Isolation and expression of a novel alternatively spliced mu opioid receptor isoform, MOR-1F

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Abstract The *MOR-1* gene is large, with a recent study reporting nine exons spanning 250 kb which combine to yield six different mu opioid receptor splice variants. We now report the isolation of exon 10, which is contained within yet another splice variant, *MOR-1F*, which is composed of exons 1, 2, 3, 10, 6, 7, 8 and 9. Exon 10 comprises 186 bp which predict a unique 58 amino acid sequence extending beyond exon 3. It has been mapped between exons 4 and 6 and has flanking consensus splice sequences. On Northern blot analysis, the *MOR-1F* mRNA is smaller than the other *MOR-1* mRNAs. When expressed in CHO cells, *MOR-1F* binds the mu opioid radioligand [^{3}H]DAMGO with high affinity ($K_D = 1.04 \pm 0.03$ nM). Competition studies demonstrated the selectivity of the variant for mu opioid ligands, supporting its classification within the mu opioid receptor family.

Key words: Morphine; Morphine-6β-glucuronide; Analgesia; Splicing; MOR-1; Antisense mapping

1. Introduction

Morphine elicits its effects through mu opioid receptors. Pharmacological studies have implied the presence of mu opioid receptor subtypes [1]. The antagonists naloxonazone and naloxonazine suggested the existence of mu_1 and mu_2 receptors [2–6] while more recent studies have raised the possibility of a third mu opioid receptor responsible for the actions of morphine-6β-glucuronide (M6G) and heroin [7–10]. However, only one gene has been identified which encodes a mu opioid receptor, *MOR-1* [11–14]. Antisense studies quickly established the importance of *MOR-1* in morphine analgesia in the rat [15]. However, not all antisense probes blocked morphine analgesia [9,16]. While a series of antisense oligodeoxynucleotides targeting exon 1 were active, antisense probes based upon exon 2 were not. M6G analgesia revealed a very different sensitivity profile. The exon 1 antisense probes active against morphine were ineffective against M6G. Conversely, the exon 2 antisense oligodeoxynucleotides which were inactive against morphine potently blocked M6G analgesia. These results raised the possibility that the receptor responsible for morphine and M6G analgesia might be splice variants of *MOR-1* [9,16]. Two splice variants were reported soon after the initial description of *MOR-1*. One variant lacked exon 4 [17] while a second contained an alternative exon (exon 5) in place of the original exon 4 [18]. Our group then identified an additional four exons downstream from exon 4 which combine to yield three additional slice variants [19]. We now report the presence of yet another *MOR-1* exon and a novel *MOR-1* splice variant.

2. Materials and methods

Male Crl:CD-1(ICR)BR mice (24–32 g) (Charles River Labs, Raleigh, NC, USA) were maintained on a 12 h light/dark cycle with food and water available ad libitum. Opiate drugs were provided by the Research Technology Branch of the National Institute on Drug Abuse. Halothane was purchased from Halocarbon Laboratory (Hackensack, NJ, USA).

2.1. RT-PCR cloning and genomic mapping

Mouse brain total RNA was obtained and reverse-transcribed with Superscript II (GIBCO) (Pan, Xu et al., 1999, ID: 7934). Partial cDNA sequences of *MOR-1F* were obtained by a nested PCR approach using the first-strand cDNA as template. A sense primer (sense primer A, 5′-CCC AAC TTC CTC CAC AAT CGA A-3′, which was designed from the 3′-end of exon 3) and an antisense primer (antisense primer A, 5′-GAA AGG CAT CTA CCC TCT CGC TGT-3′, which was derived from exon 9; GenBank accession number AF062752) were used in the first-round PCR, which did not produce visible bands on agarose gel. The first-round PCR product was then used as template in the second-round PCR with a pair of nested primers (sense primer B from exon 3, 5′-GGG AAC ACC CCT CCA CGG C-3′ and antisense primer B from exon 9, 5′-CCA CAC TGC TCA CCA GCT CAT CCC-3′). A new band of approximately 550 bp was purified from the agarose gel, subcloned into pCRII-TOPO vector (Invitrogen) and sequenced.

Full-length cDNA clones containing exon 10 were obtained by the RT-PCR approach. Two sense primers (A, 5′-GAG AGG AAG AGG CTG GGG CGC-3′ and B, 5′-GGA ACC CGA ACA CTC TTG AGT GCT-3′, both of which were designed from 5′-UTR of *MOR-1*) and two antisense primers (antisenses A and B, see above) were used in the nested PCR with the first-strand cDNA reverse-transcribed from mouse brain RNA as template. A 1.7 kb fragment was amplified and subcloned into pCRII-TOPO vector and sequenced. Sequence analysis of the product (*MOR-1F*, GenBank accession number AF167568) showed that it contained coding exons 1, 2, and 3 from *MOR-1* followed by exons 10, 6, 7, 8 and 9. The location of exon 10 within the MOR-1 gene was mapped in the BAC clone by long PCR using an AccuTag polymerase (Sigma, St. Louis, MO, USA).

2.2. Northern blotting analysis

Total RNA was obtained from mouse brain by the guanidinium thiocyanate phenol-chloroform extraction and Northern analysis performed [19]. In brief, 50 μg of total brain RNA was loaded, separated on a 0.8% formaldehyde agarose gel and transferred to a GenePlus membrane (New England Nuclear Corp, Boston, MA, USA). The membrane was hybridized with a ^{32}P-labeled exon 10 fragment generated by PCR with a sense (5′-GCCTGGAGCCAACAGAGGTCAAACGAAG-3′) and an antisense primer (5′-GCGGGACCAGAGAGATGGCTAGTGG-3′).

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2.3. Regional RT-PCR

Total RNA was isolated from different mouse brain regions and reverse-transcribed with Superscript II. The first-strand cDNA was amplified with a sense primer from exon 3 (sense primer A) and an antisense primer from exon 10 (5′-CTT GCT GCC TTC GTA AGG ACC TGG G-3′). RNA loading was measured by a parallel PCR with β_2-microglobulin primers (ClonTech). The PCR products were separated on a 1% agarose gel, stained with ethidium bromide and photographed with Kodak DC120 Digital Camera and Imaging System.

2.4. Expression of MOR-1F

The cDNA fragment containing the full-length *MOR-1F* in pCRII-TOPO was subcloned into pcDNA3.1 (Invitrogen). The resulting plasmid, *MOR-1F*/pcDNA3 was used to transfect CHO cells by Lipofect-AMINE reagent (GIBCO, Gaithersburg, MD, USA). Stable transformants were obtained 2 weeks after selection with G418 and screened in a [^{3}H]DAMGO binding assay. A stable clone, 10-5, showed high levels of binding ($B_{max} = 1.69 \pm 0.35$ pmol/mg protein) and was selected for further binding studies.

2.5. Receptor binding assays

Membranes were isolated from stable transfected clones and binding performed with [^{3}H]DAMGO at 25°C for 1 h in potassium phosphate buffer (50 mM, pH 7.4) containing magnesium sulfate (5 mM). Specific binding was defined as the difference between total binding and non-specific binding remaining in the presence of levallorphan (1 μM). K_D and K_i values were calculated by non-linear regression analysis (Prism, Graphpad Software, San Diego, CA, USA).

2.6. Behavioral testing

The drugs were administered subcutaneously (s.c.), intracerebroventricularly (i.c.v.) [20] or intrathecally (i.t.) [21]. Analgesia was assessed quantally as a doubling or greater of the baseline latency for the individual mouse using the radiant heat tailflick assay, with baselines ranging between 2–3 s, as previously described [9,22]. A cutoff of 10 s was imposed to minimize tissue damage. Groups were compared with the Fischer exact test.

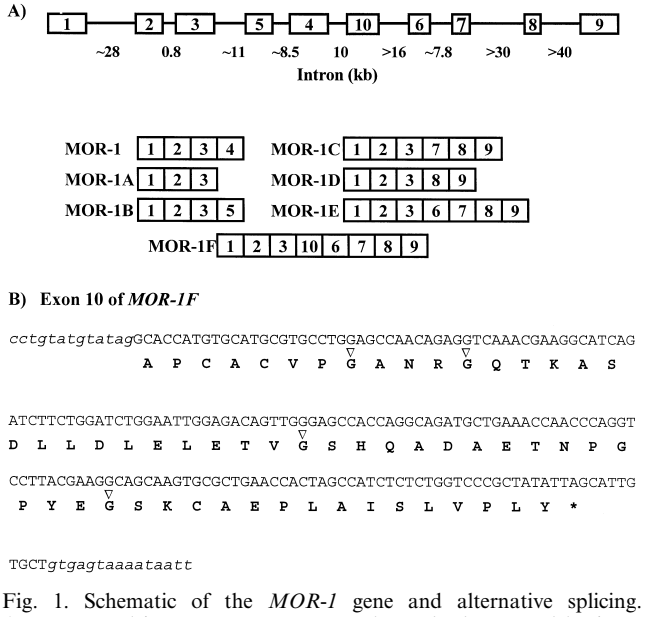

Fig. 1. Schematic of the *MOR-1* gene and alternative splicing. A: Exons and introns on *MOR-1* are shown by boxes and horizontal lines, respectively. The exons are numbered according to their discovery. The exon components of the various variants are presented. Termination codons are present in the following exons for each variant: *MOR-1*, 4; *MOR-1A*, 3; *MOR-1B*, 5; *MOR-1C*, 9; *MOR-1D*, 9; *MOR-1E*, 6; *MOR-1F*, 10. B: The nucleotide sequence of exon 10 (upper case), along with flanking intron sequence (lower case italics), is presented, along with the predicted amino acid sequence. Potential myristolation sites are indicated by ▽.

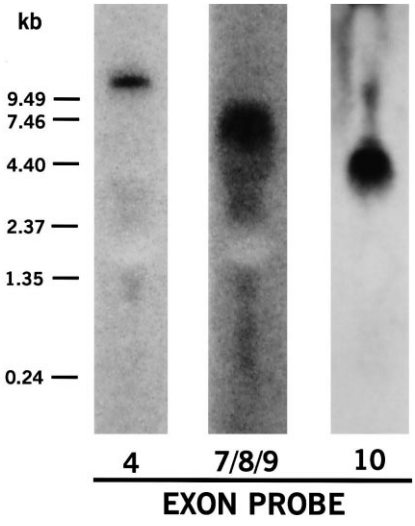

Fig. 2. Northern blot. A Northern blot was performed as described in Section 2 using a ^{32}P-labeled fragment of exon 10. Northern blots with exon 4 and 7/8/9 are from the literature [19]. The same source and amount of RNA were used for all three Northern blots, which were run on a single gel. Film exposure times did vary.

3. Results and discussion

To explore for possible additional splice variants of *MOR-1*, we performed nested RT-PCR using upstream primers in exon 3 and downstream primers in exon 9 (Fig. 1). In addition to the expected three fragments corresponding to *MOR-1C*, *MOR-1D* and *MOR-1E*, we observed a new band (~550 bp) which was larger than the predicted three fragments. Sequencing this band revealed a new 186 bp insertion (Fig. 1B) between exons 3 and 6. The new nucleotide sequence, exon 10, was mapped between exons 4 and 6 on a mouse genomic BAC clone [19] by a long PCR approach using AccuTag polymerase (Fig. 1). Sequence analysis of the BAC clone indicated that the flanking sequences of exon 10 were consistent with consensus splice junctions. The full-length cDNA for *MOR-1F* contained exons 1, 2, 3, 10, 6, 7, 8 and 9 (Fig. 1).

Northern analysis of the new exon 10 revealed a band of approximately 4.4 kb, which was significantly smaller than the exon 4 probe labeling *MOR-1* or the exon 7/8/9 probe which hybridizes to *MOR-1C*, *MOR-1D* and *MOR-1E* (Fig. 2).

The distribution of *MOR-1F* mRNA differed across brain regions as determined by RT-PCR (Fig. 3). The highest levels appeared to be in the hypothalamus, with moderate levels in the PAG and thalamus. Although lower levels were seen in the other regions, *MOR-1F* mRNA was seen in all of them. However, the expression of *MOR-1F* appeared to be restricted to the brain, with no observable expression in other peripheral tissues, such as heart, spleen, lung, liver, muscle and testes (unpublished observations).

Translation of the sequence predicted a novel 58 amino acid sequence extending beyond the sequence encoded by exon 3. The termination codon was located within exon 10, with the downstream exons 6, 7, 8 and 9 all in the 3′-untranslated region. There was no amino acid sequence overlap between *MOR-1F* and the other known *MOR-1* variants. The amino acid sequence encoded by exon 10 was acidic, with a calculated isoelectric point (p*I*) of 4.47, a value similar to that of *MOR-1D* (4.44) and *MOR-1* (3.46), but appreciably lower

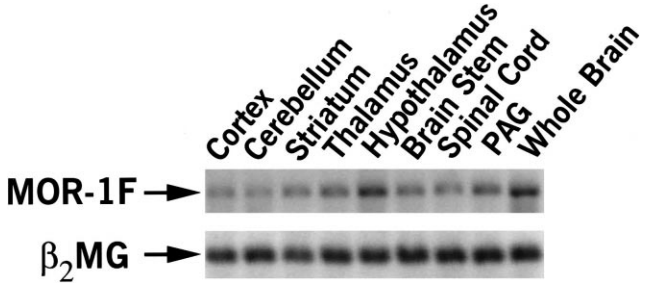

Fig. 3. Regional distribution of the MOR-1 and MOR-1F mRNA. Total RNA was isolated from the indicated brain regions and subjected to RT-PCR, as described in Section 2. RNA loading was assessed with parallel reactions with a pair of β_2-microglobulin primers, as described in Section 2.

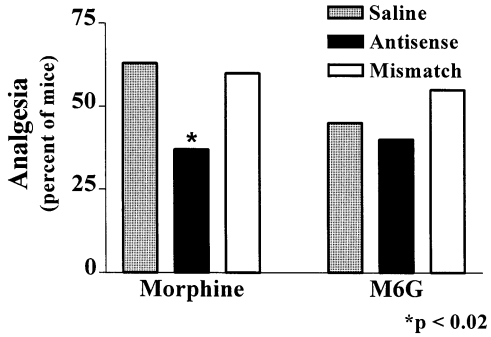

Fig. 4. Antisense mapping exon 10. An antisense oligodeoxynucleotide primer (10 μg, i.c.v.) targetting exon 10 (5′-CTTGCTGCCT TC GTAAGGACCTGG-3′), a mismatch control (5′-CTTG**TC**GCT-**CTCTG**AAGAGCCTGG-3′) or saline were administered to groups of mice ($n \geq 25$) on days 1, 3 and 5 and analgesia assessed with either morphine or M6G on the day after the last treatment. Significance was determined by Fisher exact test.

than that of *MOR-1E* (9.74) and *MOR-1C* (7.03). The new sequence contained no putative protein kinase C or casein phosphorylation sites, but there were four potential myristolation sites, raising the possibility of an association of the C-terminal tail with the membrane.

Cells stably expressing *MOR-1F* bound the mu opioid [^{3}H]DAMGO quite potently, with an affinity similar to the other *MOR-1* variants (Table 1) [19]. Competition studies also revealed a selectivity pattern consistent with a mu opioid receptor. Although mu opioids such as morphine, DAMGO, the endomorphins, β-endorphin and naloxone all competed binding quite potently, the delta-selective opioid DPDPE and the kappa$_1$-selective compound U50,488H were both inactive at high concentrations (Table 1). Dynorphin A and DSLET revealed weaker affinities for *MOR-1F* than any of the other variants tested.

To assess the importance of *MOR-1F* in mu opioid analgesia, we next treated groups of mice with an antisense oligodeoxynucleotide targeting exon 10 (Fig. 4). Prior studies with other exons had indicated that these probes typically blocked either morphine or M6G analgesia [9,16,19]. The antisense probe based upon exon 10 was inactive against M6G analgesia and it had only a modest effect against morphine when compared to the saline controls. This decrease was marginally significant and less than that seen in antisense mapping studies looking at exons 4, 6, 7, 8 and 9. Thus, these findings suggest that mu receptors containing exon 10 have, at most, a limited role in morphine analgesia.

The identification of the new exon illustrates a continuously increasing complexity of the *MOR-1* gene. There are now seven variants differing only at the tip of the C-terminus. The roles of all these variants remain unknown. They all bind morphine and other mu opioids quite potently while showing no appreciable affinity for selective delta and kappa ligands. Although the endogenous opioid peptides do show subtle binding differences among the variants, their significance is not clear. The expression of the variants varies among brain regions and even within cells [19]. For example, the striatum has high levels of *MOR-1* and little detectable

Table 1
Selectivity of MOR-1 splice variants in transfected CHO cell membranes

	Values (nM)				
	MOR-1	MOR-1C	MOR-1D	MOR-1E	MOR-1F
Saturation studies (K_D)					
[^{3}H]DAMGO	1.75 ± 0.44	0.57 ± 0.14	0.35 ± 0.07	0.74 ± 0.11	1.04 ± 0.04
Competition studies (K_i)					
Morphine	5.3 ± 2.0	2.4 ± 0.6	1.5 ± 0.2	2.3 ± 0.4	2.9 ± 0.5
M6G	5.2 ± 1.8	4.1 ± 1.2	4.8 ± 0.8	5.6 ± 0.7	9.6 ± 0.8
DAMGO	1.8 ± 0.5	0.9 ± 0.2	0.7 ± 0.1	1.2 ± 0.5	1.1 ± 0.2
DADLE	2.1 ± 0.3	3.2 ± 1.9	1.3 ± 0.4	2.5 ± 0.7	5.3 ± 0.8
DSLET	12.5 ± 3.6	8.1 ± 1.2	3.6 ± 0.6	6.7 ± 1.4	14.3 ± 1.8
Naloxone	4.3 ± 0.9	2.8 ± 0.8	0.9 ± 0.1	2.8 ± 1.4	2.1 ± 0.5
Endomorphin 1	2.1 ± 0.8	1.4 ± 0.4	1.8 ± 0.3	2.4 ± 0.1	2.9 ± 0.5
Endomorphin 2	4.2 ± 1.8	1.6 ± 0.2	2.0 ± 0.3	4.4 ± 0.8	4.1 ± 1.3
β-Endorphin	10.8 ± 2.9	5.8 ± 0.5	1.7 ± 0.4	5.0 ± 1.2	6.0 ± 1.6
Dynorphin A	10.9 ± 0.5	5.6 ± 0.8	2.2 ± 0.8	8.9 ± 1.1	12.1 ± 1.0
U50,488H	> 500	> 500	> 500	> 500	> 500
DPDPE	> 500	> 500	> 500	> 500	> 500

MOR-1F was stably expressed in CHO cells and a clone with high levels of expression (B_{max} = 1.69 ± 0.35 pmol/mg protein) was selected for further studies. Saturation studies with *MOR-1F* were performed with [^{3}H]DAMGO (0.2 to 4 nM) and the K_D and B_{max} values determined by non-linear regression analysis. Results are the mean ± S.E.M. of at least three independent determinations. The K_D values for *MOR-1*, *MOR-1A*, *MOR-1B* and *MOR-1C* are from the literature [19].

Competition studies were performed against [^{3}H]DAMGO (1 nM) using at least three concentrations of the indicated ligand with the same stable *MOR-1F* clone. IC_{50} values were calculated by non-linear regression analysis (Prism, Graphpad software) and converted to K_i values as previously reported [25,26]. Results are the means ± S.E.M. of at least three independent replications. Values for *MOR-1*, *MOR-1C*, *MOR-1D* and *MOR-1E* are from the literature [19].

MOR-1C [23]. Clearly, function is dependent upon the region of the brain expressing the variant, but the importance of the C-terminus in coupling to G-proteins raises questions about transduction mechanisms. These variants also might affect associations with other membrane-associated proteins, including dimerization with other opioid receptors [24]. Differences in putative phosphorylation sites may play a role in receptor regulation. However, all these possibilities remain speculative and remain to be explored in greater detail.

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