FEBS Letters 347 (1994) 279–283

FEBS 14174

Molecular cloning, tissue distribution and chromosomal localization of a novel member of the opioid receptor gene family

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Received 11 April 1994; revised version received 17 May 1994

Abstract

A cDNA was isolated from rat brain by low stringency hybridization with the rat μ opioid receptor cDNA. Sequence analysis of this clone indicated that it contains an open reading frame capable of encoding a 367 amino acid protein. The deduced amino acid sequence of this protein shows high degrees of homology to all three opioid receptors, μ, κ, and δ, suggesting that it is a member of the opioid receptor gene family. RNA blot analysis detected high level expression of the receptor mRNA in the brain. Southern blot analysis suggests that it is a single-copy gene, and mapping studies localized the gene on mouse chromosome 2. Despite the high sequence homologies between this protein and the other opioid receptors, expression studies of this clone in COS-7 cells did not show binding to [^{3}H]diprenorphine, a ligand that binds to the other three opioid receptors. Furthermore, co-expression of this receptor with a G protein-activated potassium channel in *Xenopus* oocytes did not show functional coupling upon stimulation with μ, κ and δ agonists. Given the similar degrees of high homology to the μ, κ and δ opioid receptors and the lack of apparent affinity for their ligands, this receptor does not appear to belong to any of the three known classes of opioid receptors. Rather, it represents a novel member of the opioid receptor gene family, not identified from previous pharmacological studies.

Key words: Opioid receptor; Molecular cloning; Tissue distribution; Chromosome mapping

1. Introduction

Exogenous opioid alkaloids and endogenous opioid peptides play a central role in mediating analgesia and many other physiological activities [1]. Opioids achieve their functions by interacting with three different types of opioid receptors, μ, κ and δ [2]. It has been suggested by pharmacological studies that different subtypes may exist within each of the major types [3]. A member from each of the three opioid receptor classes has been cloned [4–8]. Sequence comparison of these three receptors reveals highly conserved sequence similarities among them, reflecting the overlapping functions of these receptors [9]. In an effort to isolate other members of the opioid receptor gene family, we used the μ receptor cDNA to screen a rat brain cDNA library under low stringency conditions. We report here the isolation of a cDNA clone with a high degree of sequence homology to the other three opioid receptors.

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Abbreviations: RT, reverse transcription; PCR, polymerase chain reaction; DAMGO, [D-Ala^2,MePhe^4,Glyol^5]enkephalin; DPDPE, [D-Pen^{2,5}]-enkephalin; U-50488, *trans*-(±)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide methanesulfonate; U-62066, (±)-(5α, 7α, 8β)-3,4-ichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]-benzeneacetamide mesylate; *Snap*, synaptosomal associated protein; *Emv15*, ecotropic murine virus 15; *Cd40*, lymphocyte antigen 40.

2. Experimental

2.1. Library screening

1.4 kb *Hin*dIII cDNA fragment containing the complete protein coding region of the rat μ opioid receptor [4] was used to screen a rat brain cDNA library under low stringency. Hybridization and the final wash were performed at 55°C using previously described conditions [4]. Sequence analysis of 24 isolated clones showed that four identical cDNA clones were similar to the three opioid receptors, μ, κ and δ. One of four isolates was used for subsequent sequence analysis using double-stranded DNA and Sequenase version 2 (USB). Hydropathy analyses were performed and putative post-translational modification sites of the receptor protein were identified using the PCGENE program. Sequence comparisons of this clone with other three opioid receptors, as well as other G protein-coupled receptors, were performed by the BLAST program available from NIH.

2.2. RNA and DNA blot analysis

A rat multiple tissue poly(A)$^+$ RNA blot (Clontech) was used to study the tissue distribution of the putative opioid receptor under the conditions recommended by the company. The RNA blot was hybridized with a 2.0 kb *Hin*dIII fragment containing the entire protein encoding region of the putative opioid receptor clone. The final wash was performed in 0.2 × SSC and 0.1% SDS at 55°C. For Southern analysis, 30 μg of rat genomic DNA was digested with *Bam*HI, *Eco*RI and *Hin*dIII, respectively, and separated on a 0.8% agarose gel. The DNA was transferred to a Hybond-N membrane (Amersham) as described. The hybridization and wash condition was the same as for the RNA blotting.

2.3. Chromosome mapping

To map the receptor gene, two multilocus crosses were analyzed: (NFS/N or C58/J × *M. m. musculus*) × *M. m. musculus* [10] and (NFS/N × *M. spretus*) × *M. spretus* or C58/J [11]. Genomic DNAs from parental and backcross mice were used in Southern blot hybridizations, and were probed with the receptor cDNA. Progeny of these crosses have also been typed for over 575 markers, including the Chr 2 markers *Snap*, *Emv15* and *Cd40*, typed as described previously [12,13].

2.4. Transient expression in COS-7 cells

A 2.0 kb *Hin*dIII fragment encompassing the entire protein coding region of the putative opioid receptor was cloned downstream of the

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SSDI 0014-5793(94)00560-I

human cytomegalovirus promoter in a mammalian cell expression vector, pRC/CMV (Invitrogen). The recombinant plasmid was introduced into COS-7 cells by electroporation. Membrane preparation and binding assays were performed as described [4]. One nanomolar [^{3}H]diprenorphine was used in the binding assay.

2.5. Expression and electrophysiological studies in Xenopus *oocytes*

Xenopus oocytes were prepared as described [14]. The putative receptor mRNA was synthesized using T7 polymerase under the conditions described before [14]. The receptor mRNA was co-injected with a G protein-activated potassium channel mRNA into the oocytes as described [14]. Electrophysiological analysis of the oocytes was carried out by a two-electrode voltage clamping method in a solution with high potassium concentration [14]. One micromolar of different opioid agonists was used for the oocyte recording assay.

3. Results and discussion

A cDNA clone was isolated by screening a rat brain cDNA library under low stringency using the protein-coding region of the rat μ opioid receptor cDNA [4] as the radiolabeled probe. Sequence analysis of the clone showed an open reading frame capable of encoding a protein with 367 amino acids. Hydropathy plot analysis showed that the protein contains seven hydrophobic domains, a structural signature common to most G protein-coupled receptors. Sequence comparison of this clone with the three cloned opioid receptors [4–8] suggests that

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Rat putative opioid receptor                      MESLF   5
Rat μ opioid receptor         MDSSTGPGNTSDCSDPLAQASC-PA  25
Rat κ opioid receptor                  MESPIQIFRGEPGPTC  16
Mouse δ opioid receptor                          MELVPS   6

                   ^    ^          ^
XOR   PAPYWEVLYGSHFQGNLSLLNETVPHHLLLNASHSAFLPLGLKVTIVGLY  55
MOR   -GSWLNLSHVDGN-SDPCG--R-GLGGNDSLCPQTGSPSMVTAI--MA--  75
KOR   APSACLLPNS-SWFP-WAESDSNGSVGSEDQQLEP-HISPAIP-I-TAV-  66
DOR   ARAELQSSPLVNLSDAFPSAFPSAGANASGSPGARSASS-A-AIA-TA--  56

                                           *
XOR   LAVCIGGLLGNCLVMYVILRHTKMKTATNIYIFNLALADTLVLLTLPFQG 105
MOR   SI--VV--F--F------V-Y------------------A-ATS-----S 125
KOR   SV-FVV--V--S---F--I-Y------------------A--TT-M---S 116
DOR   S---AV-----V---FG-V-Y--L---------------A-ATS-----S 106
        TM1                                   TM2
                   =      *
XOR   TDILLGFWPFGNALCKTVIAIDYYNMFTSTFTLTAMSVDRYVAICHPIRA 155
MOR   VNY-M-T----TI---I--S---------I---CT------I-V---VK- 175
KOR   AVY-MNS----DV---I--S---------I----M------I-V---VK- 166
DOR   AKY-MET----ET---A-LS---------I----M------I-V---VK- 156
                           TM3
         $                                       =
XOR   LDVRTSSKAQAVNVAIWALASVVGVPVAIMGSAQVEDEEIECLVEIPAPQ 205
MOR   --F--PRN-KI---CN-I-S-AI-L--MF-ATTKYRQGS-D-TLTFSH-T 225
KOR   --F--PL--KII-IC--L---S--ISAIVL-GTK-REDVDVIECSLQF-D 216
DOR   --F--PA--KLI-IC--V---G----IMV-AVT-PR-GAVV-MLQF-S-S 206
                   TM4

XOR   DY    WGPVFAICIFLFSFIIPVLIISVCYSLMIRRLRGVRLLSGSREKD 252
MOR   W-    -ENLLK--V-I-A--M-I---T---G---L--KS--M----K--- 272
KOR   -EYSW-DLFMK--V-V-A-V------I---T---L--KS----------- 266
DOR   W-    -DT-TK--V---A-VV-I---T---G--LL---S-------K--- 253
                         TM5
         o
XOR   RNLRRITRLVLVVVAVFVGCWTPVQVFVLVQGLGVQPGSETAVAILRF C 301
MOR   --------M---------IV----IHIY-IIKA-ITI-ETTFQTVSWH- - 321
KOR   -------K----------II----IHI-I--EA--STSH-TAVLSSYY- - 315
DOR   -S------M-----GA--V--A-IHI--I-WT-VDINRRDPL-VAALHL- 303
                 TM6
                                 #            $
XOR   TALGYVNSCLNPILYAFLDENFKACFRKFCCASSLHREMQVSDRVRSIAK 351
MOR   I----T------V----------R---E--IPT-STI-Q-N-T---QNTR 371
KOR   I----T--S---V----------R---D--FPIKMRM-R-STN---NTVQ 365
DOR   I----A--S---V----------R---QL-RTPCGRQ-PGSLR-P-QATT 353
        TM7

XOR   DVGLGCKTSETVPRPA                                    367
MOR   EHPSTAN-VDRTNHQLENLEAETAPLP                         398
KOR   -PASMRDVGGMNKPV                                     380
DOR   RERVTAC-PSDG-GGGRAA                                 372
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Fig. 1. Sequence alignment of the putative opioid receptor with three other opioid receptors. The amino acids identical to the putative opioid receptor are marked by a dash (–). The seven putative hydrophobic transmembrane domains are underlined and labeled from TM1 to TM7. Gaps are introduced for alignment. Other symbols: (=), the conserved cysteine residues possibly involved with disulfide bond formation; (*), the conserved aspartic acid residues that may interact with the primary amine group found in ligands; (^), the potential N-linked glycosylation sites; ($), the putative protein kinase C sites; (o), the potential cAMP dependent protein kinase site; (#), the potential palmitoylation site. The sequence of the putative opioid receptor has been submitted to Genbank with an accession number of L28144.

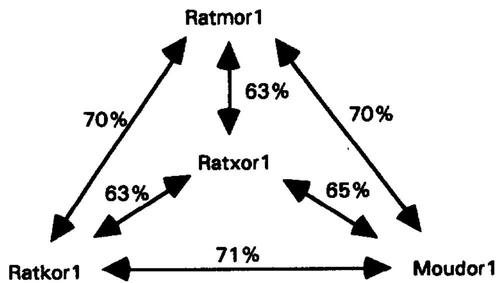

Fig. 2. Homology relationships of the putative opioid receptor with three other opioid receptors. Amino acid homology was calculated by taking into consideration both identical and similar residues. Symbols used: ratmor1, the rat μ opioid receptor [4,5]; ratkor1, the rat κ receptor [9]; moudor1, the mouse δ receptor [6,7]; ratxor1, the putative opioid receptor.

it is closely related to these opioid receptors in structure. Fig. 1 illustrates the amino acid sequence alignment of this protein with the three opioid receptors. The overall similarity of this receptor with the three opioid receptors is around 65%, and a higher level of homology is found in some of the putative transmembrane domains (75–80%) as well as in the intracellular loop between transmembrane domains 5 and 6 (~85%), a region that has been proposed to interact with G proteins [15].

Sequence comparison of this protein with other G protein-coupled receptors also detected homology to other receptors, including somatostatin receptors [16], angiotensin receptors [17] and formyl peptide receptor [18]. However, the degree of similarity of this receptor to these non-opioid receptors is considerably lower (25–45%) than that to the three opioid receptors (65%), suggesting its close relationship to opioid receptors. It is noteworthy that this clone has an almost equal degree of similarity to other three opioid receptors. Fig. 2 shows the homology relationship between this receptor and each of the three opioid receptors as well as the homology relationships among the opioid receptors. This receptor has a ~65% similarity to each of the μ, κ and δ receptors, whereas the similarity between any of the two opioid receptors is ~70%. These homology values suggest that this receptor is likely to be a novel member of the opioid receptor gene family, and it does not appear to be a subtype within one the three receptor classes, i.e. μ, κ and δ.

To determine the tissue distribution pattern of this novel receptor, RNA blot analysis was performed using RNA isolated from various rat tissues. Hybridization of the RNA blot with the protein coding region sequence of this receptor showed a high level expression in the brain (Fig. 3A). Three major transcription products were detected, with molecular sizes of about 10 kb, 7.5 kb and 3.4 kb, respectively. The different sizes of mRNAs may result from differential splicing of the same primary transcript, a phenomenon found in many other mammalian genes, including opioid receptors [6], or from multiple polyadenylation sites.

Southern blot analysis was also performed. Rat genomic DNA was digested with *Hin*dIII, *Eco*RI or *Bam*HI and hybridized with the same radiolabeled probe

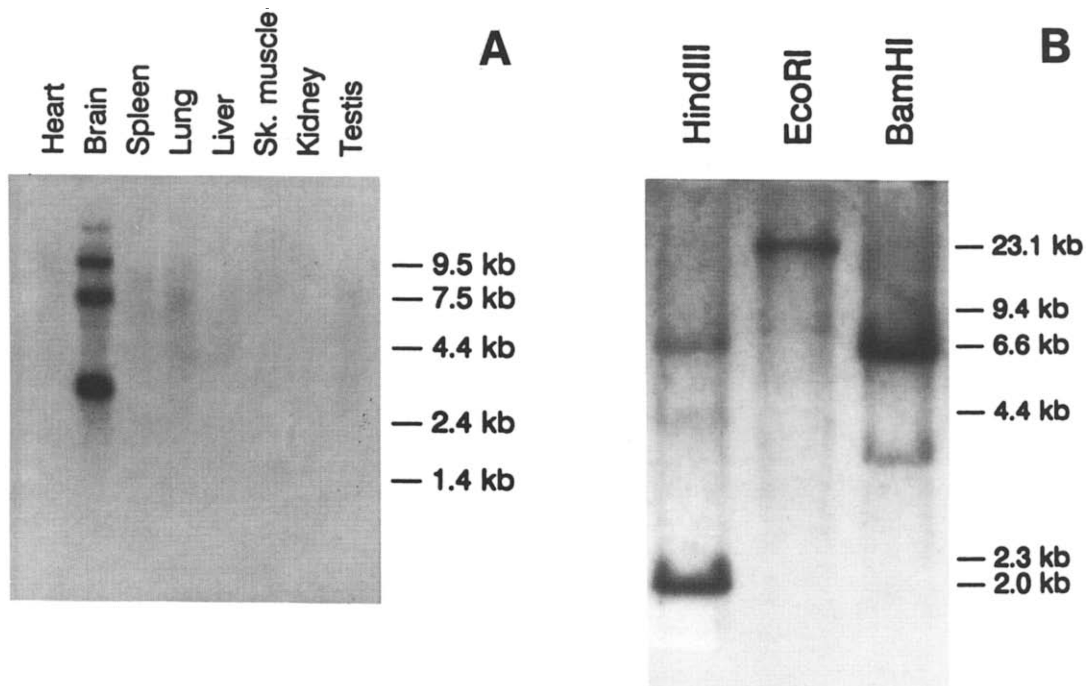

Fig. 3. RNA tissue distribution and Southern blot analysis of the putative opioid receptor. (A) RNAs from eight different rat tissues are labeled above each lane. About 2 μg of poly(A)$^+$ RNA was used for each tissue. The sizes of the RNA size marker are labeled on the left side; (B) genomic DNA Southern blot analysis. The restriction enzymes used to cut the rat genomic DNA are labeled above the corresponding lanes. λDNA digested with *Hin*dIII was used as the size marker with the sizes labeled on the left side.

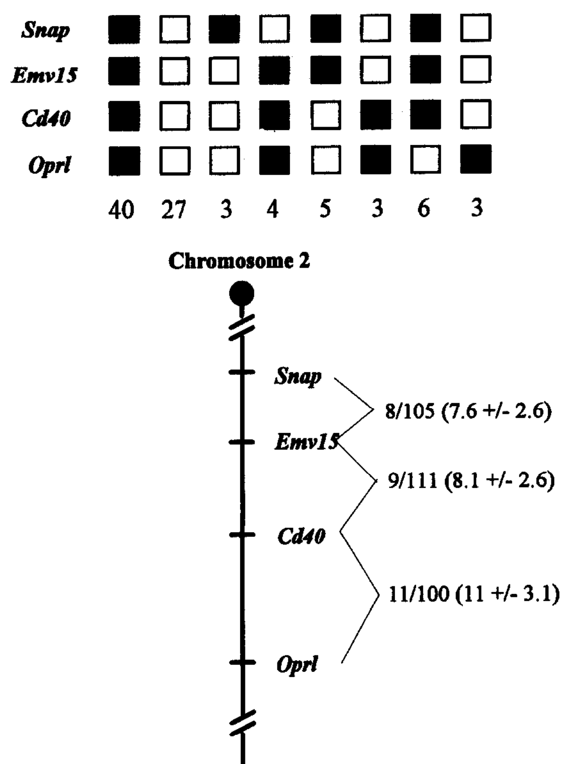

Fig. 4. Linkage of the putative opioid receptor gene *Oprl* with markers on Chr 2. (Top panel) Segregation of *Oprl* with markers on Chr 2. Black squares represent heterozygous mice; open squares are homozygous mice. The numbers under each column represent the number of progeny in each of the two backcrosses with the indicated genotype. (Bottom panel) A partial Chr 2 linkage map indicating relative marker positions. Percent recombination and standard error were calculated from the total number of mice typed for each locus pair according to Green [25]. No double recombinants were observed in this data set.

as used in the RNA blot analysis. As shown in Fig. 3B, the simple pattern of hybridizing bands in each of the restriction digestions suggests that the gene for this receptor may be a single-copy one. It is noteworthy that the strongly hybridizing band in *Hin*dIII is about 2 kb. Since that restriction analysis of the cDNA clone detected an internal *Hin*dIII site in its 3′-untranslated region (data not shown) and that the protein coding region is about 1.1 kb, this result suggests that this receptor gene is most likely a compact one with small or no introns in its protein coding region.

To determine the chromosome location of this receptor, the cDNA insert was used as a probe to analyze two multilocus crosses. Digestion with 13 different restriction enzymes failed to identify polymorphic fragments for the parental mice of our *M. m. musculus* cross [10]. Digestion with *Eco*RI produced fragments of 7.8 and 6.6 kb in *M. spretus* and 19.4 and 16.0 in NFS/N. Progeny of the *M. spretus* cross were typed for inheritance of the polymorphic fragments and the pattern of inheritance was compared with over 430 markers typed in this cross. As shown in Fig. 4, this gene, *Oprl* (opioid receptor like), maps to the distal region of Chromosome (Chr) 2 distal to *Cd40*. This region of Chr 2 is homologous to human chromosome 20 [19], suggesting a human map location for this gene.

Examination of this region of Chr 2 identified two recessive mutations, wasted (*wst*) and lethal spotting (*ls*). Homozygous wasted mice have tremor and uncoordinated body movements at 20 days of age, and they develop progressive paralysis leading to death before 30 days. Homozygous lethal spotting mice have white spotting and die in the third week of life from megacolon with deficiency of intrinsic ganglion cells in the lower colon. Since opioids have effects on both the nervous system and gastrointestinal functions, the close proximity of *Oprl* to *wst* and *ls* warrants further investigation of the potential role of the putative opioid receptor in these mutant phenotypes.

In an attempt to determine the pharmacological profile of this novel receptor, we expressed the cDNA clone in both COS-7 cells and *Xenopus* oocytes. A *Hin*dIII fragment encompassing the entire protein coding region of this receptor was subcloned in pRC/CMV vector downstream from the cytomegalovirus promoter, and this construct was used in transient expression studies in COS-7 cells. As positive controls for COS-7 expression, the rat μ opioid receptor cDNA in the same vector [4] was used in parallel transfection (data not shown). Binding assays using the COS-7 cell membranes transfected with this receptor clone and [^{3}H]diprenorphine, a ligand with high affinity for the three opioid receptors [20], did not detect any specific binding (data not shown), while the positive control showed good expression of the rat μ opioid receptor. Furthermore, mRNA of this clone was synthesized by in vitro transcription and co-injected into *Xenopus* oocytes with the mRNA for a G protein-activated potassium channel [21,22]. It has been shown that this inwardly rectifying potassium channel is functionally coupled to both δ and μ opioid receptors [14,22]. However, no potassium conductance was induced by stimulation of the oocytes with a broad range of opioid agonists, including DAMGO, morphine, DPDPE, U-50488 and U-62066 (data not shown). In parallel experiments, mRNA of the μ opioid receptor was used as a positive control and showed high-level expression. These results further suggest that this receptor may not belong to any of the three opioid receptors. It is unlikely that this receptor gene is a pseudo-gene, since it is transcribed into RNA (Fig. 3A) and we isolated the clone from a cDNA library. During the preparation of this manuscript, a report appeared about the isolation of a human cDNA clone encoding a novel member of the opioid receptor family [23]. This clone is most likely a human homologue of the one reported here, since the two clones share 94% identity at the amino acid sequence level.

Thus, we have isolated a novel seven-helix receptor

cDNA. The high degrees of sequence homology with all three types of opioid receptors and the lack of apparent affinity for many opioid ligands suggest that it encodes a novel member of the opioid receptor gene family, with previously unknown pharmacological characteristics.

Acknowledgements: J.L. is the recipient of a John B. Hickman Memorial postdoctoral fellowship from the American Heart Association, Indiana Affiliate, Inc., and A.M. is supported by an NIH training grant (T32HD07373). L.Y. is the recipient of an NIH Research Career Development Award (NS01557) and is a J. Alfred Prufrock investigator. This work was supported by National Institutes of Health Grant NS28190 (to L.Y.).

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Note added in proof

Fukuda et al. [24] reported the isolation of a rat cDNA clone that is identical to the one reported here.