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Molecular cloning and tissue distribution of a putative member of the rat opioid receptor gene family that is not a μ, δ or κ opioid receptor type

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Abstract

A novel G protein-coupled receptor was cloned by PCR and homology screening. Its deduced amino acid sequence is 47% identical overall to the μ, δ and κ opioid receptors and 64% identical in the putative transmembrane domains. When transiently expressed in COS-7 cells this receptor did not bind any of the typical μ, δ or κ opioid receptor ligands with high affinity. In situ hybridization analysis revealed that LC132 mRNA is highly expressed in several rat brain areas, including the cerebral cortex, thalamus, subfornical organ, habenula, hypothalamus, central gray, dorsal raphe, locus coeruleus and the dorsal horn of the spinal cord. Based on this distribution and its high homology with the μ, δ and κ opioid receptors, it is proposed that LC132 is a new member of the opioid receptor family that is involved in analgesia and the perception of pain.

Key words: Opioid receptor; In situ hybridization; Analgesia

1. Introduction

The endogenous opioid peptides and the opiate alkaloids can affect a wide range of physiological processes through interactions with specific receptors. The opioid receptors comprise at least three subtypes (μ, δ and κ) which are classified on the basis of their pharmacological characteristics [1,2]. While screening for novel opioid receptors using degenerate oligonucleotides based on the mouse δ-opioid receptor sequence [3,4], we identified a clone (LC132) which has high homology at the amino acid level but is different from the published μ, δ and κ opioid receptors [3–6]. Based on the high degree of amino acid conservation between LC132 and the μ, δ and κ opioid receptors in their putative transmembrane domains and the cytoplasmic loops we predicted that LC132 encodes a G protein-coupled receptor that is a previously unrecognized member of the opioid receptor family. To test this hypothesis, LC132 cDNA was expressed transiently in COS-7 cells for pharmacological evaluation, and the distribution of its mRNA in the rat central nervous system was mapped by in situ hybridization.

2. Materials and methods

First strand cDNA was synthesized from various rat brain regions and was used as a template for PCR using the degenerate oligos: 5′-ATGAATTCAC(GATC)(AG)T(GC)ATGAG(CT)GT(GC)GAC-(CA)G(CA)TA-3′ and 5′-TTGTCGAC(GA)TA(GA)AG(GA)A(CT)-(GATC)GG(GA)TT-3′. The sequences of these oligonucleotides were based on putative transmembrane domains (TM) III and VII of the mouse δ-opioid receptor [3,4]. The PCR conditions used were: 94°C 1 min (denaturation); 50°C for 1.5 min (annealing) and 72°C for 1.5 min (extension). This cycle was repeated 35 times. PCR products in the size range from 400 bp to 750 bp were purified from a 1.0% agarose gel using Prep-A-Gene (Bio-Rad), digested with *Eco*RI and *Sal*I and subcloned into the vector pBluescript (Stratagene). The full-length mouse δ-opioid receptor was recloned from NG108-15 cDNA by PCR, radiolabeled with [α-^{32}P]dCTP by random priming (Prime It; Stratagene) and used to probe nylon membranes (Colony/Plaque Screen; NEN) on which recombinant bacteria had been grown. The filters were allowed to hybridize with the probe DNA (1×10^6 cpm/ml) overnight in 35% formamide, 5 × SSC, 1% SDS and 5 × Denhardt's at 37°C with slow shaking. They were then washed twice at room temperature in 2 × SSC, 0.1% SDS followed by several washes at 55°C and exposed to Kodak XAR-5 film for 2 days with an intensifying screen at −70°C. The inserts of the hybridizing clones were sequenced by the dideoxy chain termination method [7].

To obtain a full-length clone the LC132 probe was used to screen an oligo(dT)-primed rat brain cDNA library in λGT11. The hybridization conditions used were as described above except that hybridization was in the presence of 50% formamide and the final wash was in 0.5 × SSC, 0.1% SDS at 55°C. Several positive λGT11 clones were plaque-purified, analyzed by restriction enzyme digestions, subcloned into pBluescript and subjected to double-stranded DNA sequencing.

The 1.3 kb coding region of LC132 was cloned into the eukaryotic expression vector RcRSV (Invitrogen). The calcium phosphate precipitation method [8] was used to introduce this construction (LC132-RcRSV) into COS-1 and COS-7 cells. As positive controls for expression, cells were transfected in parallel with RcRSV constructs containing either rat μ [6,9], rat κ ([18,19] and our unpublished clone) or mouse δ [3,4] opioid receptor cDNAs. Stable G418-resistant Ltk$^-$ and CHO cell lines expressing either LC132 or another opioid receptor were also established using the same RcRSV vector (which contains a selectable marker for neo^r). The stable cell lines that were used in the binding studies were selected following Northern blot analysis of LC132 mRNA expression. Preparation of the cell membranes and protein determinations have been previously described [10]. Final protein concentrations were adjusted to 15–80 mg per binding assay tube.

The in situ analysis of LC132 mRNA was performed on 30 μm thick

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sections prepared from freshly dissected adult male rat brain and spinal cord. An ^{35}S-labeled antisense riboprobe corresponding to the first 100 N-terminal amino acids of the receptor was prepared as previously described [11]. This region was chosen because it is poorly conserved between LC132 and the μ, δ and κ opioid receptors. As a negative control the complementary sense strand was labeled with [^{35}S]UTP and reacted with coronal rat brain sections. The slide-mounted sections were first exposed to Cronex film for 5 days, then dipped in emulsion and exposed for 2 weeks.

3. Results and discussion

Using a strategy that combined PCR, degenerate oligonucleotide primers based on the mouse δ-opioid receptor [3,4] and low stringency homology screening we identified a fragment (spanning a region from putative TMIII to TM VII) of a putative G protein-coupled receptor the amino acid sequence of which was on average 67% identical to the rat μ, the rat κ and the mouse δ opioid receptors [3–6]. A probe made from this 600 bp fragment was then used to isolate LC132, a 3.1 kb cDNA, from an oligo(dT)-primed rat brain library. When Northern blots of total RNA prepared from rat thalamus and rat locus coeruleus were probed with LC132, an mRNA of approximately 3.2 kb was detected

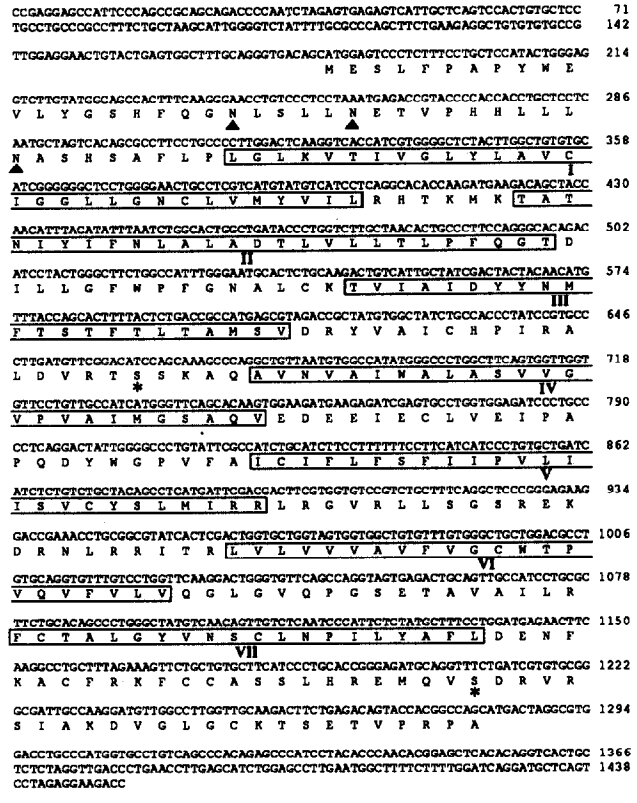

Fig. 1. The cDNA and predicted amino acid sequence of the LC132 receptor. The putative transmembrane domains are boxed and numbered with Roman numerals. Triangles indicate potential N-linked glycosylation sites and the asterisks indicate potential phosphorylation targets of protein kinase C. The nucleotide sequence has been assigned GenBank accession number U01913.

(data not shown) suggesting that the cDNA was nearly full-length.

Within the 3.1 kb LC132 cDNA one long open reading frame of 367 amino acids was identified (Fig. 1). Hydropathy analysis of this sequence predicted seven putative transmembrane domains, a characteristic feature of G protein-coupled receptors. An analysis of LC132's deduced amino acid sequence revealed three consensus sequences for N-linked glycosylation at its amino-terminus. As with the μ, κ and δ opioid receptors, LC132 contains cysteine residues in the receptor's putative extracellular loops I and II which may be involved in the stabilization of the receptors' tertiary structure [12]. Furthermore, there are several cysteine residues in LC132's carboxy-terminus that are potential targets of palmitoylation [13]. In addition, the receptor encoded by LC132 also shares with μ, κ and δ opioid receptors several consensus sequences for phosphorylation by protein kinase A and protein kinase C, as well as serines and threonines that are potential targets of receptor kinases, in the putative cytoplasmic loops II and III and the carboxy-terminus [14,15].

The alignment of LC132 with the amino acid sequences of the μ, δ and κ opioid receptors revealed that 145 amino acids, including nine cysteine residues, are absolutely conserved among all four receptors (Fig. 2). Overall the four receptors are 47% identical, however, when only their transmembrane domains are compared LC132 is about 64% identical to the rat μ, rat κ and mouse δ opioid receptors. Interestingly, when LC132 was aligned with the published G protein-coupled receptors, only the somatostatin receptor SSTR1 possessed significant identity: 32% overall and 51% within the putative transmembrane domains [16].

In addition to the high degree of amino acid identity in their putative transmembrane domains the LC132, μ, δ and κ receptors also possess extensive conservation in their first, second and third putative cytoplasmic loops. These domains are critical for productive receptor–G protein interactions [17]. As a consequence of this extensive conservation the receptor encoded by LC132 probably couples to the same second messenger pathways as the other opioid receptors, i.e. inhibition of adenylyl cyclase, activation of inward rectifying potassium channels and inhibition of N-type calcium channels. When taken together, all of the structural information is consistent with LC132 being a member of the same gene family as the μ, δ and κ opioid receptors.

To pharmacologically characterize the receptor encoded by LC132 its cDNA was cloned into the RcRSV expression vector. This construction was used to transiently express the LC132 receptor in COS-1 and COS-7 cells. In addition this same construction was used to stably express the receptor in Ltk$^-$ and CHO cells. As positive controls for expression rat μ [6,9], mouse δ [3,4] and κ [18,19] opioid receptor cDNAs were cloned into

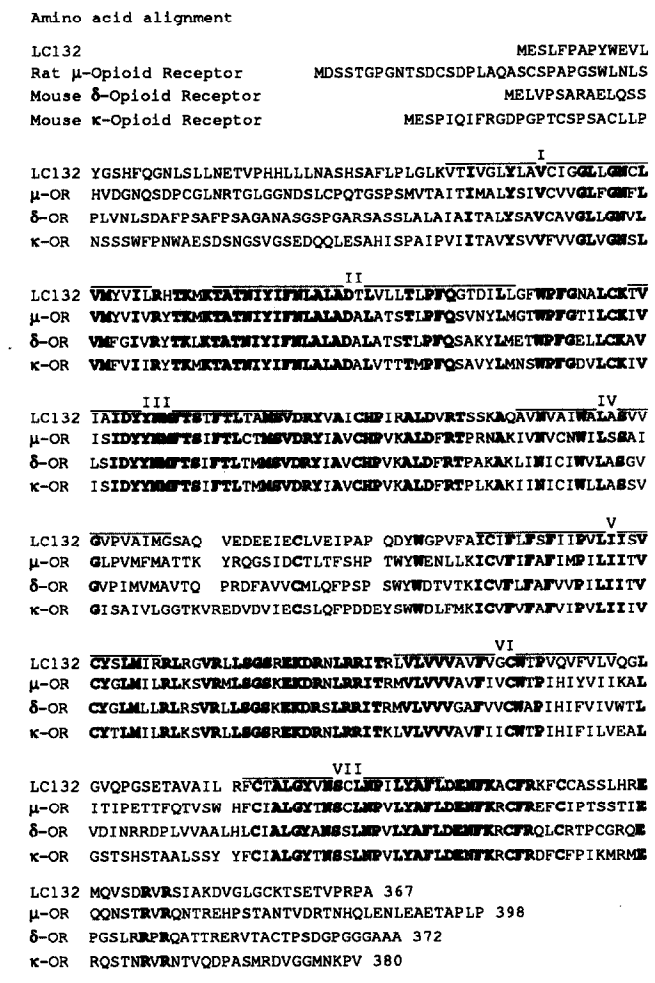

Fig. 2. Alignment of the deduced amino acid sequence of the putative LC132 receptor with the μ, δ and κ opioid receptors. Gaps were introduced to maximize the alignment. The amino acids that are conserved among all four receptors are in boldface print. The putative transmembrane domains are overlined and numbered with Roman numerals. Only the complete sequence of LC132 is shown. The numbers at the end of the μ, δ and κ opioid receptor sequences indicate the number of amino acid residues in the respective protein.

RcRSV and these constructs were both transiently and stably expressed in parallel with LC132RcRSV. For the binding assays membrane fractions prepared from LC132- and the μ, δ and κ opioid receptor-expressing cells were incubated with a variety of radioligands. In spite of repeated efforts none of the membranes prepared from any of the LC132-expressing cells (expression was evaluated by Northern blotting experiments, data not shown) specifically bound [^{3}H]DAMGO, [^{3}H]DPDPE, [^{3}H]U-69,593, [^{3}H]diprenorphine, [^{125}I]β-endorphin, [^{3}H]dihydromorphine, [^{3}H]ethylketocyclazocine, [^{3}H]-dextrorphan or [^{3}H]baclofen. In contrast, when membranes prepared from cells transfected with either the rat μ, κ or mouse δ opioid receptors were used in control binding experiments they routinely demonstrated robust and specific binding of [^{3}H]DAMGO, [^{3}H]U-69,593 and [^{3}H]DPDPE, respectively (data not shown). Interestingly, LC132-expressing membranes did possess low levels of specific [^{3}H]naloxone and [^{3}H]bremazocine binding. Unfortunately, the level of binding was so low it was not possible to pharmacologically characterize LC132 using these compounds. When the results of the binding studies are considered along with the structural conservation that LC132 shares with the μ, δ and κ opioid receptors, LC132 appears to be a G protein-coupled receptor that is a product of a previously unrecognized member of the opioid receptor gene family but with a pharmacology that is not that of a classical opioid receptor. It remains to be determined whether LC132's endogenous ligand is one of the many proteolytic fragments derived from the opioid peptide precursors proopiomelanocortin, proenkephalin or prodynorphin, or a ligand that remains to be identified.

In an attempt to identify specific brain regions that express LC132 the distribution of its mRNA was determined in the central nervous system of the adult male rat by in situ hybridization. A freshly dissected rat brain and a portion of cervical spinal cord were cut into 30 μm sections and hybridized to an antisense [^{35}S]UTP-riboprobe that corresponded to the first 100 N-terminal amino acids of LC132. This region was chosen because it is one of the least conserved domains between the opioid receptors and LC132. As a negative control several additional sections were hybridized with a sense riboprobe. To illustrate the distribution of LC132 mRNA throughout the central nervous system six coronal sections from a complete survey were selected (Fig. 3). In the most rostral section (Fig. 3A) the paraventricular (PVN) and ventral medial hypothalamic nuclei (VMH), the periventricular thalamic nucleus (PVA), the amygdala (Amg), and the piriform cortex (Pir) are densely labeled, while the reticular (Rt) and ventrolateral (VL) thalamic nuclei are less so. It is also worth noting the diffuse but layered pattern of LC132 labeling in the cortex. Of particular interest is the patchy labeling pattern that is visible in the globus pallidus (GP) compared to the lack of label in the caudate putamen (CPu). The lack of CPu labeling is in striking contrast to the striatal distribution of μ, δ or κ opioid receptor mRNAs (our unpublished results). Not shown in this section is the intense labeling of the subfornical organ. The patchy labeling of the globus pallidus (GP) and the diffuse and layered hybridization of LC132 in the cortex continues to be visible in the next section (Fig. 3B), while the medial habenula (MHb), hippocampus, ventral medial hypothalamus (VMH), zona incerta (ZI), and the medial nuclei of the amygdala, posterior (Me) and posteroventral (MePV) parts are densely labeled. There is a striking lack of LC132 hybridization in the ventromedial (VM) and mediodorsal (MDC) thalamic nuclei. At the level of the midbrain (Fig. 3C) the densest labeling is found in the dentate gyrus (DG) as well as CA1, CA2 and CA3 neurons, however, significant hybridization continues to be

evident throughout the cortex, in the periaqueductal gray (PAG) and the zona inserta (ZI). Note the absence of any signal in the fornix (fx). Fig. 3D is a coronal section through the midbrain but caudal to Fig. 3B and illustrates the dense labeling of the dorsal raphe (DR), pontine gray (PG), and the superior colliculus (SC). Additional hybridization appears as a diffuse signal in the periaqueductal gray (PAG) and the pontine reticular nucleus (PRNT). The intense labeling of the locus coeruleus (LC) is the predominant signal in the pons (Fig. 3E), however, the nucleus raphe magnus (RM), pontine central gray (PCG), olivary nuclei (ON) and the anterior aspect of the ventral cochlear nucleus (VCOa) are, all be it diffusely, labeled significantly. At the level of the cervical spinal cord a characteristic patchy signal arranged in an H-shaped pattern is seen in both the anterior (AGH) and posterior (PGH) gray horns (Fig. 3F). Note the absence of staining in the lateral (LF) and anterior funiculi (AF). The sections incubated with the sense LC132 riboprobe failed to hybridize to the probe (data not

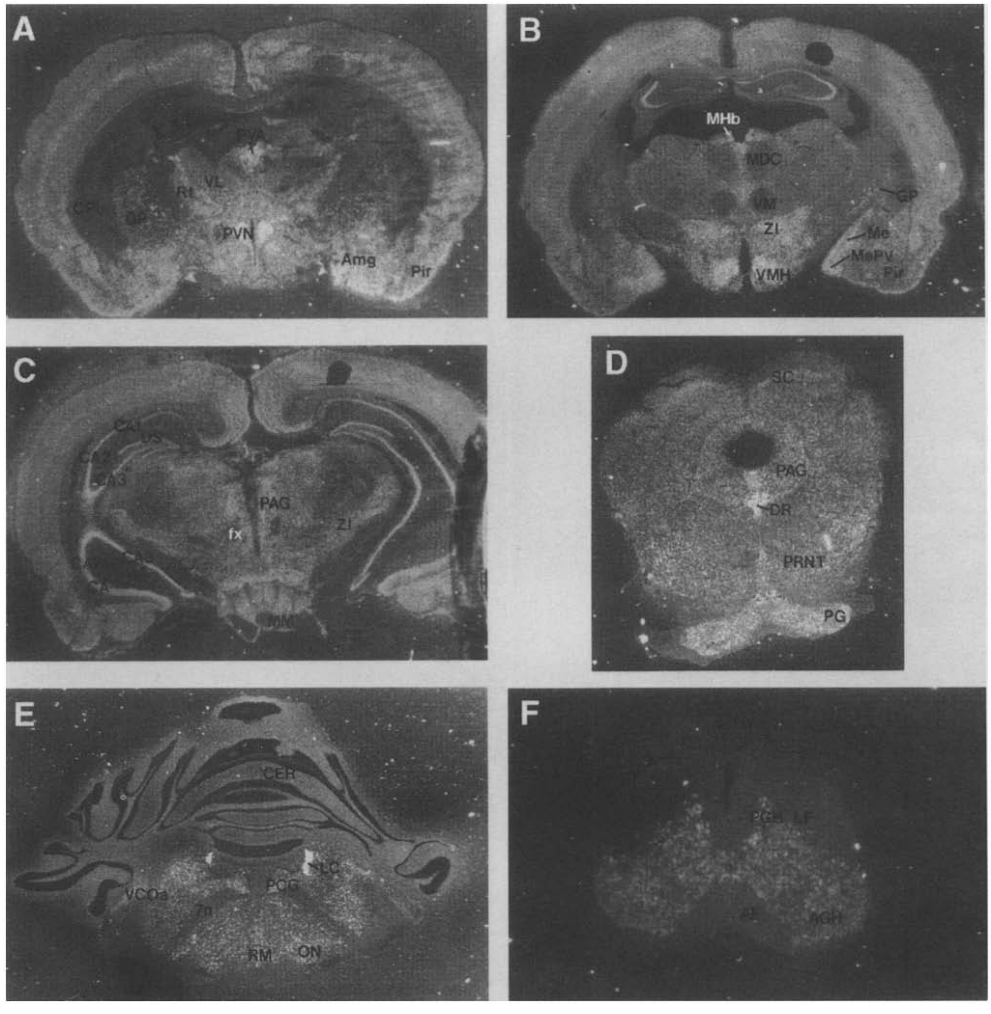

Fig. 3. Distribution of LC132 mRNA in the rat central nervous system and cervical spinal cord. The most rostral coronal section (A) is through the diencephalon. The paraventricular (PVN) and ventral medial hypothalamic nuclei (VMH); the periventricular thalamic nucleus (PVA); the amygdala (Amg); and the piriform cortex (Pir) are densely labeled while the reticular (Rt) and ventrolateral (VL) thalamic nuclei are less so. The cortex is also significantly labeled in a diffuse but layered pattern. Of particular interest is the patchy labeling pattern in the globus pallidus (GP) as compared to the lack of label in the caudate putamen (CPu). The patchy globus pallidus (GP) labeling and the diffuse and layered hybridization of LC132 in the cortex continues to be visible in (B) while the medial habenula (MHb), hippocampus, ventral medial hypothalamus (VMH), zona incerta (ZI), and the medial nuclei of the amygdala, posterior (Me) and posteroventral (MePV) parts are densely labeled. There is a striking lack of LC132 hybridization in the ventromedial (VM) and mediodorsal (MDC) thalamic nuclei. At the level of the midbrain (C) the densest labeling is found in the dentate gyrus (DG) as well as CA1, CA2 and CA3 neurons while significant hybridization continues to be evident throughout the cortex, in the periaqueductal gray (PAG) and the zona inserta (ZI). Note the absence of any signal in the fornix (fx). (D) A more caudal coronal section through the midbrain in which dense labeling of the dorsal raphe (DR), pontine gray (PG), and the superior colliculus (SC) is seen. Additional hybridization appears as a diffuse signal in the periaqueductal gray (PAG) and the pontine reticular nucleus (PRNT). The intense labeling of the locus coeruleus (LC) is the predominant signal in the pons (E), however, the nucleus raphe magnus (RM), pontine central gray (PCG), olivary nuclei (ON) and the anterior aspect of the ventral cochlear nucleus (VCOa) are also significantly labeled. At the level of the cervical spinal cord (F) a characteristic patchy signal arranged in an H-shaped pattern is seen in both the anterior (AGH) and posterior (PGH) gray horns. There is no LC132 reactive sequence in the lateral (LF) and anterior funiculi (AF).

shown), consistent with our interpretation that the signal we detected with the antisense probe was specific.

In summary we have cloned a novel rat G protein-coupled receptor that shares extensive amino acid sequence identity with the δ, κ and μ opioid receptor types. Although a labeled ligand has not been identified the dense expression of LC132 mRNA in limbic, cortical and spinal cord nuclei is consistent with an abundant receptor that mediates analgesia and the perception of pain. When the identification of its endogenous ligand has been accomplished the role of this previously unknown, yet abundant, member of the opioid receptor gene family will become clearer.

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Note added in proof

During the preparation and review of this manuscript Mollereau et al. [20] reported the cloning of a human receptor that is 92% identical to LC132 and Fukuda et al. [21] reported a rat amino acid sequence that is identical to LC132.