RESEARCH COMMUNICATION

Cloning and expression of a cDNA encoding a mouse brain orphanin FQ/nociceptin precursor

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By using a reverse transcription–PCR approach we have cloned a peptide precursor from mouse brain which contains the sequence of orphanin FQ/nociceptin. The mouse sequence of orphanin FQ/nociceptin is identical at the amino acid level with that isolated from rat and porcine brain. Northern analysis of the mRNA encoding the precursor reveals a single band of approx. 1 kb, with the highest levels in the brain and much lower levels in kidney and spleen. Southern analysis is consistent with a single gene. The precursor peptide from mouse contains two other

putative peptides. Upstream from the orphanin FQ/nociceptin is a 41-amino-acid peptide which is almost identical, except for a six-amino-acid insertion, with the corresponding 35-amino-acid peptide predicted from the rat sequence. Interestingly, the mouse contains a triple AEPGAD repeat within this peptide that is not seen in the rat sequence. Downstream from the orphanin FQ/nociceptin sequence is another 17-amino-acid peptide which is identical with that found in the rat.

INTRODUCTION

Soon after the cloning of a delta opioid receptor [1,2], another member of the opioid receptor family was reported from human (opioid-receptor-like clone, ORL-1) and mouse (κ_3 -related opioid receptor clone, KOR-3) and other species [3–14]. The relationship of this receptor to the traditional opioid receptors has been unclear. Evidence suggests that it is related to the κ_3 opioid receptor [3–5]. A monoclonal antibody against the natively expressed κ_3 receptor recognized this clone [15], and six different antisense oligodeoxynucleotides based upon the second and third coding exons of this clone selectively blocked the analgesic actions of the κ_3 drug naloxone benzoylhydrazone [3–5]. Yet this clone does not appear to be the κ_3 receptor. In contrast with the activity of the antisense probes which targeted the second and third coding exons, five of six directed against the first coding exon were inactive, raising the possibility that the clone and the κ_3 receptor result from alternative splicing of the gene. This remains to be demonstrated. Furthermore, this clone does not bind opioids with the anticipated affinity of a traditional opioid receptor. Thus the pharmacological significance of this receptor has remained unclear.

Two groups have now isolated the same novel peptide from rat (nociceptin) [16] and porcine (orphanin FQ) brain which appears to be an endogenous ligand for the ORL-1/KOR-3 receptor [17]. The heptadecapeptide is highly related to dynorphin A. Instead of the typical Tyr-Gly-Gly-Phe- motif at the N-terminus, orphanin FQ/nociceptin contains Phe-Gly-Gly-Phe and it has similar basic residues near the C-terminus. Although many neurotransmitter peptides are identical among species, others, such as β -endorphin, show differences. We have cloned the precursor peptide for orphanin FQ/nociceptin from the mouse.

EXPERIMENTAL

Materials

Oligodeoxynucleotides were synthesized by GIBCO

(Gaithersburg, MD, U.S.A.). Restriction and modification enzymes were from Promega (Madison, WI, U.S.A.) and GIBCO (Gaithersburg, MD, U.S.A.). DNA sequencing reagents were from USB (Cleveland, OH, U.S.A.) and Perkin-Elmer (Foster City, CA, U.S.A.). Gene-Screen Plus membranes and all radiochemicals were from du Pont–New England Nuclear (Boston, MA, U.S.A.). All other chemicals and reagents were from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

PCR cloning and sequence analysis

Four oligodeoxynucleotides were designed on the basis of the nucleotide sequence of rat nociceptin [16] at positions 62–83 (sense primer 1, 5'-CGGCTCCGGGCAGCTTCAACCT-3'), 195–218 (sense primer 2, 5'-GCTCACGTCCTGCTCTTTACCA-3'), 528–547 (antisense primer 1, 5'-GGGTTGCAGTGCCGGTGCAG-3') and 646–667 (antisense primer 2, 5'-GACATGCTGTGGGGAGGTGCCG-3') and used in PCRs to amplify cDNA fragments encoding the mouse brain orphanin FQ/nociceptin precursor. The template was first-strand cDNA reverse-transcribed with random hexamers from mouse brain total RNA prepared as described [4]. PCR reactions were carried out in a Perkin-Elmer thermal cycler 480 for 25–30 cycles using Vent DNA polymerase (New England Biolabs, Beverly, MA, U.S.A.) to obtain high-fidelity amplification. Each cycle consisted of a melting step (20 s at 94 °C), an annealing step (30 s) at various temperatures depending upon the primers and a 2 min extension step (2 min at 72 °C). Multiple bands were obtained in the first round of PCR using sense primer 1 and antisense primer 2, but the second round PCR using the first round PCR product as template gave a single fragment with sense primer 2 and antisense primer 1, which was subcloned into the Bluescript plasmid (Stratagene, La Jolla, CA, U.S.A.). The clones were sequenced in both directions, using either Sequenase version 2 (USB) or a Amplicycle sequencing kit (Perkin-Elmer). The DNA sequence was analysed using the personal computer package DNANALYZ (Gregory Wernke, University of Cincinnati, Col-

Abbreviations used: KOR-3, κ_3 -related opioid receptor clone; ORL-1, opioid-receptor-like clone.

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The nucleotide sequence shown in Figure 1 is deposited with the GenBank Nucleotide Sequence Database under the accession no. U44027.

lege of Medicine, Cincinnati, OH, U.S.A.) and CLONE and ALIGN programs from Scientific and Educational Software.

Northern- and Southern-blot analysis

Polyadenylated RNAs were isolated from mouse total RNAs using oligo(dT) chromatography (Pharmacia). Mouse genomic DNA was purified from mouse liver tissue as described [4] and was digested with appropriate restriction enzymes. Northern and Southern blotting were performed as described in [4]. A ^{32}P -labelled, 372 bp fragment generated by PCR with sense primer 2 and antisense primer 1 was used in Northern and Southern blotting.

RESULTS

To isolate cDNA encoding the mouse orphanin FQ/nociceptin precursor, we used a PCR cloning strategy based upon the rat nociceptin cDNA sequence. Although the multiple bands were picked up by the first round PCR with sense primer 1 and antisense primer 2, the second round PCR using nested primers gave a single fragment similar in size to the rat cDNA (results not shown). Analysis of four different clones revealed identical sequences. The nucleotide and predicted amino acid sequences of the mouse clones (Figure 1) show very high similarity to those of

GCTCAGTCCGCTGCTCTTTACCAGCCAAAGGCCGCGAGATGCAGCACCTGAAGAGAAT	60
L T S A A L Y Q P K A S E M Q H L K R M	20
L T S A A L Y Q S K A S E M Q H L K R M	rat

GCCGCGTGTCCGGAGCTTGGTGCAAGTGCAGAGCTGGCGCAGATGCTGAGCC	120
P R V R S L V Q V R D A E P G A D A E P	40
P R V R S V V Q A R D A E P E A D A E P	rat

TGGCGCAGATGCTGAGCTGGCGCAGATGACGCTGAGGAGGTGGAGCAGAAGCAGTGCA	180
G A D A E P G A D A E E V E Q K Q L Q	60
V A D E - - - - - A D E V E Q K Q L Q	rat

GAAAAGTTTGGGGGCTTACCGGGGCCGGAATCAGCCCGAAGTTGGCCAACAGAA	240
K R F G G F T G A R K S A R K L A N Q K	80
K R F G G F T G A R K S A R K L A N Q K	rat

GCGGTTCACTGAGTTTATGAGGCGATACCTGGTCCTGAGCATGCAGTCAAGTCAACGCGG	300
R F S E F M R Q Y L V L S M Q S S Q R R	100
R F S E F M R Q Y L V L S M Q S S Q R R	rat

GCGCACCTTGCACCAATGGTAATGTGTAGCCAGAAGGAGCCCTCCAGCTGCACCGG	360
R T L H Q N G N V *	109
R T L H Q N G N V *	rat

CCACTGCAACCC	367

Figure 1 Partial nucleotide and predicted amino acid sequences for the mouse orphanin FQ/nociceptin precursor

The predicted mouse amino sequences are shown in single-letter code below the nucleotide sequences, which are numbered at the right. The rat amino acid sequence is given in the third line for comparison. The orphanin FQ/nociceptin peptide sequence is underlined. The 41-amino-acid putative neuropeptide sequence is marked by the broken line and the other 17-amino-acid putative neuropeptide by the dotted line. The putative Lys-Arg motifs for proteolytic processing are indicated by **bold italics**. The triple AEPGAD repeat is shown with a thin solid line. The stop codon is shown by an asterisk.

Figure 2 Northern and Southern analyses of mouse orphanin FQ/nociceptin precursor

(A) Polyadenylated RNA (15 μg) from each tissue was used in Northern-blot analysis, as described in the Experimental section. The single band corresponding to the precursor mRNA and the two size markers are indicated by arrows. (B) Each lane was loaded with 10 μg of mouse genomic DNA digested with the indicated enzyme, as described in the Experimental section.

the rat nociceptin precursor. The mouse heptadecapeptide sequence is identical with the nociceptin sequence in the rat and the porcine orphanin FQ sequence. The Lys-Arg motifs and basic amino acids for putative proteolytic processing [18] which define two other putative neuropeptides are also highly conserved between the mouse and rat. Like the rat, the mouse precursor has

two additional putative peptide products. The sequence upstream of the orphanin FQ/nociceptin is 88 % identical at the amino acid level with the rat version, with the exception of a six-amino-acid insertion present in the mouse sequence. Interestingly, the translated mouse sequence contains a triple AEPGAD repeat. At the nucleotide level, the second two sequences encoding AEPGAD are identical, but the first differs from the others by a single base. Downstream from the orphanin FQ/nociceptin sequence there is another heptadecapeptide which is identical with the one found in the rat.

Probing polyadenylated RNA from mouse brain and other tissues at high stringency with the cloned cDNA fragment gave a single band of approx. 1 kb in the brain on Northern-blot analysis (Figure 2A). The levels in the brain were greater than in the other tissues. The RNA loading of the various tissues was relatively equal, as determined by probing the blot with a ^{32}P -labelled β_2 -microglobulin PCR fragment (results not shown). If over-exposed, the gel reveals light bands of similar size in mRNAs from spleen and kidney (results not shown). Southern analysis (Figure 2B) of genomic DNA digested with *HindIII*, *EcoRV*, *XbaI*, *BamHI* and *ApaI* reveals a single band, suggesting a single gene encoding the orphanin FQ/nociceptin. Digestion with *PstI* yields three bands.

DISCUSSION

The discovery of orphanin FQ/nociceptin [16,17] has opened a major new area of research. The peptide has a number of similarities with dynorphin A, but it does not bind to the traditional opioid receptors. Pharmacologically, orphanin FQ/nociceptin has actions which appear to be opposite to those of the opioids. Whereas typical opioids prolong latencies in established analgesic tests, such as the hotplate and tailflick assays, orphanin FQ/nociceptin reduces these latencies, suggesting that it may be nociceptive. Structurally, the peptide is closely related to dynorphin A, but they show very different receptor-binding profiles. Orphanin FQ/nociceptin does not label opioid receptors, and opioid peptides have low affinity for the KOR-3/ORL-1 receptor. The amino acid sequence of orphanin FQ/nociceptin is conserved among at least three species: rat, pig and mouse.

The cloned precursor from the rat suggested two additional peptides, one of 35 amino acids and the other of only 17 [16]. The mouse clone also contains similar peptides. The second heptadecapeptide is identical between the two species. The larger peptides in both species also are quite similar, except for an insertion in the mouse clone of six amino acids in the middle of the mouse peptide. It will be interesting to see whether these peptides are physiologically active and, if so, whether the insertion in the mouse peptide changes the activity and/or selectivity. Unlike that of the rat, the mouse peptide contains a triple AEPGAD repeat. The significance of this triple repeat is unclear, but a search of several databases reveals no other peptides containing this sequence.

The KOR-3 clone has been observed in a number of non-neuronal tissues, including an osteoblast cell line [19] and immune cells [14], including the B-cell Raji cell line [20]. This raises the

possibility of a broader hormonal role for orphanin FQ/nociceptin beyond the nervous system. Northern analysis reveals a single transcript of approx. 1 kb in the brain. We also find lower levels of the mRNA in kidney and spleen, consistent with a broader physiological role of the peptide.

Southern analysis is consistent with a single gene. The presence of a *PstI* site in the cDNA encoding the precursor explains two bands. The third band raises the possibility of another *PstI* site within an intron sequence.

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