J Mol Evol (1996) 43:179–184

JOURNAL OF MOLECULAR EVOLUTION

Mu Opioid Receptor-Like Sequences Are Present Throughout Vertebrate Evolution

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Received: 5 January 1996 / Accepted: 7 March 1996

Abstract. The sequence of the mu opioid receptor is highly conserved among human, rat, and mouse. In order to gain insights into the evolution of the mu opioid receptor, polymerase chain reaction (PCR) was used to screen genomic DNA from a number of different species using degenerate oligonucleotides which recognize a highly conserved region. DNA was assayed from representative species of both the protostome and deuterostome branches of the metazoan phylogenetic tree. Mu opioid receptor-like sequences were found in all vertebrate species that were analyzed. These species included bovine, chicken, bullfrog, striped bass, thresher shark, and Pacific hagfish. However, no mu opioid receptor-like sequences were detected from protostomes or from any invertebrates. The PCR results demonstrate that the region of the mu opioid receptor gene between the first intracellular loop and the third transmembrane domain (TM3) has been highly conserved during evolution and that mu opioid receptor-like sequences are present in the earliest stages of vertebrate evolution. Additional opioid receptor-like sequence was obtained from mRNA isolated from Pacific hagfish brain using rapid amplification of cDNA ends (RACE). The sequence of the Pacific hagfish was most homologous with the human mu opioid receptor (72% at the amino acid level between intracellular loop 1 and transmembrane domain 6) although over the same region high homology was also observed with the delta opioid receptor (69%), the kappa receptor (63%), and opioid receptor-like (ORL1) (59%). The hagfish sequence showed low conservation with the mammalian opioid receptors in the first and second extracellular loops but high conservation in the transmembrane and intracellular domains.

Key words: Mu opioid receptor gene — Vertebrates — Molecular evolution

Introduction

In mammals, opioid receptors are abundant in the central and peripheral nervous system and are the targets of both opiate drugs and a family of endogenous opioid peptides including the enkephalins, endorphins, and dynorphins (Mansour et al. 1988; Olson et al. 1994). This receptor family is involved in the modulation of a number of functions such as pain, stress, cardiovascular and immunological responses, development, neuroendocrine regulation, and reward and affective behavior (Olson et al. 1994). Four structurally homologous members of the opioid receptor family have been cloned; they are designated delta (δ) (Evans et al. 1992; Kieffer et al. 1992), mu (μ) (Chen et al. 1993a; Thompson et al. 1993; Wang et al. 1994a), kappa (κ) (Yasuda et al. 1993; Chen et al. 1993b), and opioid receptor-like (ORL1) (Mollereau et al. 1994; Meunier et al. 1995). The mu opioid receptor is particularly interesting since it is the principal site of activity for highly addictive and clinically used drugs such as morphine and fentanyl (Olson et al. 1994). The human (Wang et al. 1994a), rat (Chen et al. 1993a), and mouse (Kaufman et al. 1995) mu opioid receptor cDNA

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Table 1. Primer sequences used for PCR and RACE

Primer	Nucleotide	Amino acids
R1	5′ -GAAGAC(GTC)GC(AC)ACCAACATCTA	Lys-Thr-Ala-Thr-Asn-ILe-Tyr
R2	5′ -GT(GA)AACAT(GA)TT(GA)TAGTA(GA)TC	Thr-Phe-Met-Asn-Tyr-Tyr-Asp
Ra	5′ -AA(GA)AC(GATC)GC(GATC)AC(GATC)AA(TC)AT(ATC)TA	Lys-Thr-Ala-Thr-Asn-ILe-Tyr
Rb	5′ -GT(GA)AACAT(GA)TT(GA)TA(GA)TA(GA)TC	Thr-Phe-Met-Asn-Tyr-Tyr-Asp
H1	5′ -ATCCTCAACCTTGCCTTGGCC	Ile-Leu-Asn-Leu-Ala-Leu-Ala
H2	5′ -GGCCTATGCAAGATCATCCTC	Gly-Leu-Cys-Lys-Ile-Ile-Leu
A21	5′ -pCTGGAAAGGTCGACTCGAGTC	
A25	5′ -pGACTCGAGTCGACCTTTCCAGCACA	

R: degenerate primers. H: sequences from hagfish

clones display high amino acid sequence conservation with the exception of the N-terminal extracellular domain.

In order to study the evolution of the mu opioid receptor, we screened genomic DNA of representative species from the protostome and deuterostome branches of the metazoan phylogenetic tree with polymerase chain reaction (PCR) using degenerate oligonucleotides. We now report partial sequences of the mu-like opioid receptors in many lower vertebrates. In addition, we have obtained a larger fragment of this receptor from Pacific hagfish with rapid amplification of cDNA ends (RACE).

Materials and Methods

DNA Sources. Pacific hagfish (*Eptatretus stoutii*) was supplied by Pacific Biomarine (Venice, CA). Amphioxus (*Branchiostoma floridae*), acorn worm (*Saccoglossus kowalevskii*), and sea urchin (*Arbacia punctulata*) were obtained from Marine Biological Lab (Woods Hole, MA). Bovine (*Bos taurus*), chicken (*Gallus domesticus*), and earthworm (*Lumbricus terrestris*) were supplied by local retail outlets. Bullfrog (*Rana catesbeiana*) was obtained from Nasco (Fort Atkinson, WI). Striped bass (*Morone saxatilis*) and thresher shark (*Alopias vulpinus*) were obtained from local commercial fisherman. Shrimp (*Alpheus heterochaelis*), ribbed mussel (*Modiolus modiolus*), tunicate (*Polyandrocarpa maxima*), and flat worm (*Bdelloura candida*) were obtained from the Gulf Specimen Company (Panacea, FL). Fruit fly (*Drosophila melanogaster*) and *Caenorhabditis elegans* were gifts from colleagues at UCLA.

Genomic DNA Isolation. Tissues samples, either fresh or stored at −70°C, were ground in liquid nitrogen in a mortar and pestle. The resulting homogenate was incubated at 55°C in 100 mM EDTA, 0.5% SDS, 10 mM Tris-HCl, pH 8, and 100 μg/ml proteinase K for 3 h, followed by extraction with phenol, phenol/chloroform/isoamyl alcohol (25:24:1, v/v), and chloroform/isoamyl alcohol (24:1, v/v). The nucleic acids were precipitated with 0.2 vol of 10 M ammonium acetate and 2 vol of ethanol, followed by resuspension in TE, pH 8.0.

Oligonucleotide Primers. R1, R2, Ra, and Rb primer sequences were selected after alignment of cDNA sequences of the opioid receptors from human, rat, and mouse. H1 and H2 primers were selected from the partial sequence of the hagfish mu opioid receptor. Adapter sequences A25 and A21 are longer versions of *Bst*XI adapters.

cDNA Preparation. Total RNA from hagfish brain was isolated by a single-step acid guanidinium thiocyanate-phenol-chloroform procedure (Chomczynski and Sacchi 1987). Poly(A)$^+$ RNA was isolated using Oligotex (Qiagen Inc., Chatsworth, CA), and cDNA was prepared by the Gubler and Hoffman (1983) method using the Copy Kit (Invitrogen Co., San Diego, CA) with random hexamer primers.

PCR. PCR amplification was performed in a DNA Pacer thermocycler (Bellco Biotechnology, Vineland, NJ) in 50-μl reaction mixtures containing 50 mM KCl, 20 mM Tris-HCl, pH 8.4, 2 mM $MgCl_2$, 50–200 pmol of each primer, and 0.1–1 μg of template DNA. Taq polymerase (1–2 units) was added during an initial 4-min denaturation step at 94°C and was followed by 30–40 cycles of amplification. Cycling parameters were 94°C for 1 min, 45–57°C for 1 min, and 72°C for 40 s.

RACE. Poly (A)$^+$ RNA (2 μg) was purified and transcribed into cDNA as described above. After ligation with the phosphorylated adapters A25 and A21, excess adapters were removed by Sephadex G-50 chromatography. The cDNA pool (1 μl) and 100 pmol of each hagfish mu-specific primer (either H1 or H2) and adapter (A21) were added into 50 μl of PCR cocktail as described above. Cycling parameters were 94°C for 1 min, 63°C for 1 min, and 72°C for 3 min, followed by a 10-min final extension at 72°C. H1 and A21 primers were used for the primary PCR of 25 cycles; then 2% of this mixture was amplified by H2 and A21 primers for 15 cycles.

Cloning and Sequencing of Amplified DNA Fragments. After amplification, the resulting PCR and RACE products were gel purified, blunt-ended with Pfu DNA polymerase, and then cloned into pCR-Script Sk(+) (Stratagene, La Jolla, CA). Alternatively, PCR products were cloned directly into pCRII vector (Invitrogen Corp., San Diego, CA). After transformation into competent cells, positive clones containing inserts were selected by IPTG and X-Gal. Inserts were sequenced in both directions using Sequenase (United States Biochemical, Inc.), and sequence alignments were performed using MacVector (IBI Inc, New Haven, CT) and Megalign (DNASTAR, Madison, WI). All sequences will be submitted to GeneBank.

Results

Isolation of Homologous Mu Opioid Receptor Fragments from Genomic DNA

We tested our PCR protocol on human, rat, and mouse genomic DNA and verified its ability to recover known mu opioid receptor sequences. The degenerate primers used for the PCR amplifications are shown in Table 1. These primers were chosen to recognize a fragment of all opioid receptor types in an attempt to optimize mu opioid

receptor detection in lower species. We obtained 162-bp fragments from bovine, chicken, frog, and bass but not from shark or hagfish using the R1 and R2 primers. However, using the Ra and Rb primers, which are more degenerate (Table 1), specific fragments were obtained from both shark and hagfish. A 162-bp DNA fragment was the major PCR product from genomic DNA of all the vertebrate species analyzed. A number of additional bands were also observed, presumably due to the degeneracy of the primers, but none were found to be specific. No specific fragments were obtained from any protostome or invertebrate species analyzed.

Sequence Analysis of Amplified Genomic DNA Fragments

Multiple recombinant clones were sequenced for each species, and occasional differences between clones were attributed to Taq polymerase errors. The predicted amino acid sequences (Fig. 1) are shown for the mu opioid receptor from six vertebrates aligned with the published sequences of human, rat, and mouse. The 162-bp DNA fragment encodes 54 amino acids from the first intracellular loop to TM3. Over the 54-amino-acid region examined, 38 residues are conserved among all the vertebrates that were analyzed. Amino acid sequence identities range from 100% (between some mammalian species) to 72% (between bass and hagfish). The mu opioid receptors from mammalian species that were analyzed share at least 98% amino acid sequence identity, but the percent identity is lower among the nonmammalian species. The nucleotide sequences show more substantial differences between vertebrate species (Table 2), with many third base changes encoding conserved residues (data not shown).

RACE Cloning of Hagfish Mu cDNA

To further characterize the hagfish opioid receptor, RACE was performed from cDNA isolated from hagfish brain (see Materials and Methods). We used double-stranded cDNA with adapter sequences at both ends instead of single-stranded cDNA with oligo-dT. The nested primers used for 3′ RACE, H1 and H2 (see Table 1 and Fig. 2), were synthesized from within the 162-bp sequence previously amplified from hagfish genomic DNA. The product of these primers was predicted to generate a fragment with a 48-bp overlap with the 162-bp fragment (Fig. 2); 3′ RACE identified a 471-bp fragment, which extended 3′ downstream from TM3, and was subsequently cloned and sequenced. Several attempts using RACE to obtain cDNA clones extended in the 5′ direction and longer fragments in the 3′ direction were unsuccessful.

Sequence Determination of Amplified cDNA Fragment

The 471-bp fragment obtained from hagfish brain cDNA by RACE suggests that a mu-like opioid receptor is expressed in hagfish brain. Including the 162-bp sequence from the genomic DNA, a 585-bp nucleotide sequence was obtained. This sequence encodes 195 amino acids from the first intracellular loop to TM6, and is highly homologous with the human, rat, and mouse mu sequences (Fig. 3). The amino acid sequence of this hagfish opioid receptor is 72% identical to mu, 69% to delta, 63% to kappa, and 59% to ORL1 when compared with its human counterparts. Interestingly, amino acids that were different in the hagfish sequence from the mammalian mu sequence were often present in the other members of the opioid receptor family. Most of the amino acid substitutions throughout this sequence are conservative, except in the second extracellular loop, where the hagfish receptor sequence diverges markedly from the mammalian mu sequence, resulting in a significantly more highly charged extracellular domain. An additional residue is found in the hagfish sequence at the equivalent position of the exon 2/exon 3 boundary that is present in the mammalian and rodent mu opioid receptor sequence (Min et al. 1994; Wang et al. 1994a). Of note was that all consensus phosphorylation sites are conserved.

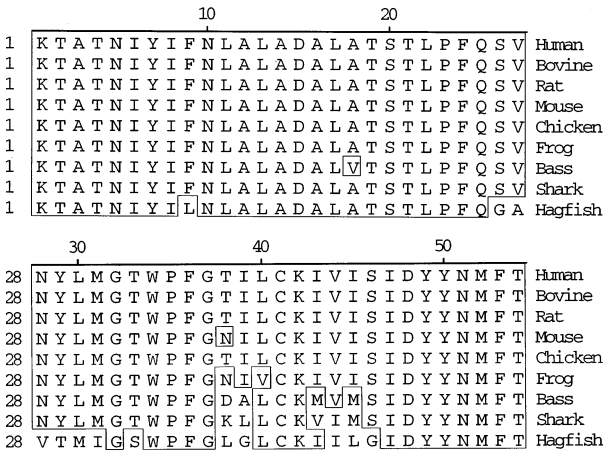

Fig. 1. Deduced amino acid sequence alignments of mu opioid receptor fragments in the region spanning intracellular loop 1 to transmembrane domain 3 from nine vertebrates. Identical amino acids are *boxed.*

Discussion

Members of the G-protein receptor family, which include the opioid receptors, have been identified in species throughout evolution including *Dictyostelium* (Klein et al. 1988). In the present study, we have successfully employed a PCR strategy to identify mu opioid receptor sequences in lower species. Because the mRNA level of mu opioid receptor in murine brain is present at very low levels (Kaufman et al. 1995) and brain tissue is often difficult to obtain in lower species, we targeted our analysis to genomic DNA. The region we chose to amplify was a 162-bp fragment representing sequence from

Table 2. Percent amino acid and nucleotide identity among vertebrate Mu-receptor fragments in the region spanning intracellular loop 1 to transmembrane domain 3[a]

	Bovine	Rat	Mouse	Chicken	Frog	Bass	Shark	Hagfish
Human	100 (90)	100 (92)	98 (91)	100 (86)	96 (80)	91 (79)	91 (79)	76 (65)
Bovine		100 (88)	98 (86)	100 (87)	96 (83)	91 (78)	91 (80)	76 (70)
Rat			98 (94)	100 (83)	96 (80)	91 (80)	91 (81)	76 (70)
Mouse				96 (82)	98 (81)	91 (83)	91 (85)	76 (67)
Chicken					96 (82)	91 (76)	91 (76)	76 (68)
Frog						89 (75)	89 (76)	74 (60)
Bass							91 (85)	72 (68)
Shark								76 (70)

[a] Percent identity was calculated using Megalign (DNASTAR). The number shown is percent amino acid identity, followed by nucleotide identity in parentheses

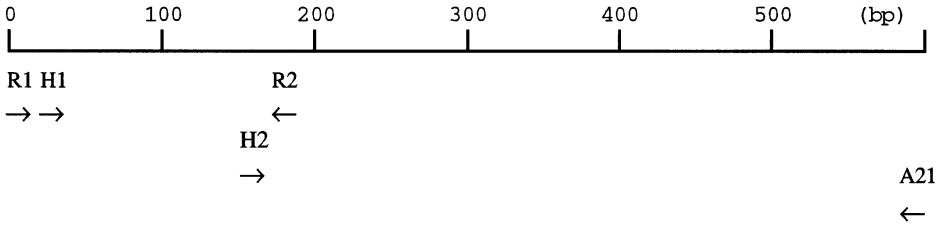

Fig. 2. Schematic map of the oligonucleotide primers used to amplify mu opioid receptor-like sequences from vertebrate tissues.

the first intracellular loop to TM3. This region is highly conserved among members of the opioid receptor family and contains no introns in the murine (Min et al. 1994) or human (Wang et al. 1994a) mu genes.

The results demonstrate that the degenerate primers designed from the mammalian mu opioid receptors can be used to obtain mu opioid receptor coding sequences from a wide variety of vertebrate species. Comparison of the amino acid sequences deduced from the PCR products between lower vertebrates with those of previously characterized mammalian receptors suggests that the mu-like opioid receptor, in the region amplified, is highly conserved throughout vertebrate evolution. Within the amplified region are two aspartate residues in TM2 and TM3 which are considered to be important for opioid receptor function. Site-directed mutagenesis and deletion studies on the rat mu opioid receptor showed that both of these aspartate residues in the TM domains may be involved in binding to mu-selective ligands (Surratt et al. 1994). We found these two aspartic acids to be conserved in all vertebrate species that were analyzed.

The lowest species from which we detected a mu opioid receptor-like fragment was in the Pacific hagfish. The hagfish and the lampreys represent the only living members of the most primitive vertebrate class—agnathans. Agnathans diverged from the main line of vertebrate evolution approximately 470 million years ago and occupy a key position in metazoan evolution (Raven and Johnson 1989). When we first sequenced the 162-bp PCR fragment from hagfish genomic DNA, it was difficult to distinguish whether it was the mu or another opioid receptor. Therefore we synthesized additional primers from the genomic sequence and used RACE (Frohman et al. 1988) to obtain additional sequence using cDNA obtained from hagfish brain. A sequence of a 585-bp RACE fragment confirmed that the hagfish PCR product most closely resembled the mu receptor sequence compared to other members of the opioid receptor family. Absolute identification of the hagfish sequence as a mu receptor was unclear because the homology between the hagfish sequence and the human mu, delta, kappa, and ORL1 sequences was similar. Thus this hagfish opioid receptor may be a mu opioid receptor or alternatively an ancestor of the opioid receptor family.

There were a number of interesting comparisons between the hagfish and the mammalian mu receptor sequences. In general, the TM domains and intracellular domains were highly conserved or contained conservative substitutions that maintained hydrophobicity and charge. All potential phosphorylation sites were conserved in the second and third intracellular loops. However, the extracellular domains showed substantially less conservation. In the second extracellular loop, striking differences were observed between the hagfish and mammalian mu receptors, especially in the distribution of charged amino acids. Of note was that two of these amino acid differences were identical to the sequence found in ORL1. Since the second extracellular loop has been associated with opioid peptide interactions (Wang et al. 1994b), it is likely that these differences will affect ligand selectivity. Furthermore, an additional asparagine residue was observed in the second extracellular loop of the hagfish cDNA, making the hagfish one amino acid longer in this region of the receptor. The position of this

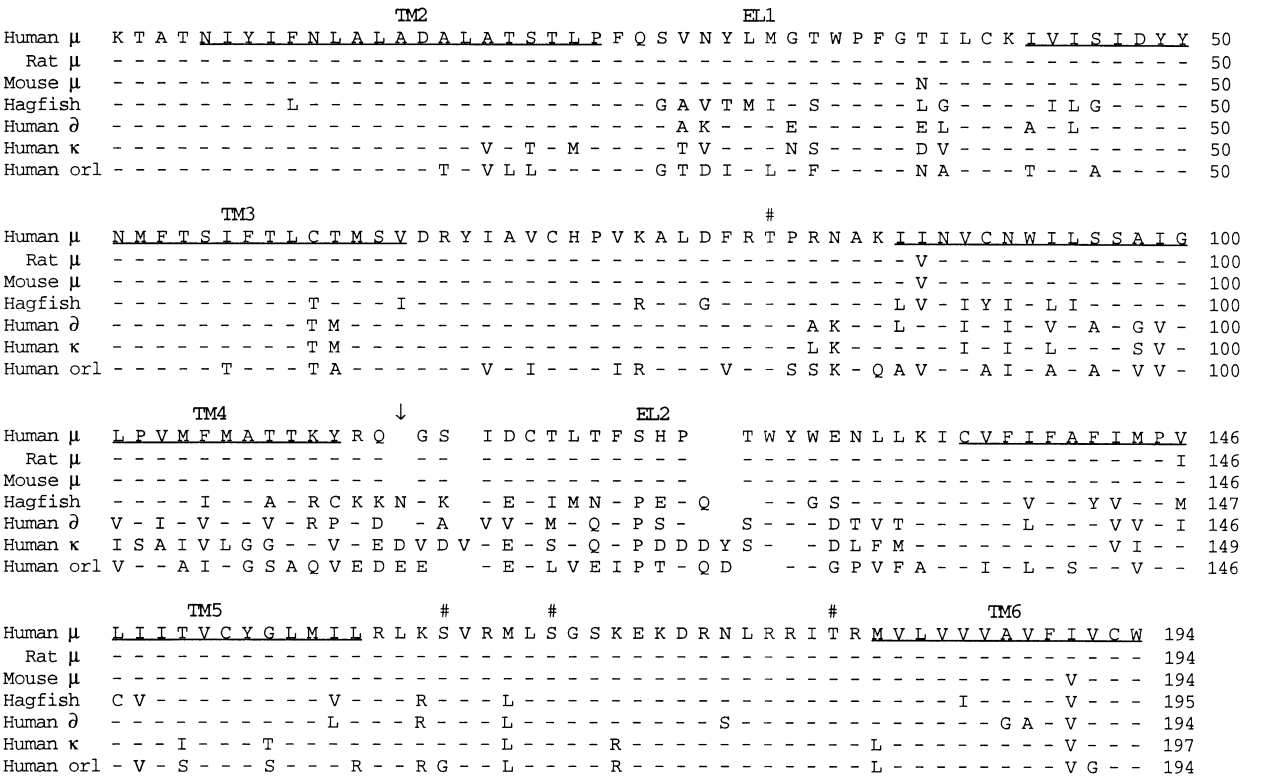

Fig. 3. Deduced amino acid sequence alignments of hagfish mu-like opioid receptor with selected mammalian opioid receptors. Identical residues are indicated with *dashes.* Transmembrane domains are *underlined.* Potential phosphorylation sites are indicated with #. Exon 2/exon 3 boundary in the mu opioid receptor is indicated with ↓. *EL*: excellular loops.

additional inserted residue was at the exact position of the exon2/exon3 boundary in the human and mouse gene (Min et al. 1994; Wang et al. 1994a). This would indicate that the splice junction itself, or less likely, the splicing apparatus, is not conserved across vertebrate evolution.

We were unable to obtain a full-length cDNA for the mu-like opioid receptor from hagfish brain using RACE, which is unfortunate because this receptor would provide an interesting model in which to study binding and signal transduction. The hagfish sequence fragment identified in this study suggests that although there is evolutionary conservation in areas of the receptor involved in signal transduction and regulation, differences in areas of the receptor involved in peptide binding may result in the primitive vertebrate mu-like receptors having pharmacological profiles different from their mammalian counterparts.

The PCR strategy that was employed in this study detected mu receptor-like sequences only in the vertebrates. No mu receptor-like sequences were amplified in any invertebrates, protostomes, bilateria, or even in amphioxus, which is close to the transition between invertebrates and vertebrates. Earlier studies have identified opioid binding sites in protostomes (Kream et al. 1980; Santoro et al. 1990; Stefano et al. 1993); however, some of these sites appear to favor opiate alkaloids rather than opioid peptides and thus may not correspond to the cloned opioid receptor family. Interestingly, protostomes appear to express enkephalin or β-endorphin-like immunoreactive opioid peptides (Alumets et al. 1979; Martin et al. 1979; Cooper et al. 1993), which raises the possibility that a separate set of receptors may have evolved in the protostome and deuterostome branches to interact with these ligands.

It is quite possible that the degenerate primers would not recognize the sequences of species which are a very large evolutionary distance from mammals, and thus it does remain possible that the mu opioid receptor gene evolved before the earliest stages of vertebrate evolution. Our results provide critical information on the evolution of mu opioid receptors in vertebrates, but additional studies will be necessary to elucidate the origin of the opioid receptor family.

Acknowledgments. This research was supported by NIDA Center grant #DA-05010 and by the W.M. Keck Foundation.

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