

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

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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.

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'-CCC GGATCC 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 μ l PCR reaction mixture contained 1.3 μ g template DNA, 1 μ M of each primer, 200 μ M 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 *Cla*I and *Bam*HI and then subcloned into the *Bam*HI and *Acl*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 $3 \times$ SSPE (1 \times SSPE is 0.15 M NaCl, 0.01 M NaH_2PO_4 , 1.3 mM EDTA, pH 7.4), and 1 \times Denhardt's solution (0.02% each of polyvinyl-

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any specific functional 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

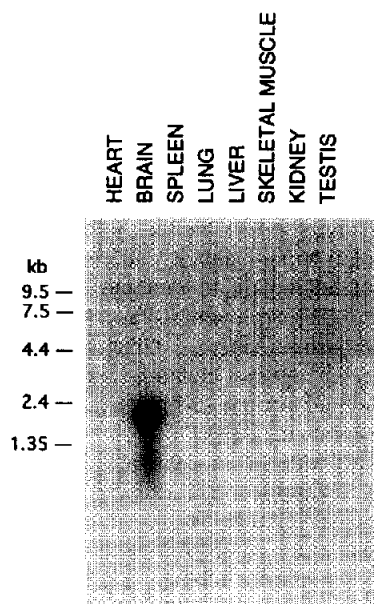


Fig. 3. Northern hybridization analysis of the candidate receptor rCNL3 mRNA expression. Each lane contained 2 μ g of poly(A)⁺ RNA of rat tissues. Positions of RNA molecular weight markers are indicated. A ³²P-labeled fragment of rCNL3 cDNA was used for hybridization. The filter was washed at high stringency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

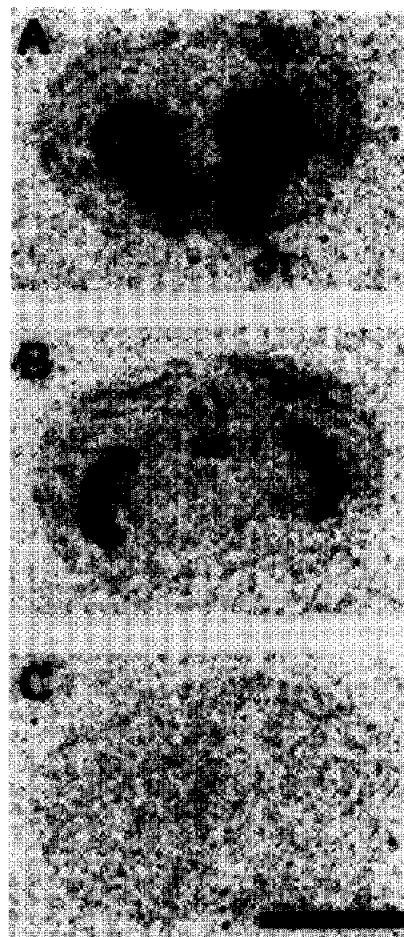


Fig. 4. In situ hybridization analysis of the distribution of rCNL3 mRNA in the brain. Coronal sections of rat brain were hybridized with either antisense (A and B) or sense (C) riboprobes of rCNL3. After washing under high stringency conditions, the slides were exposed to a Fuji imaging plate for 7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

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.

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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