombinant pBS-CB2 plasmid DNA was iso-

lated using the Qiaprep Spin Miniprep kit. Sequence analysis of pBS-CB2 confirmed the integrity of the PCR-amplified sequence.

To generate the donor plasmid for site-specific transposition of the hCB2 gene into the baculovirus shuttle vector (bacmid), hCB2 cDNA was digested from pBS-CB2 with *Eco*RI and *Not*I and ligated into *Eco*RI and *Not*I sites in pFastBac1 (Life Technologies) (see Fig. 2). The resultant pFastBac-hCB2 was replicated in XL-1 Blue MCF′ cells cultured in LB broth containing tetracycline (15 μg/mL) and ampicillin (100 μg/mL) at 37° for 16 hr at 200 rpm on a New Brunswick Scientific G10 Gyrotory Shaker.

Transformation of Competent Escherichia coli *Cells with pFastBac-hCB2 DNA*

Competent DH10Bac *E. coli* cells (Life Technologies) were used to produce an hCB2 recombinant bacmid. These bacterial hosts possess a bacmid that contains a mini-attTn7 target site, a lacZα gene, and a helper plasmid that encodes transposition proteins. Cells were transformed with the pFastBac-hCB2 donor plasmid, which contains a mini-attTn7 element to allow for site-specific transposition of hCB2 into the bacmid, using the manufacturer's recommendations. Transformed cells were streaked onto LB agar plates containing kanamycin (50 μg/mL), gentamicin (7 μg/mL), tetracycline (10 μg/mL), isopropyl-l-thio-β-D-galactoside (IPTG, 40 μg/mL), and Bluo-gal (300 μg/mL) and incubated at 37° for 48 hr. White colonies, indicative of hCB2 insertion and lacZα disruption, were restreaked to verify colony phenotype and then were cultured at 37° for 16 hr with shaking at 250 rpm in 5 mL of LB broth containing kanamycin, tetracycline, and gentamicin at the concentrations indicated above. High-molecular-weight recombinant bacmid DNA was isolated as follows: 1×10^7 cells were pelleted and resuspended in Solution I (15 mM of Tris–HCl, pH 8.0, 10 mM of EDTA, 100 μg/mL of RNase A), solubilized in Solution II (0.2 N of NaOH, 1% SDS), and proteins were precipitated with 3 M of potassium acetate (pH 5.5). Human CB2 bacmid DNA was precipitated from the supernatant using isopropanol, washed with 70% ethanol, and dissolved in TE buffer (10 mM of Tris, 1 mM of EDTA).

Southern Analysis of Recombinant hCB2 Bacmid DNA

Southern analysis was performed on isolated recombinant bacmid DNA to verify the presence of the hCB2 insert. Bacmid DNA was blotted onto a nitrocellulose membrane (Micron Separations Inc.) in 20× SSC, and UV-cross-linked in a Stratalinker 1800 (Stratagene). The blot was incubated (37°, 16 hr) in 1x prehybridization buffer containing 50% formamide, 5× SSC, 0.1% SDS, 0.1% BSA, 0.1% polyvinylpyrrolidone, 0.1% Ficoll, 0.1 M of NaH_2PO_4, and 0.25 mg/mL of total yeast RNA. Blots were incubated at 37° for 18 hr in hybridization buffer consisting of prehybridization buffer supplemented with 5% dextran sulfate and nick-translated hCB2 cDNA as described below. Blots were washed using the following regimen: four 5-min washes in 2x SSC and 0.2% SDS at 43°; one 40-min wash in 2× SSC and 0.2% SDS at 43°, and one 20-min wash in 0.5× SSC and 0.2% SDS at 43°. Autoradiography was carried out at −80° overnight using XAR imaging film (Eastman Kodak Co.).

Production of hCB2 Recombinant Baculovirus (AcNPV-hCB2)

Transfection of Sf9 insect cells with recombinant hCB2 bacmid was performed to generate recombinant baculovirus. Dilutions of recombinant bacmid were mixed with a cationic lipid (CellFectin, Life Technologies), and lipid–DNA complexes were allowed to form for 45 min at room temperature. Six well plates seeded with Sf9 cells (5×10^5 cells/mL in 2 mL of Sf-900 II SFM) were inoculated with the lipid–DNA complexes. Transfection was allowed to proceed for 5 hr at 28°. Then the medium was removed, and infected cells were cultured for an additional 48 hr in fresh SFM. Following incubation, supernatants were recovered, clarified by centrifugation, and used as a primary viral stock. To amplify the virus, 0.5 mL of AcNPV-hCB2 stock was used to inoculate a 25-cm^2 flask of Sf9 cells (3×10^6 cells) for 1 hr. Following inoculation, 4.5 mL of fresh SFM was added, and the cells were cultured at 28° for 48 hr. The medium was harvested following centrifugation (250 *g*, 5 min), and the virus titer was obtained by plaque assay following the method of O'Reilly *et al.* [17]. Then large scale recombinant hCB2 baculovirus stocks were produced by inoculation of Erlenmeyer flask cultures (50 mL, 9×10^5 Sf9 cells/mL, MOI of 0.1). Following incubation (48 hr, 28°), shaker cultures were clarified by centrifugation, supernatants were collected, and sterile BSA was added to a final concentration of 2% to protect the virus from denaturation. Virus stock suspensions were stored at 4° for immediate use and at −80° for long-term storage. Virus stock preparations were assessed for infectious virus titer by plaque assay. AcNPV-β-gal, expressing β-galactosidase, was obtained from Invitrogen. AcNPV-rCB1, expressing the rat neural cannabinoid receptor, was prepared as described previously [15].

Generation of hCB2 Probe for Northern and Southern Analysis

The hCB2 cDNA fragment derived from pBS-CB2 (*Eco*RI, *Not*I digestion) was gel purified and isolated using the Jetsorb Gel Extraction Kit (PGC Scientific). The probe was ^{32}P-labeled ([^{32}P]dCTP; DuPont/NEN) by nick-translation (Life Technologies, Inc.) to yield a specific activity of $>10^8$ dpm/μg.

Northern Analysis

Total RNA was isolated from uninfected, AcNPV-hCB2-infected, and AcNPV-β-gal-infected cells (MOI of 0.1, 48 hr pi [post-infection]) using TRIzol Reagent (Life Technologies). RNA (20 μg/lane) was separated by means of electrophoresis (30 V, 18 hr) on a 1% agarose-formaldehyde gel. Equivalent loading and the quality of the RNA were determined by ethidium bromide staining of the gel. The RNA was blot-transferred onto a nitrocellulose membrane and cross-linked using a Stratalinker 1800. Hybridization was performed using the methodology described for Southern blot analysis.

Production of Rabbit Anti-hCB2 Antibody

Affinity-purified anti-human CB2 antibody (anti-hCB2.CV) was produced to a carboxy-terminal immunodominant region in human CB2. The peptide CVRGLGSEAKEEA PRSS, which corresponds to amino acids 320–336 of the intracellular carboxy terminus, was synthesized using solid-phase F-moc chemistry and purified by HPLC to greater than 90% using a Vydac c18 reverse-phase column. The peptide was coupled via a cysteine thiol to either KLH or BSA using MBS (Bioaffinity Systems, Inc.). New Zealand white rabbits were injected with KLH-coupled peptide in complete Freund's adjuvant and received booster inoculations of the same immunogen in incomplete adjuvant on days 14, 28, and 48. Production bleeds were collected at days 52, 56, and 60, and were screened by ELISA for reactivity to the homologous peptide linked to BSA as an antigen source. The crude sera were shown to have reciprocal dilution titers of greater than 200,000 using an $O.D._{492}$ of 0.200 as the baseline for a positive signal. Anti-peptide antibody was purified from antiserum by affinity chromatography using the homologous peptide coupled to a thiol-reactive gel (Quality Controlled Biochemicals, Inc.). Affinity-purified antibody was diluted to a stock concentration of 1 mg/mL and was stored at −80°.

SDS–PAGE and Western Immunoblotting

Sf9 cells infected at an MOI of 0.1 with AcNPV-hCB2 were harvested at various periods pi by centrifugation, and washed once with PBS, pH 7.4. Protein lysates were prepared by solubilization in ice-cold TSA solution (2 mM of Tris–HCl, pH 8.0, 140 mM of NaCl) and an equal volume of lysis buffer (TSA solution, 2% Triton X-100, 5 mM of iodoacetamide, 0.2 U/mL of aprotinin, 1 mM of PMSF) for 1 hr at 4° with shaking. Following solubilization, a 0.2 volume of 5% sodium deoxycholate was added, and the mixture was incubated on ice for 10 min. The lysate was centrifuged (2800 *g*, 10 min), and the protein concentration was determined using the BCA Protein Assay (Pierce). Protein samples were mixed 1:1 with sample buffer (0.05 M of Tris, pH 6.8, 1% SDS, 1% β-mercaptoethanol, 10% glycerol, 0.05% bromophenol blue) and heated (95°, 5 min) prior to loading (25 μg/well) onto a 1.5-mm thick 10% polyacrylamide gel. The samples were electrophoresed for 4 hr at 30 mA/gel constant current in a cooled (10°) chamber. Following electrophoresis, proteins were transferred overnight at room temperature onto a PVDF-Plus membrane (Micron Separations, Inc.) at 90 mA using a Bio-Rad Blot Cell (Bio-Rad Laboratories). The membrane was blocked with rocking at room temperature overnight in Blocker Casein in TBS (Pierce) supplemented with 0.05% Tween-20. The PVDF-Plus membrane was incubated (room temperature, 2 hr) with affinity-purified rabbit anti-hCB2.CV, preabsorbed with an equal volume of uninfected Sf9 cells (1 hr) and diluted 1:100 in Blocker Casein in TBS supplemented with 0.05% Tween-20. The blot was washed (three times, 10 min each) in TBS (10 mM of Tris, 0.9% NaCl, pH 7.4), and then incubated (room temperature, 1 hr) with horseradish peroxidase-conjugated goat anti-rabbit IgG (heavy and light chains) (Cappel) diluted 1:25,000 in TBS, pH 7.4, containing Blocker Casein and 0.05% Tween-20. Following washing (six times, 10 min each) in TBS, pH 7.4, enhanced chemiluminescence was performed using SuperSignal CL-HRP (Pierce). The blot was exposed on Kodak XAR imaging film.

Microfiltration Bio-Dot Immunoblotting

Uninfected Sf9 cells, or cells infected at an MOI of 0.01 and 0.1 with AcNPV-hCB2, were harvested at 12-hr intervals from 36 to 72 hr pi by centrifugation, and cell pellets were washed once with PBS, pH 7.4. Protein lysates were prepared by solubilization as described above. Protein lysates were diluted to a concentration of 16 μg/100 μL and two-fold serial dilutions were prepared in 1% BSA-TBS and loaded onto a 96-well microfiltration apparatus (Bio-Rad Laboratories). The nitrocellulose membrane (8 × 11 cm, unsupported, Micron Separations, Inc.) was prewetted with TBS (20 mM of Tris, 500 mM of NaCl, pH 7.5) containing 20% methanol (5 min), equilibrated in TBS (15 min), and incubated with the protein samples (100 μL/well) under conditions of gravity filtration for 30 min. The blot was washed twice with TTBS (20 mM of Tris, 500 mM of NaCl, 0.05% Tween-20, pH 7.5) under vacuum filtration. Affinity-purified rabbit anti-hCB2.CV antibody (preabsorbed with uninfected Sf9 cells and diluted 1:100 in 1% BSA-TBS) was added to each well (100 μL/well) and allowed to incubate for 30 min followed by gravity filtration, and two vacuum filtration washes in TTBS. The membrane was removed from the apparatus, blocked in Pierce Blocker Casein in TBS for 1 hr, and then incubated (room temperature, 1 hr) with horseradish peroxidase-conjugated goat anti-rabbit IgG diluted 1:50,000 in TBS (pH 7.4), containing Blocker Casein and 0.05% Tween-20. Following washing (six times, 10 min each) in TBS, pH 7.4, enhanced chemiluminescence and autoradiography were performed as indicated above.

Immunofluorescence

Uninfected, AcNPV-hCB2-infected and AcNPV-β-gal-infected Sf9 cells (MOI of 0.1) were harvested at various times pi and collected onto microscope slides (600 rpm, 2 min) using a Cytospin 2 cytocentrifuge (StatSpin Inc.). Slides were air-dried (30 min), fixed in absolute acetone (10 min), and stored at room temperature in a dessicator. For immunofluorescence staining, slides were rehydrated in PBS (3 min), blocked for 30 min in 1% BSA in PBS, and incubated (60 min) with affinity-purified anti-hCB2.CV antibody in a humidified chamber. Then slides were washed in PBS (three times) and incubated (60 min) with FITC-labeled goat anti-rabbit IgG (1:32 dilution in PBS, 0.01% Evans Blue, H + L; Cappel) at room temperature. The slides were rinsed in PBS (three times), mounted in Aquamount (Lerner Laboratories), and examined with an Olympus BHA Microscope equipped with a model BH2RFL reflected fluorescence attachment and a model PM-10AD photo-micrographic system (Olympus Corp.).

Transmission Electron Microscopy

Uninfected, AcNPV-β-gal-infected, or AcNPV-hCB2-infected Sf9 cells (MOI of 0.1) were harvested at various time periods pi by centrifugation and were washed twice in PBS and once in 0.1 M of sodium cacodylate buffer, pH 7.2. Cell pellets were fixed (27°, 24 hr) in 2.5% glutaraldehyde in 0.1 M of sodium cacodylate buffer, post-fixed for 1 hr in 2% osmium tetroxide in 0.1 M of cacodylate buffer, washed twice with 0.1 M of cacodylate buffer, dehydrated through a graded series of ethanol, and embedded in Poly-Bed 812 resin (Polysciences, Inc.). Thin sections (gray interference color) were stained with saturated aqueous uranyl acetate and lead citrate and were examined in a Zeiss EM10 transmission electron microscope (Carl Zeiss) operating at an accelerating voltage of 80 kV. In all experiments, a minimum of 100 cell profiles was examined.

Radioligand Binding

The current assay was modified from that described for a brain membrane preparation [18]. Uninfected, AcNPV-hCB2-infected, and AcNPV-β-gal-infected Sf9 cells (MOI of 0.1, 72 hr pi) were harvested in Solution One (320 mM of sucrose, 2 mM of EDTA, 5 mM of $MgCl_2$) by centrifugation. The cell pellet was homogenized in 10 mL of buffer A (320 mM of sucrose, 50 mM of Tris–HCl, 1 mM of EDTA, 5 mM of $MgCl_2$, pH 7.4), and then centrifuged (1600 *g*, 10 min), the supernatant was saved, and the pellet was washed once in buffer A with subsequent centrifugation. The combined supernatants were centrifuged at 100,000 *g* for 60 min. The pellet was resuspended in 3 mL of buffer B (50 mM of Tris–HCl, 1 mM of EDTA, 3 mM of $MgCl_2$, pH 7.4) to yield a protein concentration of approximately 1 mg/mL [19]. The tissue preparation was divided into equal aliquots, frozen on dry ice, and stored at −70°. Binding was initiated by the addition of 50 μg of membrane protein to silanized tubes containing [^{3}H]CP 55,940 (102 Ci/mmol, DuPont/NEN) and a sufficient volume of buffer C (50 mM of Tris–HCl, 1 mM of EDTA, 3 mM of $MgCl_2$, and 5 mg/mL of fatty acid free BSA, pH 7.4) to bring the total volume to 0.5 mL. The addition of 1 μM of unlabelled CP 55,940 was used to assess nonspecific binding. Following incubation (30°, 1 hr), binding was terminated by the addition of 2 mL of ice-cold buffer D (50 mM of Tris-HCl, pH 7.4, plus 1 mg/mL of BSA) and vacuum filtration through Whatman GF/C filters (pretreated with polyethyleneimine, 0.1%, for at least 2 hr). Tubes were rinsed with 2 mL of ice-cold buffer D, which was also filtered, and the filters were subsequently rinsed twice with 4 mL of ice-cold buffer D. Before radioactivity was quantitated by liquid scintillation spectrometry, filters were shaken for 1 hr in 5 mL of scintillation fluid.

CP 55,940 and all cannabinoid analogs were prepared by suspension in assay buffer from a 1 mg/mL ethanolic stock without evaporation of the ethanol (final concentration of no more than 0.4%). Saturation experiments were conducted with six concentrations of [^{3}H]CP 55,940 ($N = 3$) ranging from 100 pM to 5 nM. Competition assays were conducted with 1 nM [^{3}H]CP 55,940 and six concentrations of displacing ligands (WIN 55,212-2, $N = 4$; SR 141716A, $N = 3$). The B_{max} and K_d values obtained from Scatchard analysis of saturation binding curves [20, 21] were determined by the KELL package of binding analysis programs for the Macintosh computer (Biosoft). Displacement IC_{50} values were originally determined by unweighted least-squares linear regression of log concentration-percent displacement data and then converted to K_i values using the method of Cheng and Prusoff [22].

RESULTS

Production of hCB2 Recombinant Baculovirus (AcNPV-hCB2)

A human CB2 recombinant baculovirus was constructed using a Bac-to-Bac expression system (Life Technologies). To optimize recombinant protein expression, the hCB2 cDNA present in the plasmid MC36F1 was modified using a PCR strategy to remove the 5′ non-coding sequence (Fig. 1). Sequencing of the resultant plasmid (pBS-CB2) indicated that no mutations were incorporated into the hCB2 coding sequence. The modified hCB2 cDNA within pBS-CB2 then was subcloned into pFastBac1, a donor plasmid required for the site-specific transposition of hCB2 into a bacmid construct (Fig. 2). DH10Bac *E. coli* cells, containing a bacmid with baculovirus DNA, a mini-attTn7 transposition site, and a lacZα gene, were transformed with pFastBac-hCB2 and grown on Bluo-gal selective plates. White colonies resulting from disruption of the lacZα gene and insertion of the hCB2 cDNA were identified and restreaked to ensure clonal isolation. Southern blot analysis of bacmid DNA preparations using hCB2 derived from pBS-CB2 as a probe confirmed the presence of hCB2 (data not shown). The purified hCB2-containing bacmid then

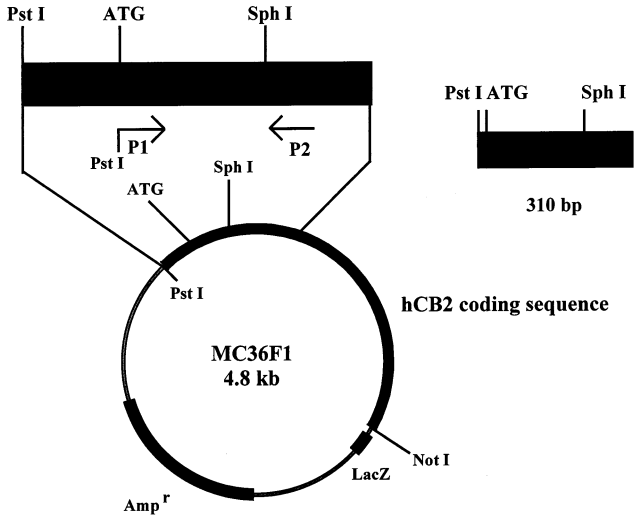

FIG. 1. PCR amplification of the start of the human CB2 receptor coding sequence. PCR amplification using primers P1 and P2 allowed for generation of a 310-bp fragment containing the initiation codon and the start of hCB2. This fragment was employed to replace coding sequence in MC36F1, which was extracted in the digestion utilized to remove the 5′ noncoding sequence. The resultant plasmid was designated pBS-CB2.

was used to transfect Sf9 cells to generate recombinant hCB2 baculovirus (AcNPV-hCB2).

Assessment of AcNPV-hCB2 for Replication Competence and for Recombinant hCB2 Gene Expression

AcNPV-hCB2 was assessed for replication competence by plaque assay. The AcNPV-hCB2 stock was shown to have an infectivity titer of 2×10^9 pfu/mL. Northern blot analysis was performed to confirm that hCB2 transcripts were being generated by AcNPV-hCB2-infected Sf9 cells. Infection of Sf9 cells with AcNPV-hCB2 resulted in the hyperproduction of a unique 2.3-kb transcript corresponding to the predicted size of the recombinant hCB2 transcript (Fig. 3), using the location of 18S rRNA as a relative marker.

Assessment of AcNPV-hCB2-Infected Sf9 Cells for CB2 Receptor Protein Expression

Microfiltration immunoblotting of homogenates of infected and uninfected Sf9 cells was performed using anti-

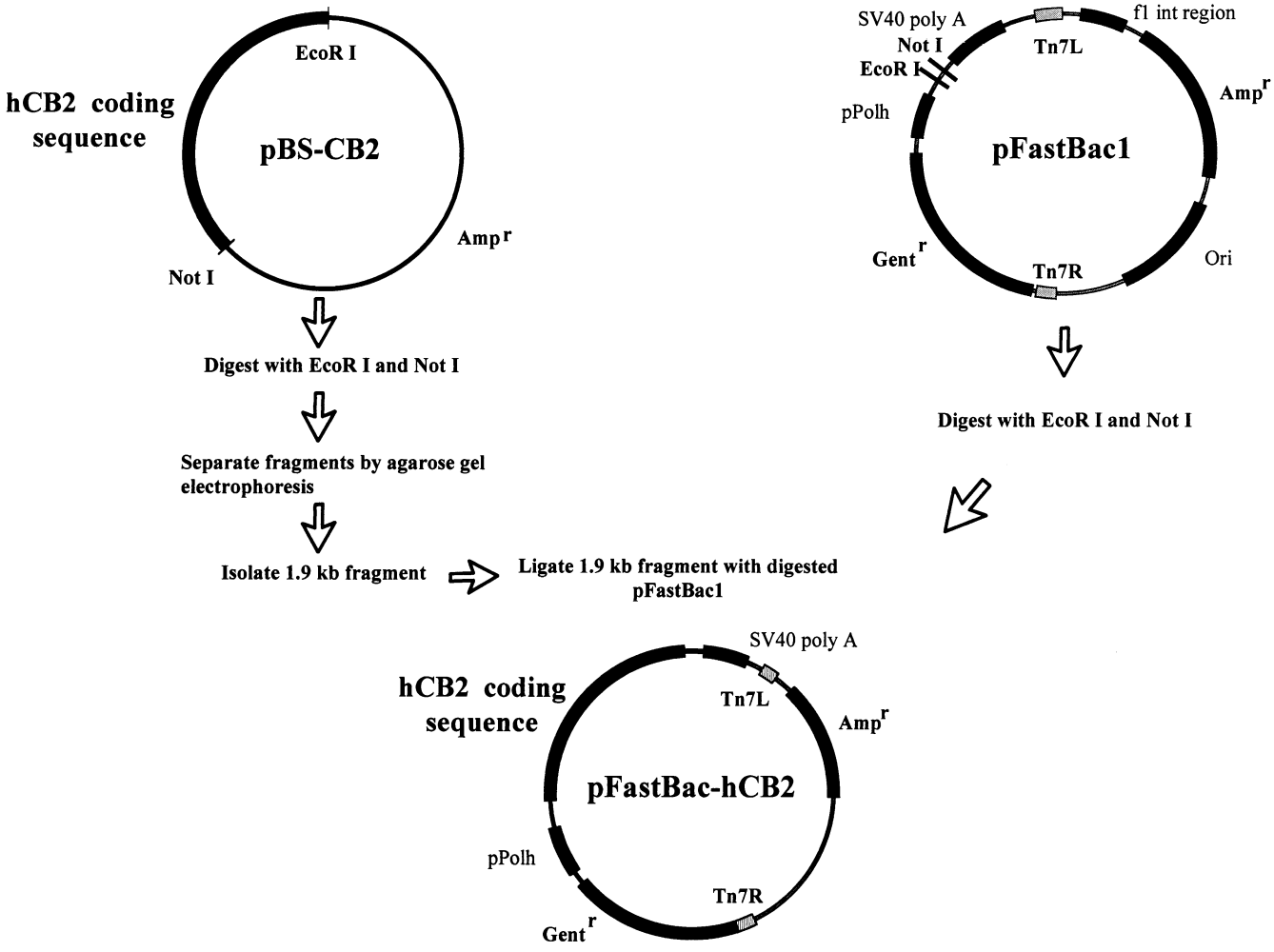

FIG. 2. Construction of the pFastBac-hCB2 donor plasmid for site-specific transposition of the hCB2 into bacmid DNA. The hCB2 cDNA within pBS-CB2 was subcloned into pFastBac1 to produce the transposition donor plasmid pFastBac-hCB2.

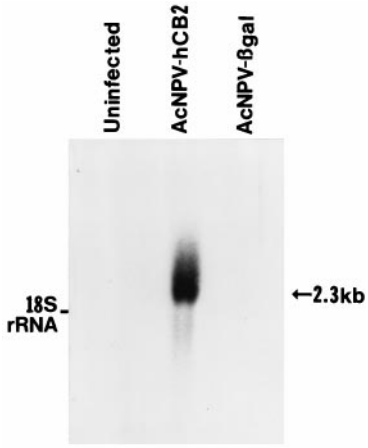

FIG. 3. Northern analysis of total RNA obtained from uninfected, AcNPV-hCB2-infected, and AcNPV-β-gal-infected Sf9 cells. A unique 2.3-kb transcript was observed from total RNA obtained from AcNPV-hCB2-infected Sf9 cells. The size of the AcNPV-hCB2 transcript was extrapolated in relation to the location of the 18S rRNA marker.

hCB2.CV antibody to determine the conditions of optimal hCB2 receptor expression (Fig. 4). Low level expression of hCB2 receptor was observed at all time points in Sf9 cells infected at an MOI of 0.01 (data not shown). No signal was observed from homogenates of uninfected Sf9 cells. In contrast, high levels of hCB2 receptor protein were observed from homogenates of cells infected with AcNPV-hCB2 at an MOI of 0.1 (Fig. 4). Positive immunostaining at a detection level of 2 μg total cellular protein was observed at 36 hr pi. Maximal immunostaining for hCB2 receptor was detected at 48 and 60 hr pi to a detection level exceeding 60 ng of total cellular protein. By 72 hr pi, lower levels of immunospecific staining were obtained. At this

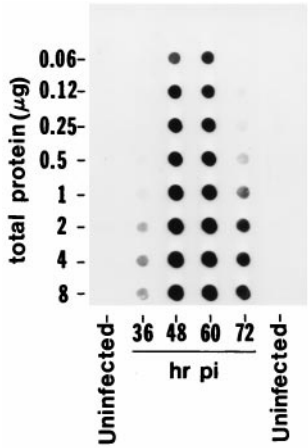

FIG. 4. Immunodot blot of whole cell homogenates of uninfected and AcNPV-hCB2-infected Sf9 cells (MOI of 0.1). Infected cell homogenates (8 μg of total protein) were subjected to two-fold dilutions and incubated with affinity-purified rabbit anti-hCB2.CV. Immunoreactive complexes were identified by means of enhanced chemiluminescence using horseradish peroxidase-conjugated goat anti-rabbit IgG. Maximal levels of hCB2 receptor were expressed at 48 and 60 hr pi.

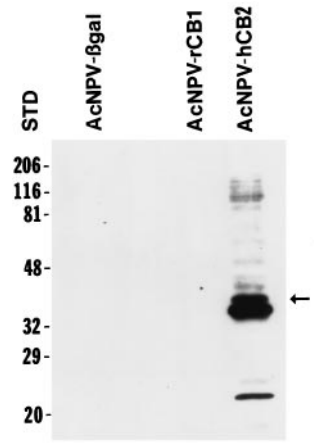

FIG. 5. Western immunoblot analysis of whole cell homogenates of AcNPV-β-gal-infected, AcNPV-rCB1-infected, and AcNPV-hCB2-infected Sf9 cells (MOI of 0.1). Homogenates were obtained from cells at 72 hr pi in order to compensate for the differential time course of virus-specified protein expression by the disparate constructs. Lanes were loaded with constant protein (25 μg). Note the absence of signal in the lanes containing homogenates of AcNPV-β-gal-infected and AcNPV-rCB1-infected Sf9 cells. The arrow denotes the major protein species in the 40 kDa relative molecular mass range consistent with that predicted for hCB2.

latter time point, detectable immunostaining was observed at a level of 250 ng total cellular protein.

To establish whether the relative molecular weight of the immunospecific species was consistent with that predicted for the hCB2 receptor, Western immunoblotting analysis was performed (Fig. 5). Homogenates of AcNPV-hCB2-infected, AcNPV-rCB1-infected, and AcNPV-β-gal-infected cells (MOI of 0.1) were obtained at 72 hr pi. This late time point pi was selected to compensate for differential time course of expression of the disparate constructs. The predominant immunoreactive species had a relative molecular mass of 40 kDa, which is consistent with that predicted for the hCB2 receptor protein following extrapolation of its coding sequence from the cDNA (39.68 kDa). Less intense immunoreactive species were observed having relative molecular masses of less than 40 kDa, and apparently represent degraded hCB2. In addition, species exceeding 40 kDa in relative molecular mass and representing potentially aggregated and glycosylated receptors were seen. No signal was observed in the lanes containing proteins from homogenates of AcNPV-rCB1-infected or AcNPV-β-gal-infected Sf9 cells, supporting the conclusion that the AcNPV-hCB2 recombinant baculovirus expresses hCB2.

Immunofluorescence of AcNPV-hCB2-Infected Cells

To confirm that AcNPV-hCB2-infected Sf9 cells expressed the hCB2 receptor protein, and to determine the cellular localization of expressed protein, immunofluorescence was performed. Infected Sf9 cells were harvested at time periods ranging from 12 to 72 hr pi, collected onto glass slides, and examined by immunofluorescence using rabbit anti-

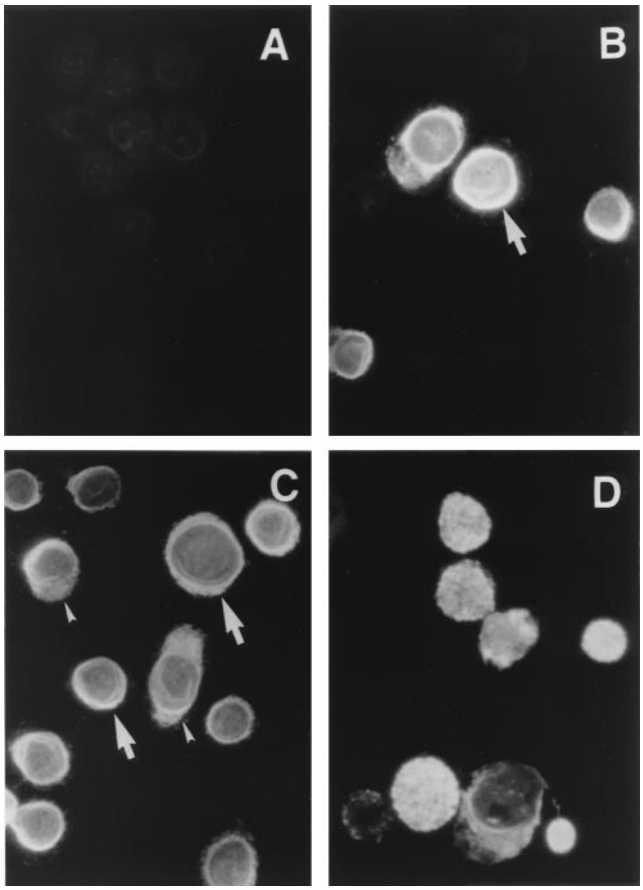

FIG. 6. Immunofluorescence staining in AcNPV-hCB2-infected Sf9 cells (MOI of 0.1). Cells were harvested onto slides by centrifugation at 12, 24, 36, 48, 60, and 72 hr pi, fixed in absolute acetone, and incubated with affinity-purified anti-hCB2.CV antibody followed by goat anti-rabbit IgG (H and L) conjugated to fluorescein isothiocyanate. (A) No immunofluorescence was observed within AcNPV-hCB2-infected Sf9 cells at 12 hr pi. (B) Approximately 10% of cells exhibited cytoplasmic perinuclear immunostaining at 36 hr pi (large arrow). (C) By 48 hr pi, approximately 80% of cells exhibited immunofluorescence. Cytoplasmic immunostaining circumscribing the nucleus (arrow) and in a perinuclear "signet-ring" arrangement (arrow-head) was observed. (D) By 72 hr pi, approximately 100% of cells demonstrated intense immunofluorescence, which encompassed the entire cytoplasm. However, approximately 20% of cells exhibited a cytoplasmic punctate pattern consistent with recombinant virus-induced late gene product expression and attendant host cell dissolution (400×).

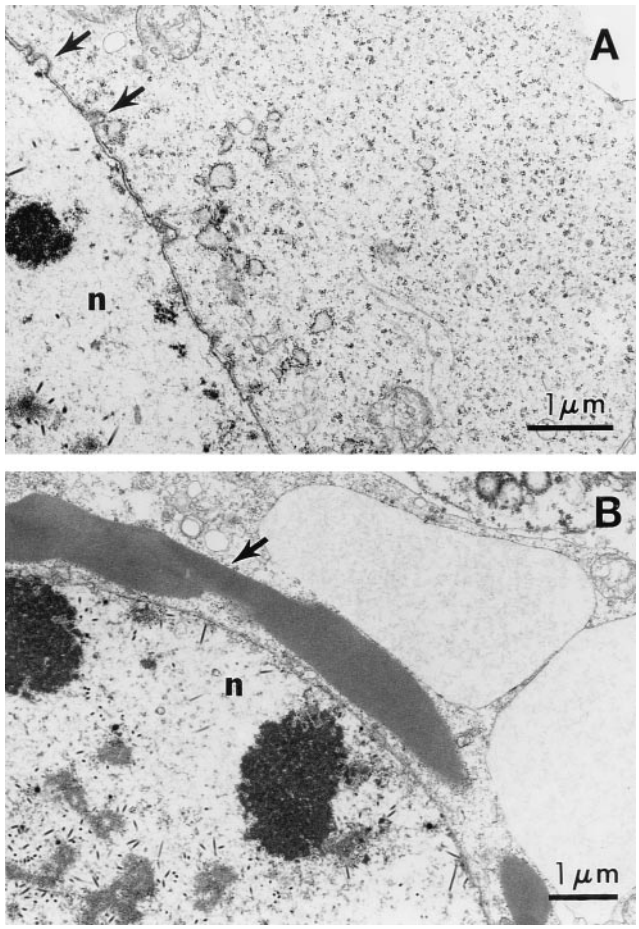

FIG. 7. Transmission electron micrographs of AcNPV-hCB2-infected Sf9 cells (MOI of 0.1). (A) Cells infected at 24 hr pi with AcNPV-hCB2 contained intranuclear baculovirus particles and exhibited nuclear membranous extensions (arrows) consistent with relatively early recombinant virus-induced cytopathogenesis. (B) Cells infected at 48 hr pi with AcNPV-hCB2 exhibited a high nuclear-to-cytoplasmic ratio, active regions of intranuclear virus transcription, intranuclear baculovirus particles, and large proteinaceous inclusions in a perinuclear arrangement (arrow) within the cytoplasm. n = nucleus.

hCB2.CV as the primary antibody (Fig. 6). No fluorescence was observed in the cell monolayers at 12 hr pi. At 36 hr pi, approximately 10% of cells exhibited cytoplasmic fluorescence in a perinuclear band. At 48 and 60 hr pi, an intense perinuclear cytoplasmic immunofluorescence pattern was observed in greater than 90% of cells. Approximately 30% of cells at these two time periods pi exhibited a cytoplasmic perinuclear pattern, which was characterized by a "signet-ring" effect. At 72 hr pi, approximately 100% of cells were positive for fluorescence. The majority of these (i.e. approximately 80%) exhibited an immunofluorescence pattern that encompassed the entire cytoplasm. The remaining cells demonstrated a punctate cytoplasmic immunofluorescence, which is consistent with the induction of late virus-induced cytopathology. In addition, at 60 and 72 hr pi, many cells were circumscribed with immunostaining, suggestive of hCB2 expression on the cell surface. Uninfected Sf9 cells incubated with anti-hCB2.CV exhibited no fluorescence. Similarly, AcNPV-hCB2-infected cells at 72 hr pi, incubated with preimmune IgG obtained from the same rabbit used to elicit anti-hCB2.CV, exhibited no fluorescence (data not shown).

Transmission Electron Microscopy

Transmission electron microscopy was undertaken to monitor the cellular effects associated with infection and hyperproduction of the hCB2 receptor (Figs. 7 and 8). Uninfected Sf9 cells exhibited intact cytoplasmic and cell surface membrane structures and a normal nuclear-to-

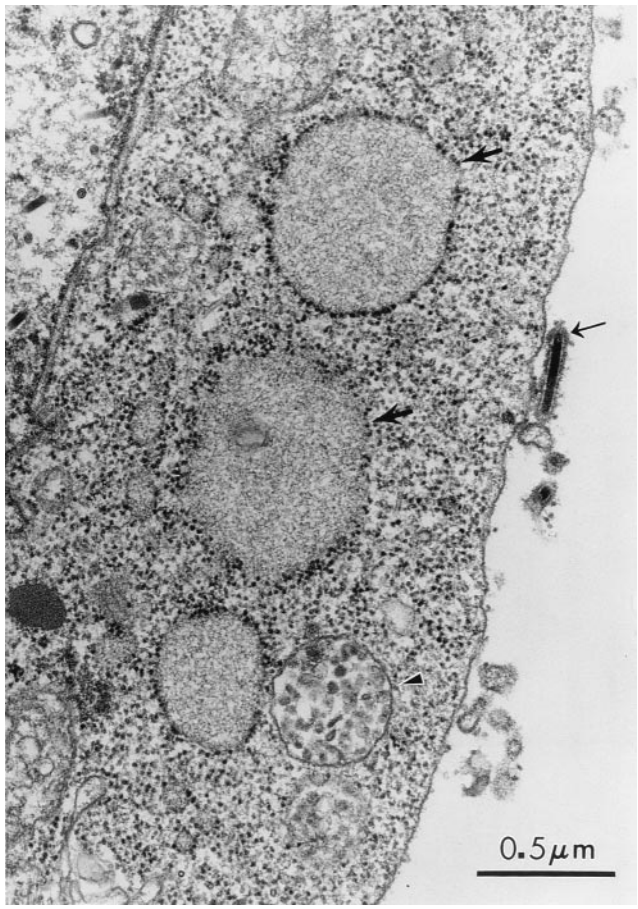

FIG. 8. Transmission electron micrograph of AcNPV-hCB2-infected Sf9 cell at 60 hr pi. Note the presence of accumulations of proteinaceous material in remnant vesicles, putatively packed with hCB2 receptor protein (large arrows). The thin arrow denotes an extracellular baculovirus particle, while the arrowhead depicts an intracytoplasmic virus inclusion.

cytoplasmic ratio of approximately 1:1 by area. In contrast, cells infected with AcNPV-hCB2 demonstrated features typical of recombinant baculovirus infection by 24 hr pi (Fig. 7A). These included intranuclear accumulations of electron-dense material representing areas of apparent active viral transcription, and enlargement of the nuclear compartment resulting in an increased nuclear-to-cytoplasmic ratio. In approximately 20% of the cells, the nucleus encompassed most of the cellular area such that the cytoplasmic compartment consisted of a thin band circumscribing the nucleus. At 48 and 60 hr pi (Figs. 7B and 8), large accumulations of virus-associated structures, intranuclear virus particles, and cytoplasmic proteinaceous inclusions were observed. At 72 hr pi, the ultrastructural features associated with virus infection, that were observed in cells at 48 hr pi, persisted. However, at this relatively late stage pi, extensive cytoplasmic vacuolization was observed in approximately 30% of the cells, and dissolution of the plasma membrane was noted in approximately 10% of the cells. In addition, aggregates of intracytoplasmic virus, and virus particles at the cell surface, were evident (Fig. 8). Electron-dense cytoplasmic accumulations, consistent with glycoprotein expression occurred in a cytoplasmic perinuclear pattern reminiscent of that observed by immunofluorescence. These electron-dense accumulations appeared in association with cytoplasmic membranous structures including both smooth vesicles and vesicles complexed with ribosomal structures. In addition, numerous accumulations of proteinaceous-like material in remnant vesicles of the rough endoplasmic reticulum were present (Fig. 8). These remnant vesicles of the rough endoplasmic reticulum compacted with putative proteinaceous material were observed at 48, 60, and 72 hr pi.

Binding Assay Characteristics

Binding assays were performed to determine if recombinant hCB2 was capable of binding ligands with similar affinities as those of native hCB2 protein. Total binding to P2 membranes from the hCB2-infected Sf9 cells (MOI of 0.1, 72 hr pi) was found to be linear at protein concentrations from 40 to 200 μg/mL (data not shown). Specific binding reached a plateau at protein concentrations above 160 μg/mL. Therefore, 100 μg/mL of recombinant hCB2 baculovirus-infected cell membrane protein was used in all subsequent assays. Specific binding to hCB2 membranes averaged 83% at a radioligand concentration of 1 nM. Uninfected and AcNPV-β-gal-infected Sf9 cells displayed no specific binding within the same range of protein concentrations.

Characterization of [^{3}H]CP 55,940 Binding to hCB2-Infected Cells.

Computer analysis of saturation data for the AcNPV-hCB2-infected cells indicated a K_d of 2.24 ± 0.76 nM, a B_{max} of 5.24 ± 0.24 pmol/mg protein, and a Hill coefficient of 0.997 ± 0.01 (mean ± SEM, $N = 3$). A representative Scatchard–Rosenthal plot is shown in Fig. 9.

Competition Studies

To identify the pharmacological specificities of the hCB2 receptor expressed in insect cells, WIN 55,212-2 ($N = 4$) and SR 141716A ($N = 3$) were tested for inhibition of [^{3}H]CP 55,940 binding. The resulting displacement curves are shown in Fig. 10. SR 141716A, the CB1-selective antagonist, competed for [^{3}H]CP 55,940 binding only at a high concentration (100 μM, where 40–50% displacement was observed). These data established the identity of this receptor as CB2, and is consistent with data from CB2 CHO cells [23]. WIN 55,212-2 displayed high affinity for the CB2 receptor expressed in baculovirus-infected insect cells (K_i = 7.15 ± 2.63 nM).

DISCUSSION

The discovery of cDNA clones coding for the CB1 and CB2 cannabinoid receptors [13, 14] has provided valuable

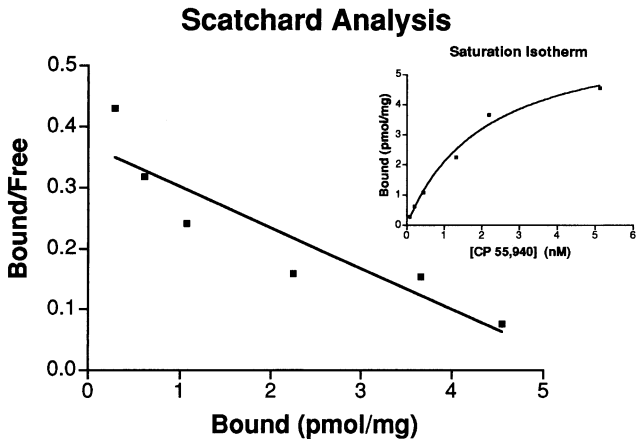

FIG. 9. Scatchard–Rosenthal analysis of [^{3}H]CP 55,940 receptor binding in membrane preparations of Sf9 cells infected with AcNPV-hCB2 (MOI of 0.1, 72 hr pi). Inset: Saturation binding isotherm of specific binding from membranes prepared from these cells. In this representative experiment, B_{max} was 5.48 pmol/mg protein, and K_d was 1.18 nM. Data points are the means of triplicate determinations.

insight concerning the expression of their respective messages and has allowed for extrapolation of basic biochemical and biophysical features. However, direct characterization of the receptors as protein entities and definition of their ligand binding sites have remained elusive. Such analysis is dependent on the availability of the purified protein, which exhibits post-translational features comparable to those found in the native state. Because the CB2 receptor is expressed at low levels in immunocytes [7, 8], the probability of its isolation from native tissues is unlikely. Strategies that employ recombinant DNA expression using bacterial and stably-transfected mammalian cell systems have attendant limitations. On the other hand, baculovirus expression systems offer certain advantages relevant to the production of cannabinoid receptor proteins. First, as a eukaryotic system, post-translational processing is similar to that in mammalian systems. Second, this system has been shown to allow for the relatively high level expression of G protein-coupled receptors [24], including that of the CB1 receptor [15].

In the present investigation, we report on the development of a recombinant baculovirus expression system capable of producing high levels of hCB2, which exhibits post-translational and binding properties approximate to those of the native hCB2. A Bac-to-Bac system that utilized site-specific transposition for insertion of hCB2 cDNA was employed for construction of the recombinant baculovirus. To allow for optimal recombinant receptor protein production, a PCR strategy was employed to remove the 5′ noncoding sequence from the hCB2 cDNA. The noncoding sequence was minimized in order to remove the sequence that resulted in a transcription stop in the recombinant plasmid and to circumvent potential translational regulation of protein expression [25]. Purified recombinant bacmid DNA, confirmed by Southern analysis to contain hCB2, was used to transfect insect cells to produce the recombinant AcNPV-hCB2 baculovirus. This strategy, which utilized site-specific transposition of the hCB2, resulted in the construction of a recombinant AcNPV-hCB2 baculovirus, and circumvented the need for extensive plaque purification since hCB2 insertion was readily visualized at the bacterial level using blue-white colony screening.

To establish whether the recombinant AcNPV-hCB2 was replication competent, titration plaque assays were performed. *In vitro* infectivity titers exceeding 10^9 pfu/mL were obtained, verifying the replication competence of the recombinant virus. Then studies were performed to determine whether the recombinant AcNPV-hCB2 had the capacity to express high levels of specific hCB2 gene product. To address this issue, expression of hCB2 mRNA and receptor protein were assessed. Northern analysis revealed the production of a unique transcript corresponding approximately to the 2.3-kb size expected of the recombinant hCB2 transcript. Thus, Sf9 cells infected with AcNPV-hCB2 were capable of expressing human peripheral cannabinoid receptor mRNA.

To determine if the expression of hCB2 mRNA resulted in the subsequent production of recombinant hCB2 receptor protein, immunodot blot and Western immunoblot studies were conducted. Affinity-purified antibody elicited

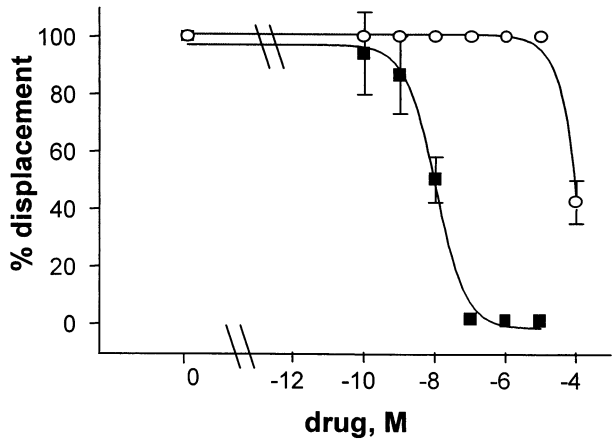

FIG. 10. Displacement of [^{3}H]CP 55,940 binding by WIN 55,212-2 and SR 141716A in membrane preparations of Sf9 cells infected with CB2 baculovirus. Data shown are the mean (± SEM) of three (SR 141716A) and four (WIN 55,212-2) experiments performed in triplicate.

against a peptide sequence comprised of amino acids 320–336 of the intracellular carboxy terminus of the human peripheral cannabinoid receptor was used. Immunodot blot analysis revealed that optimal levels of hCB2 protein were obtained at 48 and 60 hr pi, followed by decreased production at 72 hr pi. In addition to time-dependent expression, the immunoblot studies indicated that yields of receptor protein were dependent on the input multiplicity of recombinant virus infection (MOI). Based on immunoreactivity, optimal levels of receptor protein were observed at 48 and 60 hr pi when cells were infected at an MOI of 0.1. However, diminished levels of hCB2 were observed when cells were infected at higher multiplicities. On the other hand, relatively low levels of receptor were detected at all time points pi in cells infected at lower multiplicities (i.e. <0.1). Thus, at high multiplicities, multicopy virus gene expression within individual cells results in accelerated production of receptor protein. Such accelerated biosynthesis, concomitant with rapid depletion of pools of intracellular amino acids and disruption of cellular membranes could effect cessation of further virus-specified gene expression. On the other hand, at a low MOI (i.e. less than 0.1), an insufficient number of host cells may be infected to yield relatively high levels of gene product. Temporal and input multiplicity dependence of virus-specified gene product expression is a characteristic feature of virus systems. This dependency may be especially germane to that of the expression of G protein-coupled receptors, such as cannabinoid receptors, which are localized in membranes and possess seven hydrophobic transmembrane domains. The observations at 72 hr pi are consistent with recombinant rCB1 baculovirus-specified gene expression wherein cellular dissolution and disaggregation occur as a result of virus-induced cytopathogenesis [15]. Indeed, alterations of cell surface membranes, with attendant defects in selective permeability, have been proposed as the mode by which certain viruses effect the shutdown of host cell macromolecular synthesis [26–28].

Western immunoblot analysis on homogenates of cells infected at the optimal MOI (i.e. 0.1) revealed the presence of immunoreactive bands of relative molecular masses ranging from 25 to 100 kDa. The presence of multiple immunoreactive protein bands is consistent with high level expression of hydrophobic protein by recombinant baculovirus [15]. The major immunoreactive band of 40 kDa most likely represents fully synthesized, unglycosylated receptor recombinant protein and is in agreement with that predicted for hCB2 based on extrapolation of its relative molecular mass (i.e. 39.68 kDa) from the corresponding cDNA. The immunoreactive species of relative molecular masses exceeding 40 kDa may represent glycosylated receptor or receptor in aggregated form. The presence of aggregated receptor is not unexpected due to the highly hydrophobic nature of this protein. The presence of immunoreactive species of relative molecular mass of less than 40 kDa may be due to degradation of receptor during the solubilization process. Confirmation that the recombinant protein expressed represents hCB2 is supported by the observation that no immunoreactive bands were observed in homogenates of β-gal recombinant baculovirus-infected cells or AcNPV-rCB1-infected cells.

The immunofluorescence data, indicative of hCB2 expression within AcNPV-hCB2-infected Sf9 cells, were in agreement with the receptor protein expression data obtained by immunodot blot and western immunoblot analyses. The level and intensity of immunofluorescence observed in infected cells at different times pi approximated that of immunoreactivity for protein assessed by immunodot blot at the same time points pi. The immunofluorescence data also provided support for the aggregation of expressed receptor, as observed by western immunoblot analysis. At earlier times pi (i.e. 36 and 48 hr pi), a granular punctate distribution of hCB2 was observed within infected cells. However, at later times pi (i.e. 60 and 72 hr pi), large accumulations and clusters of immunofluorescence within the cytoplasm were observed. The appearance of these receptor accumulations may be due to overproduction of protein, resulting in formation of inclusion bodies.

In addition, the immunofluorescence experiments provided insight regarding the post-translational modification and compartmentation of hCB2 in recombinant virus-infected cells. A cytoplasmic perinuclear circumscription pattern of expression was observed at early periods pi (i.e. 24 and 36 hr). This pattern was followed by a cytoplasmic and reticulated "signet-ring" distribution that radiated outward from the nuclear compartment at 48 hr pi. Intense immunofluorescence encompassing the entire cytoplasm was noted at relatively late time periods pi (i.e. 60 and 72 hr pi). At this time point many cells also demonstrated a punctate, cytoplasmic immunofluorescence consistent with late virus-induced cytopathology. The immunofluorescence observations suggest that the hCB2 is associated with cellular membranes and undergoes glycosylation within Sf9 cells. The appearance of a "signet-ring" conformation, in particular, is consistent with virus-specified protein localization and glycosylation within the Golgi apparatus [29]. In addition, the reticulated cytoplasmic pattern of staining is suggestive of hCB2 interaction with cytoplasmic membrane structures. Collectively, these observations indicate that hCB2 receptor expression in the recombinant virus-infected cells is accompanied by appropriate post-translational modifications and occurs in association with cellular membranes.

Transmission electron microscopy was performed in order to monitor cellular effects associated with recombinant protein expression under conditions of low MOI (i.e. 0.1). Profiles of recombinant virus-infected Sf9 cells exhibited an increased nuclear to cytoplasmic ratio, without other distinctive stigmata of infection, when compared with those of uninfected cells at 12 hr pi. However, by 48 hr pi, greater than 90% of cells infected with AcNPV-hCB2 exhibited distinctive intranuclear structures characteristic of baculovirus infection. These included electron-dense condensations indicative of active regions of intranuclear virus-

transcription, accumulations of intranuclear filamentous elements, aggregates of intranuclear unenveloped virus, and the presence of individual extracellular enveloped baculovirus particles. Moderate cytoplasmic membranous perturbation and intracytoplasmic vacuolization concordant with events associated with virus infection were noted. The observed limited cellular disruption consequent of virus infection is reflective of the low input MOI (i.e. 0.1) that was used to infect cells. These results indicate that low multiplicity conditions for infection of Sf9 cells with the recombinant hCB2 baculovirus circumvent the extensive membrane perturbation and intracytoplasmic vacuolization that occur in cells infected at higher multiplicities (i.e. MOI >1). These observations are in agreement with those obtained previously for production of the rat CB1 receptor using a baculovirus expression system [15]. In those studies, cytopathological events, including extensive cytoplasmic vacuolization and membrane dissolution, concomitant with a low frequency of structurally defined intracellular baculovirus particles, were found to be associated on a temporal basis with diminished production of recombinant CB1.

The pattern of electron-dense depositions at the ultrastructural level, suggestive of the localization of recombinant hCB2, was in agreement with that obtained for hCB2 by immunofluorescence. Electron-dense cytoplasmic accumulations circumscribing the nucleus, and in apparent association with membranous structures, were observed at 36 and 48 hr pi. After these time periods, the electron dense accumulations were found to extend throughout the cytoplasm. In addition, particularly at 60 and 72 hr pi, remnant vesicles of the endoplasmic reticulum packed with proteinaceous material were observed. The putative accumulation of hCB2 within these remnant vesicles is in agreement with the phenomenon of "ER-overload response," which has been observed in human embryonic kidney cells transfected with an adenovirus gene [30]. It has been postulated that ER overload occurs in a pathway through which stressed cells respond to unfolded proteins, especially proteins with transmembrane domains, which are localized in the endoplasmic reticulum until they are transported to the cell surface [31].

To determine if the hCB2 recombinant protein was capable of binding cannabinoids, radioligand binding studies were performed. Radioligand binding studies of the AcNPV-hCB2-infected cells confirmed the presence of a functional CB2 binding site. Saturable, high-affinity binding was observed using [^{3}H]CP 55,940 as a radioligand. Infected cells demonstrated high levels of [^{3}H]CP 55,940 binding (B_{max} of 5.24 ± 0.24 pmol/mg protein). No specific binding was detected in uninfected or β-galactosidase-infected Sf9 cells. SR 141716A, the CB1-selective antagonist, did not displace [^{3}H]CP 55,940 until a concentration of 100 μM, which established the presence of a CB2 receptor as opposed to a CB1 receptor [32]. The CB2 receptor expressed in baculovirus-infected Sf9 cells had high affinity for WIN 55,212-2, as would be expected for the CB2 receptor subtype [23, 33].

It is characteristic of viruses that production of virus-specified gene products under conditions of one-step growth during the logarithmic phase of the replication cycle is related directly to ambient temperature, input multiplicity of infection, and time post-infection. Thus, appropriate modulation of these factors allows for optimization of expression levels of fully processed virus-specified proteins. Application of this strategy is especially relevant to recombinant baculovirus expression of cannabinoid receptors, which are highly hydrophobic and are translocated to the plasma membrane in the native state in eukaryotic cells. In the present investigation, an MOI of 0.1, 28°, and 60 hr pi proved optimal for the expression of high levels of hCB2 receptor protein, which retained the CB2 binding site based on radioligand binding. These results indicate that this baculovirus system may be applicable for the high level expression of hCB2 and its subsequent preparative isolation for biochemical and biophysical characterization. In addition, this system offers the potential for isolation of homogeneous receptor in sufficient quantities to allow for mapping of the ligand binding site. In this context, the baculovirus expression system may prove ideal, since expression of hCB2 in native tissue is at low levels and its preparative isolation is not feasible. Furthermore, since the recombinant hCB2 baculovirus exhibits CB2 binding properties, it could serve as a reproducible source of purified protein for binding studies following coreconstitution with various purified G proteins in phospholipid vesicles. Such reconstituted receptors have been shown to retain regulatory activity and appropriate selectivity among G proteins [24].

In conclusion, a baculovirus system has been developed that allows for the expression of relatively high levels of hCB2 exhibiting CB2 binding properties. Studies are in progress to isolate preparative levels of the recombinant hCB2 receptor protein and to define its basic biochemical and biophysical features.

The authors thank Dr. Larry Melvin for the synthesis of CP 55,940; Dr. John Lowe for the synthesis of SR 141716A; and Dr. Susan Ward for provision of WIN 55,212-2. The authors also thank Ms. Cathy Boothe and Ms. Judy Williamson for excellent technical assistance. This research was supported by National Institutes of Health/NIDA awards DA05832, DA05274, DA09158, and TA32DA07027.

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