FEBS Letters 351 (1994) 375–379

FEBS 14501

Molecular cloning of a novel candidate G protein-coupled receptor from rat brain

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Received 12 July 1994

Abstract A PCR cloning strategy using primers designed from sequences selectively conserved among a cannabinoid receptor and two orphan receptors, was used to isolate novel G protein-coupled receptors. rCNL3, a 1.75 kb cDNA encoding a 363 amino acid protein, was isolated from a rat cerebral cortex library. Sequence analysis showed that rCNL3 possesses a number of structural characteristics of G protein-coupled receptors and has 61% amino acid identity (from transmembrane region one through the carboxyl-terminus) with two other candidate G protein-coupled receptors. Therefore, these three receptors may comprise a receptor subfamily with identical or closely related endogenous ligands. Northern and in situ hybridization experiments demonstrated that rCNL3 mRNA is expressed in the rat brain, with a prominent distribution in striatum.

Key words: G protein-coupled receptor; Cloning; Rat brain; Striatum; Retrosplenial cortex

1. Introduction

G protein-coupled receptors mediate cellular responses elicited by a wide variety of extracellular stimuli [1,2]. These stimuli range from photons, odorants and ions to cytokines, hormones and neurotransmitters. Consequently, G protein-coupled receptors are very important for the normal functions of many systems, including vision, smell, immune response, endocrine function and neurotransmission. Abnormalities in the structures and functions of G protein-coupled receptors are responsible for certain diseases [3,4]. Molecular cloning of novel G protein-coupled receptors may contribute to a better understanding of the normal functions of these receptors and the roles that these receptors might play in certain diseases.

Molecular cloning studies have revealed that many G protein-coupled receptors have common structural features, including seven hydrophobic putative transmembrane domains, six hydrophilic loops, an extracellular amino-terminus, and an intracellular carboxyl-terminus [2,5]. G protein-coupled receptors can be grouped into at least three superfamilies, namely the secretin/VIP receptor family, the metabotropic glutamate receptor family, and the rhodopsin receptor family which contains the vast majority of the known receptors. Among these superfamilies, there is little sequence homology. Within each family, however, certain amino acid residues are well conserved, for example, the sequence motif E/DRY at the end of the third transmembrane domain in the rhodopsin receptor family. By exploiting the sequence homologies within each receptor superfamily, approaches based on low-stringency hybridization and polymerase chain reaction (PCR) have been used to clone new members of G protein-coupled receptors [6–8]. Many of these candidate receptors later have been shown to have important functions [6,9,10]. In this paper, we report the cloning of a novel candidate G protein-coupled receptor that is localized predominantly to rat striatum.

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2. Materials and Methods

2.1. cDNA cloning and sequencing

A cDNA library for rat cerebral cortex consisting of 7×10^6 clones was constructed in the mammalian expression vector pCD [11]. DNA prepared from this library or rat genomic DNA was used as a template for PCR. Degenerate PCR primers (CN4 and CN7) were designed based on conserved amino acid sequences of SKR6, edg-1 and 6-7 (Fig. 2). SKR6 was the only cannabinoid receptor that had been cloned when this project was initiated [6]. edg-1 is a published orphan receptor [12]. 6-7 is an orphan receptor (Bonner and Brownstein, unpublished, GenBank Accession Number U12184) which is virtually identical to R334, a published orphan receptor [19], except R334 has a different 5′-untranslated region and several apparent sequencing errors. In the region of the upstream PCR primer used for this study, the sequence of 6-7 is different from R334, probably due to a pair of frame shifts within R334. This pair of PCR primers was designed to isolate those potential receptor cDNAs that might be related to SKR6, edg-1, or 6-7. The sequence for the upstream primer (CN4) was 5′-CCCGGATCC CCI NTI NIN GGI TGG AA(C/T) TG-3′. The sequence for the downstream primer (CN7) was 5′-GGGATCGAT IA(A/G) IG(C/T) (A/G)TA IAT IA(C/T) NGG (A/G)TT-3′. The 100 μl PCR reaction mixture contained 1.3 μg template DNA, 1 μM of each primer, 200 μM each of dATP, dTTP, dCTP and dGTP, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 mM MgCl and 2.5 units of *Taq* DNA polymerase (Perkin Elmer Cetus). PCR conditions were: 1 min at 93°C, 2 min at 50°C, and 2 min at 72°C. After 33 cycles, the products were incubated for 7 min at 72°C. The PCR-amplified products of interest were purified on a 2% NuSieve gel (FMC Bioproducts), digested with *ClaI* and *Bam*HI and then subcloned into the *Bam*HI and *Acc*I sites of pUC18 for sequencing. For making sequencing templates, DNA was amplified directly from individual colonies using primers pUC18rev2 (5′-ACA CAG GAA ACA GCT ATG ACC ATG ATT ACG AA-3′) and pUCfor2 (5′-GTG AAA TAC CGC AGA GAT GCG-3′). The PCR-amplified products were then purified on Magic columns (Promega) and sequenced using a cycle sequencing protocol and the ABI universal dye sequencing primers. The sequences were analyzed on a 373A autosequencer (Applied Biosystems) and assembled with the Seqman program (DNASTAR).

To isolate a full-length cDNA for the new candidate G protein-coupled receptor rCNL3, nick-translated probes for Southern and colony hybridization were made from the partial-length rCNL3 cDNA clone generated by PCR [13]. Pools and subpools of the rat cerebral cortex library were screened by Southern hybridization as described previously [14]. The full-length clone was finally isolated by colony hybridization. Southern and colony hybridizations were performed at 60°C in 3 × SSPE (1 × SSPE is 0.15 M NaCl, 0.01 M NaH_2PO_4, 1.3 mM EDTA, pH 7.4), and 1 × Denhardt's solution (0.02% each of polyvinyl-

SSDI 0014-5793(94)00888-4

pyrolidone, bovine serum albumin, and Ficoll). The washing conditions were 60°C in 0.5 × SSPE. In order to obtain the entire cDNA sequence of rCNL3, subclones of this cDNA were made in pUC18 and both strands of the cDNA were sequenced.

2.2. Northern hybridization

For northern hybridization, a 422 base pair *NaeI–NsiI* fragment of the rCNL3 cDNA corresponding to bases 1,136–1,558 was labeled with [^{32}P]dCTP by nick-translation. A rat multiple tissue northern blot containing 2 μg of poly(A)$^+$ RNA in each lane (Clontech) was prehybridized in a solution containing 5 × SSPE, 10 × Denhardt's, 100 μg/ml salmon sperm DNA, 50% formamide and 2% SDS for 3 h at 42°C. The blot was then hybridized in the same buffer with 5×10^6 cpm/ml of nick-translated probe for 20 h at 42°C. The blot was washed to a final stringency of 0.1 × SSPE, 0.1% SDS at 60°C and exposed to X-ray film for up to 2 weeks. The blot was also exposed to Fuji BAS-III imaging plates and images were analyzed on a BAS 2000 phosphor-imaging system (Fuji Biomedical).

2.3. In situ hybridization

To construct a template for making riboprobes, a 422 bp *NaeI–NsiI* fragment of the rCNL3 cDNA (the same fragment used for Northern hybridization) was subcloned into the *SmaI* and *PstI* sites of pGEM4Z vector (Promega). ^{35}S-labeled cRNA probes were transcribed from the linearized template using 20 μM ^{35}S-UTP (1,000–1,500 Ci/mmol, New England Nuclear), with either T7 (for anti-sense probe) or SP6 (for sense probe) RNA polymerase.

Serial 12-μm-thick frozen sections of Sprague–Dawley rat brain were cut on a cryostat-microtome and thaw-mounted onto gelatin-coated glass slides. Tissue pretreatment, hybridization and washing were performed as described previously [15]. The sections were washed to a final stringency of 0.1 × SSC at 65°C. Air-dried slides were exposed to Fuji BAS-III imaging plates for 7–10 days and images were analyzed. Subsequently, slides were coated with Ilford D5 nuclear track emulsion and stored at 4°C for 4 months. The slides were finally developed in D-19 (Kodak) and counterstained with toluidine blue.

2.4. Expression and functional analysis

The full-length cDNAs of rCNL3 and 6-7, which were isolated from a rat cerebral cortex library made in the mammalian expression vector pCD [11], were transfected transiently into COS-7 cells using a DEAE-dextran method [16]. COS-7 cells were maintained in Dulbecoo's Modified Eagle's Medium containing 10% fetal calf serum. Radioligand binding or functional assays were carried out 72 h after transfection. Membrane preparation, radioligand binding, and assays of cAMP accumulation and inositol phosphate release were performed as described previously [17]. To establish cell lines stably expressing rCNL3 or 6-7, CHO-K1 cells were cotransfected with rCNL3 or 6-7 cDNA and a plasmid conferring neomycin resistance, using a calcium phosphate precipitation procedure [18]. Transfected cells were selected in medium containing the neomycin analog G418 (400 μg/ml), cell lines expressing orphan receptor mRNAs were screened by RNA dot-blot analysis and functional assays were also performed on these stably transfected cells.

3. Results

3.1. Isolation of rCNL3 cDNA

PCR amplification of rat genomic DNA or the DNA prepared from a rat cerebral cortex cDNA library resulted in products in the 420–520 base pair range. These DNA fragments were extracted from gel and subcloned into pUC18 for sequencing. In addition to SKR6, edg-1, and 6-7, two novel clones related to edg-1 and two novel clones related to 6-7 were isolated. Among these clones was rCNL3 which is related to 6-7. This partial-length rCNL3 cDNA clone was ^{32}P-labeled and used as a probe to screen the rat cerebral cortex library at high stringency. Using strategies similar to those previously described [14], a cDNA clone with a 1.75 kb insert was isolated from this library.

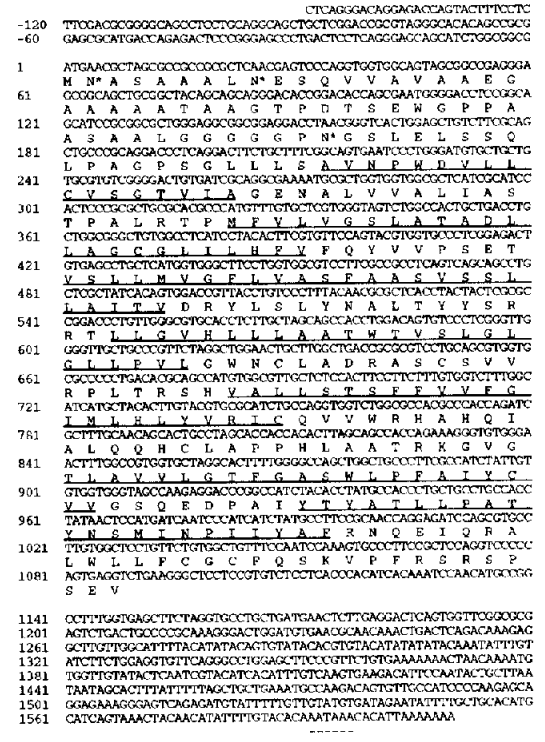

Fig. 1. Nucleotide and deduced amino acid sequence of the candidate receptor rCNL3 cDNA. Nucleotide sequences are numbered beginning with the first residue of the initiating methionine. Putative transmembrane domains are underlined. Potential N-linked glycosylation sites on the amino terminal are indicated by asterisks. A potential polyadenylation signal is indicated by a dashed line.

3.2. Structural characteristics of the rCNL3 cDNA and its protein product

Fig. 1 shows the nucleotide and deduced amino acid sequences of the rCNL3 cDNA clone (GenBank Accession Number U12006). Using the first methionine codon following an in-frame termination codon as the translational initiation site, the nucleotide sequence of the rCNL3 cDNA clone contains an open reading frame of 1,089 base pairs, followed by a 221 base pair 3′-untranslated region containing a polyadenylation signal and ending in a polyA tail. rCNL3 cDNA encodes a protein of 363 amino acid residues, with an estimated molecular weight of 38 kDa, in the absence of post-translational modifications. However, as illustrated in Fig. 1, the consensus sequences for three potential asparagine-linked glycosylation sites are present within the amino-terminus of rCNL3. A hydropathy plot [20] reveals that rCNL3 has seven putative hydrophobic transmembrane domains, connected by three extracellular and three intracellular loops. There is no discernible signal peptide sequence at the amino terminus of rCNL3.

Comparison of rCNL3 with several other G protein-coupled receptors (Fig. 2) indicated that rCNL3 has several conserved features of the 'E/DRY family' (rhodopsin-like family) of G protein-coupled receptors [2,5]. No apparent sequence homology was evident between rCNL3 and members of secretin/VIP receptor family or metabotropic glutamate receptor family. rCNL3 is most closely related to 6-7/R334 and GPCR21 (61% amino acid identity from transmembrane region one through the carboxyl-terminus), two other candidate G protein-coupled

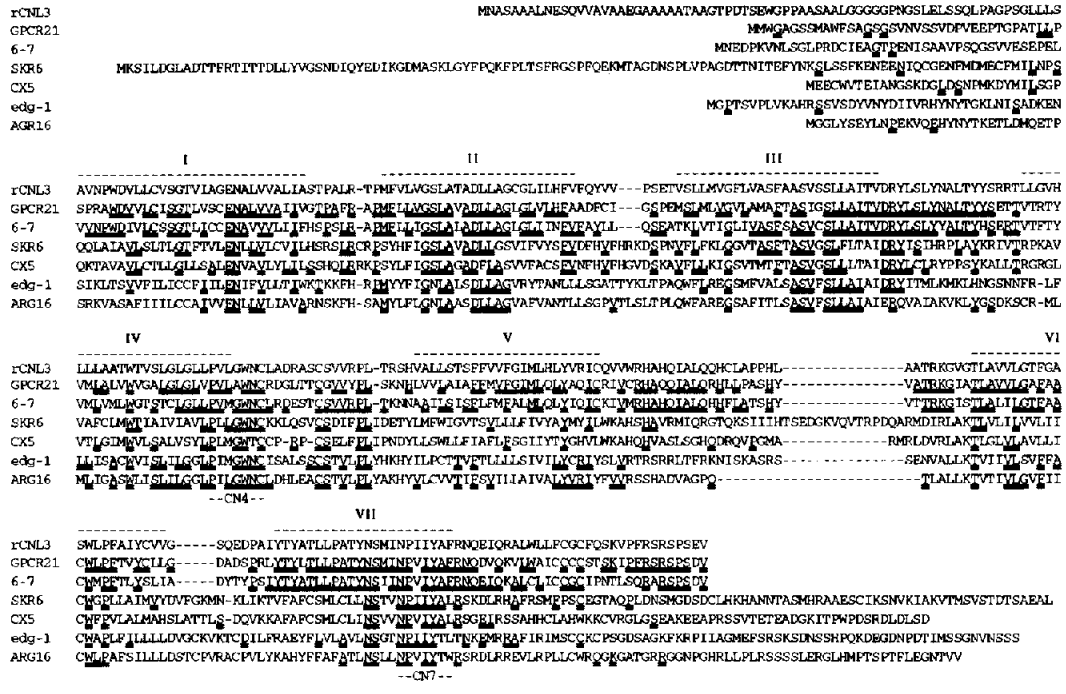

Fig. 2. Alignment of the amino acid sequence of the candidate receptor rCNL3 with six other G protein-coupled receptors. A minimal numbers of gaps (−) were introduced to allow alignment. Amino acid sequences that are identical to rCNL3 are double underlined. Putative transmembrane domains are indicated by dashed lines. The amino acid sequences corresponding to the PCR primers used in this study (CN4 and CN7) are indicated.

receptors ([19,21] and Bonner and Brownstein, unpublished). In addition, in the same region, rCNL3 is 27% identical with central and peripheral cannabinoid receptors SKR6 and CX5 [6,22], and 27% identical with two related orphan receptors edg1 and AGR16 [12,23] Compared to other G protein-coupled receptors, rCNL3, 6-7/R344, GPCR21, edg-1, AGR16 and the two cannabinoid receptors have several unique features. They all have Glu-Asn rather than Gly-Asn in transmembrane region 1, they have no Cys residue in the second extracellular loop and no Pro residue in transmembrane region 5.

3.3. Tissue distributions of rCNL3 mRNA

Northern hybridization analysis was performed to investigate the tissue distribution and the size of rCNL3 mRNA (Fig. 3). Under high stringency conditions, strong signal for a single RNA species of 2.1 kb was found in the brain after a 24 h X-ray film exposure. Following 2 weeks exposure on X-ray films or 10 days exposure on Fuji imaging plates (which is about 5 times more sensitive than X-ray film for ^{32}P), no hybridization signal was detected in heart, spleen, lung, liver, skeletal muscle, kidney and testis.

To examine in more detail the distribution of rCNL3 mRNA expression in brain, in situ hybridization histochemistry was conducted on rat brain coronal sections (Fig. 4). With the ^{35}S-labeled antisense RNA probe, hybridization signals were detected in the caudate-putamen, nucleus accumbens, olfactory tubercle and retrosplenial cortex. In contrast, with the sense probe, no significant signal was observed in any brain region. Some sections were examined at higher resolution after exposure with nuclear emulsion. Again, the labeling of cells within the caudate-putamen was especially prominent, with approximately 40% of the medium-sized neurons being labeled. We also noted occasional labeled cells within the medial amygdala and stratum radiatum of the hippocampus.

3.4. Functional analysis of rCNL3

In our attempts to identify the ligand for the two related orphan receptors, rCNL3 and 6-7, cAMP accumulation (stimulation and inhibition of adenylyl cyclase) and inositol phosphate release assays were performed with various potential ligands on rCNL3 and/or 6-7 cDNA transfected cells. In addition, radioligand binding assays were conducted on membranes prepared from these cells.

Ligands tested with cAMP accumulation assays included endogenous cannabinoid agonist anandamide and related compounds palmityl-ethanolamide, γ-linolenylethanolamide, dihomo-γ-linolenylethanolamide, adrenylethanolamide and docosahexaenyl-ethanolamide; synthetic cannabinoid agonists Win55212–2 (a prototypic aminoalkylindole) and (−)-11-hydroxy-Δ^8-tetrahydrocannabinol-3-dimethylheptyl(HU210);imidazo-line receptor agonist clonidine; neuropeptides α-melanocyte stimulating hormone (α-MSH), galanin, and calcitonin gene related peptide (CGRP).

Ligands tested with inositol phosphate release assays were, lekotrienes B4, C4 and D4, ATP, dopamine, galanin, pancreastatin, and pancreastatin (26–51).

Those tested with radioligand binding assays were Win 55,212–2, glibenclamide (a sulfonyl urea), baclofen (a $GABA_B$ receptor agonist), nicotinamide-adenine dinucleotide (NAD), 1,3,di-ortho-tolyl-guanidine (DTG) (a sigma receptor ligand), haloperidol (a sigma and dopamine receptor ligand), L-DOPA, (+)-butaclamol (a dopamine receptor ligand), clonidine and galanin.

In our assays, none of the ligands tested were able to produce

any specific funtional responses on rCNL3 or 6-7 cDNA transfected cells, or bind specifically to rCNL3 or 6-7. However, since questions such as whether a functional receptor is expressed properly and coupled properly to second messenger systems cannot be answered without identifying the ligand for the receptor, at this point we can not be completely certain that the compounds we tested are not the ligands for these candidate receptors.

4. Discussion

In our efforts to clone novel G protein-coupled receptors, particularly those which might be related to cannabinoid receptors, rCNL3 cDNA was isolated from a rat cerebral cortex library. Like other G protein-coupled receptors, the amino acid sequence of rCNL3 predicts that it consists of seven hydrophobic transmembrane domains and six connecting loops. In addition, rCNL3 also has conserved amino acid residues and motifs typical of G protein-coupled receptors. Therefore from the structural characteristics of rCNL3, we conclude that it is a new G protein-coupled receptor.

In this study, the tissue distributions of rCNL3 mRNA was first mapped by Northern hybridization analysis. Among the tissues that we examined, rCNL3 mRNA was detected only in the brain. This result indicates that rCNL3 is likely a receptor for a neurotransmitter or a neuromodulator. Furthermore, the anatomical and cellular distributions of rCNL3 mRNA in the rat brain were determined by in situ hybridization histochemistry. The fact that rCNL3 mRNA is found in striatum, retrosplenial cortex, amygdala, and hippocampus suggests that it may be involved in motor and learning functions. Medium-sized neurons in the striatum are primarily composed of two populations [24,25]. Striatopallidal neurons contain enkephalin and the D1 receptor, whereas striatonigral neurons contain substance P, dynorphin, and the D2 receptor. The observation that approximately 40% of striatal medium-sized neurons are labeled by rCNL3 probe suggests that rCNL3 is expressed in essentially all the neurons of one of the two populations. We are currently engaged in further experiments to examine this hypothesis.

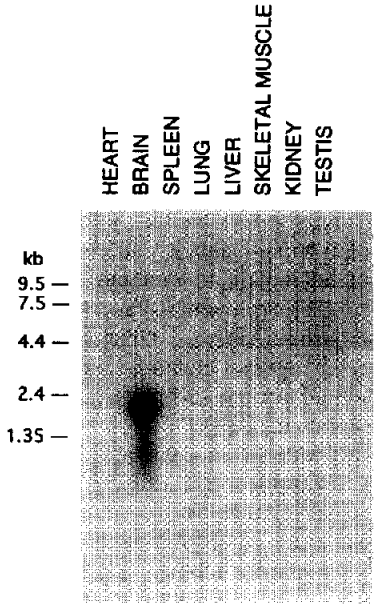

Fig. 3. Northern hybridization analysis of the candidate receptor rCNL3 mRNA expression. Each lane contained 2 μg of poly(A)$^+$ RNA of rat tissues. Positions of RNA molecular weight markers are indicated. A ^{32}P-labeled fragment of rCNL3 cDNA was used for hybridization. The filter was washed at high stringency conditions and exposed to X-ray film for 14 days.

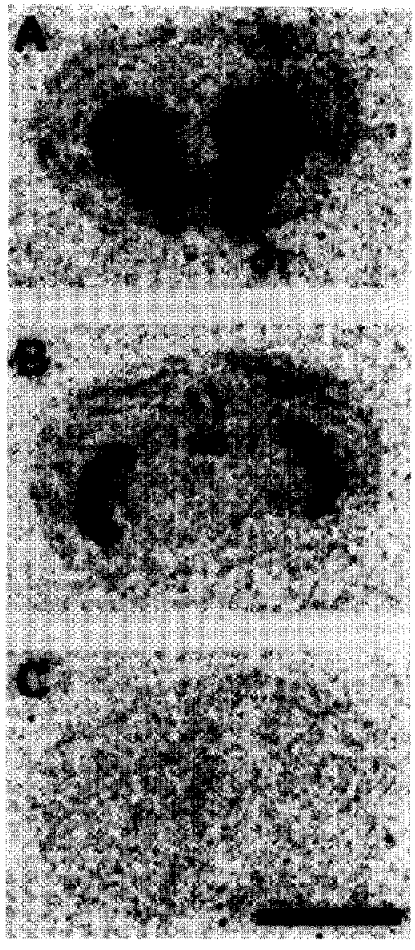

Fig. 4. In situ hybridization analysis of the distribution of rCNL3 mRNA in the brain. Coronal sections of rat brain were hybridized with either antisense (A and B) or sense (C) riboprobes of rCNL3. After washing under high stringency conditions, the slides were exposed to a Fuji imaging plate for 7 days. CP, caudate-putamen; NA, nucleus accumbens; OT, olfactory tubercle; Cx, cortex; RS, retrosplenial cortex. Bar = 5 mm.

Even though that the amino acid sequence of rCNL3 clearly demonstrates the structural features of G protein-coupled receptors, the identity of the ligand of rCNL3 could not be predicted based on the sequence similarity of rCNL3 to other G protein-coupled receptors. However, it is interesting to note that rCNL3 has high amounts of amino acid identity with two other orphan receptors, 6-7/R334 and GPCR21 ([19,21] and Bonner and Brownstein, unpublished). Therefore, these three orphans receptors may belong to a novel G protein-coupled receptor family with identical or structurally related endogenous ligands. So far, our attempts to identify the ligand for rCNL3 have not been successful. To understand the functions of rCNL3, for example what physiological roles it might play in striatum, further investigations are needed to identify its ligand and the signal transduction pathways.

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