Multiple opioid receptor-like genes are identified in diverse vertebrate phyla

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Abstract Polymerase chain reaction was used to determine whether opioid receptor-like sequences are present in species from the protostome and deuterostome branches of the metazoan kingdom. Multiple opioid receptor-like sequences were found in all vertebrates, but no specific fragments were obtained from any invertebrates. Delta, mu, kappa and ORL-1 receptors were identified from bovine DNA, and three different opioid receptor-like fragments were identified from the other vertebrates analyzed. The data suggest that the opioid receptor gene family has been highly conserved during vertebrate evolution and that, even in the primitive jawless fish, multiple members of the opioid receptor family appear to be present.

Key words: Opioid receptor gene family; Vertebrate evolution; Polymerase chain reaction; Sequence analysis

1. Introduction

Opioid drugs have many different physiological effects and the actions of these drugs are mediated through membrane-associated opioid receptors. Four members of the opioid receptor family have been cloned: delta (DOR) [1,2]; mu (MOR) [3–5]; kappa (KOR) [6,7]; and opioid receptor like (ORL-1) [8,9]. The opioid receptors are typical G-protein coupled receptors containing seven transmembrane (TM) domains. In mammalian species, all members of the receptor family have homologous protein sequences and conserved intron/exon boundaries [10]. Thus, it is anticipated that the opioid receptor family arose from a common ancestral gene via gene duplication. The endogenous ligands for these receptors are derived from four precursors: pro-orphanin FQ, pro-enkephalin, pro-dynorphin and pro-opiomelanocortin. Pro-orphanin FQ is processed into peptides selective for ORL-1 receptors, and the other three generate delta-, mu-, and kappa-selective peptides. In the frog, two additional opioid peptide precursors — pro-dermorphin and pro-deltorphin — have been detected, and their bioactive processing products (the dermorphins and deltorphins) include a D-amino acid adjacent to the *N*-terminal tyrosine residue [11,12]. Opioid peptides have been identified in a number of invertebrate species [13–15]. Moreover, there are reports of opioid binding sites in protostomes [16,17], but they exhibit unusual ligand selectivities and thus may not correspond to the cloned opioid receptor family.

We designed a polymerase chain reaction (PCR) strategy to identify all members of the opioid receptor family and used it to attempt to trace the opioid receptors through a wide evolutionary range of metazoan classes. The analysis of opioid receptors in lower species, in addition to providing phylogenetic information, may give insights into structural elements necessary for receptor function through identification of residues conserved in the receptor family during evolution.

We describe here the partial sequences of the opioid receptor gene family identified from six vertebrates including the hagfish. However, this strategy failed to identify opioid receptor-like sequences in any invertebrates.

2. Materials and methods

2.1. Samples

Genomic DNA was isolated as described [18] from the following species: bovine, *Bos taurus*; chicken, *Gallus domesticus*; bullfrog, *Rana catesbeiana*; striped bass, *Morone saxatilis*; thresher shark, *Alopias vulpinus*; Pacific hagfish, *Eptatretus stoutii*; amphioxus, *Branchiostoma floridae*; tunicate, *Polyandrocarpa maxima*; acorn worm, *Saccoglossus kowalevskii*; sea urchin, *Arbacia punctulata*; fruitfly, *Drosophila melanogaster*; shrimp, *Alpheus heterochaelis*; ribbed mussel, *Modiolus modiolus*; earthworm, *Lumbricus terrestris*; *Caenorhabditis elegans* and flat worm, *Bdelloura candida*.

2.2. Oligonucleotide primers

Degenerate oligonucleotides were designed to amplify a 162 bp fragment of all members of the opioid receptor family from human, rat and mouse. The primer sequences correspond to regions of amino acid identity in the boundary between the first intracellular loop and transmembrane domain 2 (TM2) and in transmembrane domain 3 (TM3) of the cloned mammalian opioid receptor family. (These sequences are underlined in Fig. 1.) Primers R1 and Ra encode the sequence Lys-Thr-Ala-Thr-Asn-Ile-Tyr, and primers R2 and Rb encode the sequence Asp-Tyr-Tyr-Asn-Met-Phe-Thr. The Ra and Rb primers are more degenerate versions of R1 and R2. The sequences were:

R1: 5′-GAAGAC(CGT)GC(CA)ACCAACATCTA
R2: 5′-GT(AG)AACAT(AG)TT(AG)TAGTA(AG)TC
Ra: 5′- GAA(AG)AC(GATC)GC(GATC)AC(GATC)AA(TC)AT-(TCA)TA
Rb: 5′-GT(AG)AACAT(AG)TT(AG)TA(AG)TA(AG)TC

2.3. DNA amplification

Genomic DNA (0.1–1 μg) was used with the above primers in a DNA Pacer thermocycler (Bellco Biotechnology) using Taq-polymerase. PCR reactions were carried out in 50 μl volumes containing 50 mM KCl, 20 mM Tris-HCl, pH 8.4, 2 mM $MgCl_2$, 50–200 pmol of each primer, and 2.5 U of Taq polymerase. An initial 3-min denaturation step at 94°C was followed by 30 cycle amplification. Cycling parameters were 94°C for 1 min, 55–57°C for 1 min, and 72°C for 40 s.

2.4. Cloning and sequencing of amplified fragments

After amplification, the resulting PCR products were gel-purified, blunt-ended with Pfu DNA polymerase, then cloned into pCR-Script Sk(+) (Stratagene Inc., La Jolla, CA). Alternatively, PCR products were cloned into pCRII vector with the TA Cloning Kit (Invitrogen, San Diego, CA). After transformation, clones containing inserts were selected using IPTG and X-Gal. Multiple independent clones for each species were sequenced in both directions using Sequenase (United

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States Biochemical, Cleveland, OH). Alignments and phylogenetic analyses were performed using MacVector (Oxford Molecular Group, Campbell, CA) and MegAlign (DNASTAR, Madison, WI).

3. Results and discussion

Using the PCR strategy, we were able to amplify sequences from human, bovine, rat, mouse, chicken, frog, and bass genomic DNA using the R1 and R2 primers. The sequences of human, rat and mouse receptors have been reported by other groups [1,4,8,19–21] and were not analyzed in this study. The more degenerate Ra and Rb primers were able to amplify sequences from shark and hagfish. After sequencing many clones generated by PCR of bovine DNA, four different sequences were identified which corresponded to all four receptor types (Fig. 1). Of the 56 bovine clones sequenced, 50 were KOR, three were ORL-1, two were DOR, and only one was MOR. Similarly, of 64 chicken clones analyzed, 59 corresponded to MOR, one to DOR, and three had high homology to ORL-1. This apparent primer bias shows that the technique does not seem to amplify and clone individual receptors with equal efficacy.

Three different opioid receptor-like sequences were detected in chicken, bass and frog with the R1 and R2 primers and three each in shark and hagfish using Ra and Rb primers (Fig. 1). Sequences homologous to both the delta and mu opioid receptors were identified in all four species, and the third sequence identified was most homologous to either the kappa receptor or ORL-1. This failure of the primers to obtain four receptor types from each species may be due to the above mentioned primer bias. Alternatively, it is possible that only three members of the opioid receptor family are present in the genome of these species. The identification of four bovine sequences confirms that the strategy can detect all members of the opioid receptor family, at least in mammals, with the caveat that every member is not equally represented in the cloned products.

Because multiple clones were sequenced, occasional Taq polymerase errors were found at approximately the expected rate (0.25%). For clones which had only a single isolate, the certainty of the sequence is, of course, subject to this same error rate.

3.1. Opioid receptor sequences in mammals

Among the mouse, rat, human, and bovine sequences obtained over the region we analyzed, there was between 83% and 91% identity at the nucleic acid level among the four receptor types, but the predicted amino acid sequences were 96–100% conserved. The identification of only four bovine receptors suggests that there are no other members of the opioid receptor family in the mammalian genome, and that the reported sub-types of opioid receptors are generated either by alternative splicing, post-translational modification or by non-homologous G-protein coupled receptors [22].

3.2. Opioid receptor-like sequences in chicken, frog, bass, and shark

Unambiguous DOR and MOR clones were isolated from chicken, frog, bass, and shark. However, no kappa-like sequences were found in the chicken or shark, although ORL-1-like fragments were present. There are reports that there are kappa receptors in chicken brain. The kappa selective ligand U-50,488 can induce a concentration-dependent hyperpolarization and inhibit ion conductance in chick ganglia [23]. This

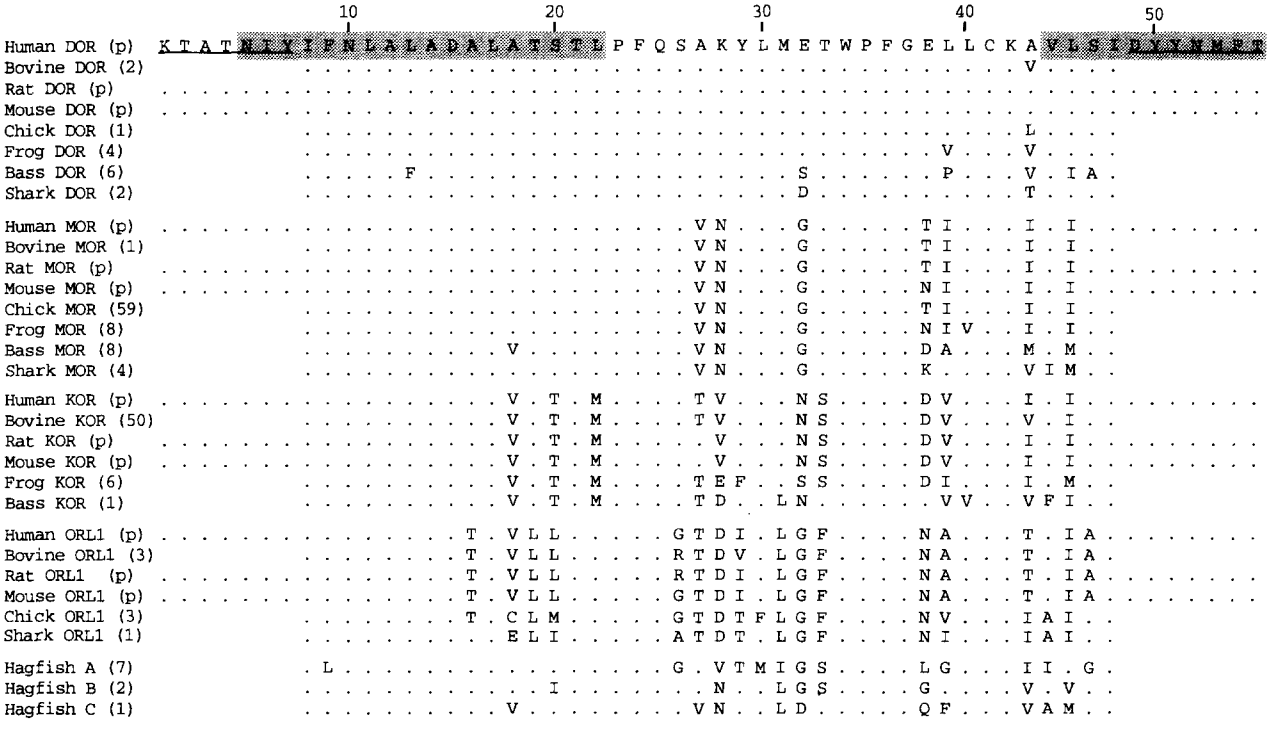

Fig. 1. Alignment of all deduced amino acid sequences of PCR fragments identified from vertebrates with the published human and rodent sequences. The value in parentheses after the name is the number of clones sequenced, or (p) for published data. Underlined are the sequences corresponding to the primers, and shaded are TM2 and part of TM3. The area in between (residues 23–43) corresponds to the first extracellular loop. Only changes from the human DOR sequence are shown.

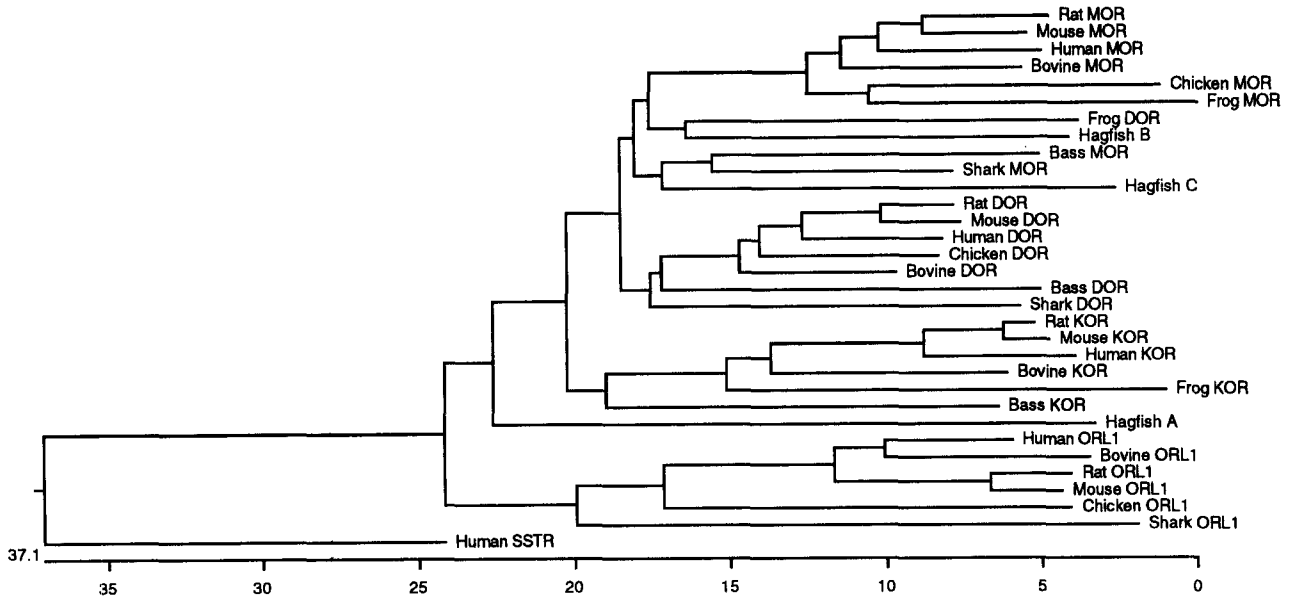

Fig. 2. Phylogenetic analysis of nucleotide sequences between primers of all opioid receptor-like sequences identified in vertebrates. Alignment was performed using MegAlign (DNASTAR). The human somatostatin receptor (Human SSTR) is shown for comparison and X axis shows number of substitution events.

drug can also impair memory formation in chicks, and the kappa selective antagonist nor-binaltorphimine enhances memory formation in a peck avoidance paradigm [24]. Furthermore, autoradiographic [25] and immunohistochemical [26] studies indicate the presence of kappa receptors in the chicken brain. This evidence for the existence of the kappa receptor in the chicken suggests that our PCR strategy simply failed to detect kappa receptors in this species. Nothing is known about opioid receptors in shark, although beta-endorphin-like peptides have been identified in one species — the spiny dogfish [27].

In contrast to the chicken and shark, KOR was found in frog and bass DNA, but ORL-1-like fragments were not. A plethora of evidence exists for the presence of opioid receptors in amphibians, and kappa-like binding sites appear to be the predominant opioid receptor in frog brain [28,29]. Additionally, genes encoding frog pro-opiomelanocortin and pro-enkephalin have been cloned, and pro-dynorphin derived peptides [30] have been identified in frogs. The deltorphans and dermorphins were identified in frog skin, and appear to be relatively selective for the delta and mu receptors, respectively.

3.3. Opioid receptor-like sequences in hagfish

The hagfish is a jawless fish which occupies a key position in metazoan evolution. This exceptionally slimy fish is one of the only surviving members of the most primitive vertebrate class, *agnathans*, which diverged from the main line of vertebrate evolution approximately 470 million years ago [31]. Three different opioid receptor-like sequences were identified from the hagfish genome (Fig. 1). These sequences are clearly members of the opioid receptor family with between 69% and 85% amino acid identity with individual members of the human opioid receptor family (Table 1). Amino acid identity among the three hagfish sequences is between 70% and 83% (Table 1), similar to the identity among the human opioid receptor family.

All three hagfish sequences are most homologous to MOR and DOR at both the amino acid and nucleotide level (Table 1), and we have named these gene fragments hagfish A, B and C, because of the difficulty in classifying them. In an earlier study, we reported the extended, but still incomplete sequence of hagfish A [18], but even with additional 3′ sequence it remained difficult to classify. It is interesting that the first extracellular loops of hagfish A, B and C are devoid of charged amino acids except for the conserved Lys residue at position 42 and an Asp residue in hagfish C at position 32. All the other vertebrate sequences except the mammalian mu receptors have multiple charges in this loop. Clearly, obtaining full-length clones from this species would provide valuable tools for structure-function studies of opioid receptors and might shed more light on the origin of the opioid receptor family. Because these structures are significantly different from their mammalian counterparts, it might be anticipated that the pharmacological profiles are different.

3.4. Lack of opioid receptor-like sequences in invertebrates

Using this strategy, no opioid receptor-like sequences were obtained from protostomes, including *C. elegans* and *Drosophila*, or from any invertebrate species tested — not even from amphioxus, a transition species between invertebrates and ver-

Table 1
Comparison of sequence identity of opioid receptor-like sequences from hagfish with human sequences in the region spanning intracellular loop 1 to TM3

	Nucleotide							Amino acid						
	Human				Hagfish			Human				Hagfish		
	δ	μ	κ	o	A	B	C	δ	μ	κ	o	A	B	C
Hagfish A	60	56	48	49	–	58	50	76	76	72	69	–	76	70
Hagfish B	72	72	55	55	–	–	65	85	85	80	74	–	–	83
Hagfish C	70	65	57	58				82	85	78	72			

Values shown are percent identity and were calculated using MegAlign (DNASTAR). Nucleotide comparisons were made in the sequence between primers. δ(DOR), μ(MOR), κ(KOR), o(ORL-1).

tebrates. It is possible that the degenerate primers may not recognize the genes from species which have a large evolutionary gap from mammals. Alternatively, lower species may not have opioid receptors homologous to the vertebrates — an independent system of opioid receptors may have evolved in the protostomes.

3.5. Delta and mu opioid receptor-like sequences

In this study, delta and mu opioid receptor-like sequences were found in all representative species of vertebrates that were analyzed, and each receptor type is highly conserved during vertebrate evolution (Fig. 1). Chicken and frog DOR and MOR showed exceptionally high homology with their mammalian counterparts (between 96 and 98% amino acid identity), and retained the same charge profile. The bass and shark sequences are slightly more divergent, with most amino acid substitutions in the extracellular loop and the C-terminal half of TM2. Although the amino acid sequences between some species are identical, the nucleotide sequences are more divergent as would be anticipated (see Fig. 2).

Bass DOR was noteworthy in a number of respects, having changes at position 13 (Leu to Phe), 32 (Glu to Ser), and 39 (Leu to Pro). Leucine at position 13 in TM2 is present in all opioid receptors that have been analyzed except the bass DOR. The Glu to Ser changes the charge profile in this loop and the Leu to Pro changes the predicted secondary structure in this region. Also of interest is that mammalian, frog and chicken MOR have a neutral Thr residue at position 38 in the first extracellular loop, whereas shark MOR contains a positively charged Lys at this position and bass MOR has a negatively charged Asp residue. These changes may affect ligand selectivity.

3.6. Kappa opioid receptor-like sequences

Kappa opioid receptor-like sequences were identified in bovine, frog and bass (Fig. 1). Frog KOR has 91% identity to the human kappa receptor and substantially less identity to the other opioid receptors ($< 82\%$). Both frog and bass KOR gain a negatively charged residue at position 28, which is a valine residue in all the mammalian kappa receptors. It is interesting that this negatively charged residue is also found in ORL-1, and it is tempting to speculate that orphanin FQ (nociceptin) may bind to these receptors, since no ORL-1 was identified in these species.

3.7. ORL-1 opioid receptor-like sequences

We identified ORL-1 sequences in bovine, chicken, and in the primitive cartilaginous fish, the thresher shark (Fig. 1). All are highly conserved, except at positions 18 and 26. At position 18 in TM2, chicken has a Cys and shark a Glu where as in all other receptors this is a Val or Ala residue. The shark is thus highly unusual in that TM2 has two negatively charged residues.

3.8. Conclusions

As shown in Fig. 1, over the 54 amino acid region from the first intracellular loop to TM3 there is high homology among the human mu, delta, kappa and ORL-1 receptors (between 70 and 87% amino acid identity). Within this region there are two aspartate residues in TM2 and TM3. Site-directed mutagenesis and deletion studies on the rat mu opioid receptor showed that both of these aspartate residues are important for binding to mu selective ligands [32], but the Asp in TM3 is not as important for DOR binding [33]. Our data suggests these two transmembrane aspartate residues are conserved in all opioid receptor members in all vertebrate species analyzed. It can be assumed that these and other conserved residues are potentially significant elements for maintaining the functions of the opioid receptor gene family under the pressure of evolution.

Opioid receptor-like sequences were identified in all classes of vertebrates, including bovine, chicken, frog, bass, shark and hagfish and demonstrate that the degenerate primers designed from the mammalian opioid receptors can be used to identify opioid receptor-like sequences in lower vertebrate species. Comparison of the DNA and amino acid sequences suggest that the opioid receptor gene family is evolutionarily old, because the partial sequences are well preserved throughout vertebrate evolution (Figs. 1 and 2). Among the members of the opioid receptor family, the delta and mu opioid receptors appear to be the most conserved in the vertebrates that were analyzed. Three opioid receptor-like sequences were found in hagfish, and although their unequivocal classification was difficult, their presence clearly indicates that even at this early stage in evolution, multiple opioid receptors are present.

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