Isolation and sequence of cDNA encoding the motilin precursor from monkey intestine. Demonstration of the motilin precursor in the monkey brain

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Abstract The motilin precursor cDNA has been isolated and sequenced from a cDNA library prepared from monkey small intestine. The sequence indicates a 345 bp open reading frame, a 63 bp 5′ untranslated region and a 154 bp 3′ untranslated region. The sequence encodes a 115 amino acid motilin precursor composed of a 25 amino acid signal peptide, the 22 amino acid motilin peptide and a 68 amino acid motilin associated peptide (MAP). Compared with the human motilin precursor cDNA, there are two amino acid substitutions in the signal peptide, one in motilin and four in the MAP. The presence of the motilin precursor in hypothalamus, hippocampus and cerebellum was demonstrated by RT-PCR.

Key words: Motilin; Brain; Precursor

1. Introduction

Motilin, a 22 amino acid polypeptide was first isolated and sequenced by Brown et al. from porcine intestine in 1973 [1]. Motilin stimulates gastrointestinal motor activity and appears to play an important physiological role in the regulation of fasting motility patterns [2].

The cDNA encoding the motilin precursor has been cloned in man [3,4], pig [5], rabbit [6], sheep [7], dog and chicken (De Clercq, unpublished data). In every species the motilin precursor consists of a signal peptide of 25 amino acids, the sequence of the 1–22 active peptide, and a motilin associated peptide (MAP) with variable length and unknown function. The gene structure of human motilin has also been elucidated [8–10]. It is located on chromosome 6 and consists of five exons and four introns. The active peptide (1–22) is encoded by exon 2 and exon 3.

Numerous immunocytochemical studies have demonstrated the presence of motilin in endocrine cells of the gut, while some studies have, less unequivocally, addressed the presence of motilin in extradigestive structures, especially the brain. Unfortunately many studies suffer from uncertainty regarding the suitability of the antisera used, as often the amino acid sequence of the motilin under consideration was unknown. More in general, immunocytochemistry does not allow one to identify with absolute certainty the molecular species present in a tissue (for a review, see [2]).

Molecular biological techniques allow one to study the expression of a given substance, but few such studies have been performed as yet with motilin. Daikh et al. [10] studied the motilin gene expression in different tissues in monkey by Northern blot hybridization using a cRNA probe complementary with a segment of the human motilin precursor. Their data show high expression levels in the duodenum, weaker levels in monkey colon, liver and adrenal, kidney and testis, while the hybridization signal was absent in the pituitary and cerebellum. Recently motilin mRNA has been isolated from the human and the rabbit brain [11], raising again the question of the presence of motilin in the central nervous system. Evidence is also emerging that motilin, besides its effect on the gastrointestinal system, which is mediated via peripheral motilin receptors, may also have a central role. Thus recently motilin binding sites have been demonstrated in the brain of the rabbit [12,13].

In order to verify the presence of motilin in the central nervous system of the monkey, we decided to elucidate first the structure of monkey motilin precursor. We here report the isolation and sequencing of the motilin precursor cDNA from monkey intestine, and the demonstration of the presence of motilin mRNA in the monkey brain.

2. Material and methods

2.1. Total RNA extraction and first strand cDNA synthesis

Intestinal tissue from rhesus monkey was obtained from Dr. A. Dubois (NIH, Bethesda, MD, USA). Total RNA was extracted from 100 mg duodenal mucosa by the TRIzol reagent procedure (GIBCO, New York, USA). First strand cDNA was synthesized from 1 μg total RNA using oligo-dT_{12-18} as primer and Superscript II RNase H^- reverse transcriptase (Gibco, New York, USA) (42°C for 1 h).

2.2. Oligonucleotides

All PCR primers and the hybridization probe were synthesized by GIBCO (New York, USA). The oligo-dT anchor primer and the PCR anchor primer were from the 3′/5′ RACE kit (Boeringer, Mannheim, Germany). Primers were designed using program Prime from the GCG program package from BEN (Belgian European Molecular Biology Net node). All oligonucleotides used are listed in Table 1.

2.3. PCR

2.3.1. PCR amplification of the middle portion of the motilin cDNA sequence. The motilin cDNA fragment was isolated from the first strand cDNA pool of monkey intestine by polymerase chain reaction (PCR) with Taq DNA polymerase (Eurogentec, Belgium) and the primers moth.for and moth.rev. These primers are located in exon 2 (forward) and exon 4 (reverse) of the human motilin gene respectively (Table 1). The PCR was performed in a 20 μl mixture containing $MgCl_2$ 3.5 mM, dNTP 0.2 mM, Taq DNA polymerase 0.5 unit (Boehringer Mannheim, Germany), 0.5 μM of each primer and 1 μl cDNA. The reaction mixture was heated at 94°C for 4 min. Amplification was performed in a OmnE cycler (Hybaid Ltd., UK) at 94°C (1 min), 55°C (1 min), 72°C (1 min) for 32 cycles, followed by an extension step at 72°C for 10 min. The PCR was analyzed by electrophoresis on a 1.5% agarose gel and Southern blot hybridization was performed with the ^{32}P-labeled motex2.for probe (Table 1).

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Table 1
DNA sequences of primers and probes

Oligonucleotide	Sequence	Positions
Moth.for[b]	5′ TGCTGGCCTCCCACCASACGGAAGCCT 3′	116–139[a]
Moth.rev[b]	5′ GGKGGCCSGGTACTTTTCCAGCTG 3′	340–363[a]
Monkey3′.for	5′ AGGATGAACTCCAGACAG 3′	325–342[a]
Monkey5′.rev3	5′ TTCGTTTCCTTCTTCCTC 3′	271–288[a]
Monkey5′.rev1	5′ TACTCCGTTCCTTTTCCTG 3′	178–196[a]
Monkey5′.rev2	5′ GGATTTCTTTTGCCCTTTAC 3′	194–213[a]
Motex2.for[b]	5′ GGAAYRAGGGCAMAAGAARTCCC 3′	191–214[a]
18s rRNA forward	5′ GGAATAATGGAATAGGACC 3′	872–890[c]
18s rRNA reverse	5′ GCTCCACCAACTAAGAAC 3′	1367–1350[c]
Monkey forward	5′ TTCGTCCCCATCTTCACCTAC 3′	139–159[a]
Monkey reverse	5′ GGTACTTTTCCAGCTGTCTG 3′	336–355[a]

[a]The numbers refer to the numbering used in Fig. 1.
[b]The degenerate primer set moth.for and moth.rev, and the probe motex2.for are based on the sequence alignment of human and porcine cDNA.
[c]The 18s rRNA primer positions refer to the human 18s rRNA cDNA sequence [15].

2.3.2. Amplification of the 3′ and 5′ cDNA ends. Amplification of the 3′ and 5′ cDNA ends was done with the 5′/3′ RACE kit (Boehringer Mannheim, Germany), using primers based upon the sequence of the middle portion of the cDNA encoding the monkey motilin precursor. Briefly, the first strand cDNA was synthesized with oligo-dT anchor primer, AMV reverse transcriptase (Boehringer Mannheim, Germany) and the deoxynucleotide mixture at 55°C for 1 h. 3′ RACE was performed using monkey3′.for and the PCR anchor primer from the kit.

The first strand cDNA to obtain the 5′ cDNA end was synthesized by using a gene specific reverse primer monkey5′.rev3, AMV reverse transcriptase and the deoxynucleotide mixture. The first strand cDNA was purified by the High Pure PCR Product Purification kit (Boehringer Mannheim, Germany). A homopolymeric A-tail was added to the 3′ end of the cDNA by terminal transcriptase (Boehringer Mannheim, Germany). 5′ RACE PCR was then carried out with a gene specific reverse primer monkey5′.rev2 and oligo-dT anchor primer. Nested PCR was performed using a gene specific reverse primer monkey5′.rev3.

Analysis of the PCRs was performed as described above except that the probes used for hybridization were moth.for for the 5′ cDNA end and moth.rev for the 3′ cDNA end.

2.4. Cloning and sequencing

PCR products that hybridized with the selected probe were ligated into the pCR 2.1 plasmid vector and transformed into *E. coli* Invα competent cells (TA cloning kit, InVitro Gen, San Diego) [14]. Screening of positive clones was performed by hybridization with a ^{32}P-labeled oligonucleotide. The plasmid DNAs from positive clones were extracted and purified by the mini Qiagen Plasmid kit manual (Qiagen, CA, USA). Sequencing was performed by a ABI377 automatic fluorescent sequencer based on the dideoxy chain-termination method (Eurogentec, Belgium). The nucleotide sequence presented has been accepted by GeneBank database under accession number AF016372.

2.5. Demonstration of the presence of the motilin precursor in the brain

Tissue from the cerebellum, the hippocampus and the hypothalamus of the rhesus monkey was obtained from Dr. A. Dubois (NIH, Bethesda, MD, USA). Total RNA was extracted by the TRIzol reagent procedure (GIBCO, New York, USA).

One μg of RNA was used to synthesize single-stranded cDNA using 200 units of Superscript II RNase H$^-$ reverse transcriptase (Gibco BRL, New York, USA) and random primers (25 μg/ml) (Gibco BRL, New York, USA). The obtained cDNA served as a template for the polymerase chain reaction. Hot start PCR was performed by heating the reaction mixture (all components except Taq DNA polymerase) to 95°C for 3 min. Then Taq polymerase was added and 32 cycles of amplification were performed (95°C for 1 min, 55°C for 1 min, 72°C for 1 min with a final extension duration of 10 min at 72°C) using 0.25 U of Taq DNA polymerase (Pharmacia Biotech., USA) and 0.5 μM of each primer. Primers (18s rRNA forward and reverse) for amplifying 18s rRNA ss-cDNA were taken from the sequence of the human 18s rRNA cDNA [15]. Primers (monkey forward and reverse) for amplifying a motilin cDNA segment were taken from the sequence of the monkey duodenal motilin precursor cDNA (Table 1). PCR products were analyzed on a 1.5% agarose gel and hybridized subsequently with a monkey motilin oligonucleotide probe (monkey-5′.rev3) at 50°C and a human 18s rRNA cDNA probe at 68°C (Table 1). The monkey oligonucleotide probe was end labeled with γ^{32}P-ATP (Amersham Life Science, Buckinghamshire, England) and T4 polynucleotide kinase (Eurogentec, Belgium). The 18s rRNA cDNA probe was labeled with rapi-probe kit (Amersham Life Science, Buckinghamshire, England) by random priming.

3. Results and discussion

3.1. Isolation and sequencing of cDNA encoding monkey intestinal prepromotilin

The sequence of prepromotilin cDNA isolated from the duodenum of the monkey was obtained in three steps. Using moth.for and moth.rev as primer in a PCR we obtained a strong visible band of about 250 bp which hybridized with the ^{32}P-labeled motex2 probe. This band was ligated and transformed as described in Section 2. The length of the cloned cDNA fragment was 248 bp and its sequence was used to design primers for the amplification of the 3′ and 5′ cDNA ends. Cloning and sequencing of the amplified 3′ and 5′ cDNA ends revealed a sequence of about 300 bp and 98 bp,

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  1  CTGAAGACAA GCAGAAAGAG ACTCCTCCAG ACCCACTCAA TACCACACGC

 51  ACGCTCTCCA ATAATGGTAT CCCGTAAGGC TGTGGCTGCT CTGCTGGTGG

 +1                                              Phe ValProIle
101  TGCATGCACC TGCCATGCTG GCCTCCCAGA CGGAAGCCTT CGTCCCCATC

     PheThrTyrGly GluLeuGln ArgMetGln GluLysGluArg SerLysGl
151  TTCACCTACG GCGAACTCCA GAGGATGCAG GAAAAGGAAC GGAGTAAAGG

     yGln
201  GCAAAAGAAA TCCCTGAGTG TATGGCAGAG GTCTGGGGAG GAAGGTCCTG

251  TAGACCCTGC GGAGCCCATC GAGGAAGAAG GAAACGAAAT GATCAAGCTG

301  ACTGCTCCTC TGGAAATTGG AATGAGGATG AACTCCAGAC AGCTGGAAAA

351  GTACCGGGCC GCCCTGGAAG GGCTGCTGAG TGAGATGCTT CCCCAGCACG

401  CAGCCAAGTG ACGGCCATGC TGGGGAGAAG GTGGACAGAT TTGGGAGGAC

451  TCTCCCGCCC AAGTGAGGCC CTGGGAATTT GCACAGCCTG CCAGCTGGGC

501  TTGGAAGAAT AACACCCTTT CCAAAGCAAA TCCCCCTCCA GCAAATAAAG

551  CATGAAATAT AC
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Fig. 1. The nucleotide sequence of monkey prepromotilin. The amino acid sequence of motilin is underlined.

Fig. 2. Multiple alignment of motilin precursor from several species. The most conserved region between species is indicated in dark background. Amino acids are numbered relative to the motilin peptide start site.

respectively. The cDNA sequences were confirmed by six independent sequences from six different PCR products. All gave identical results and the complete nucleotide sequence of the cDNA encoding the monkey motilin precursor is given in Fig. 1.

Analysis of the cDNA sequence of 562 bp revealed an open reading frame of 345 bp initiated at the 5′ end by AUG. The 5′ untranslated region consists of 63 bp. The stop codon TGA is located at position 408 and is followed by 154 bp from the 3′ untranslated region. The polyadenylation signal sequence AATAAA is located in nucleotides 544–549, 13 bp before the poly(A) tail.

The amino acid sequence deduced from the cDNA sequence indicates that the monkey motilin precursor consists of 115 amino acids. Like in other motilin precursors, the sequence starts with a 25 amino acid signal peptide followed by the 22 amino acid bioactive motilin peptide and a 68 amino acid MAP (Fig. 2).

As one may expect, the monkey motilin precursor shares good homology with the human precursor: 92% in the signal peptide, 96% in motilin and 94% homology in the MAP, while the correspondence with the rabbit precursor is somewhat less: 88%, 77% and 27% respectively.

The signal peptide is very hydrophobic, as in other peptides, and in line with the function of this part of the molecule. The signal peptide cleavage site is located at Ala^{25}. There are two amino acid differences between the signal peptide of motilin from the monkey and from man. At position −11 and −10, Val and Ala (man) are substituted by Ala and Pro (monkey). There is one amino acid difference between human and monkey motilin. At position 19 Asp (man) is replaced by Ser (monkey) (Fig. 2). As the C-terminal part of motilin does not contribute to the bioactivity, this substitution is probably functionally not important [16]. As in other motilin precursors, two Lys residues adjacent to the C-terminus form the promotilin cleavage site [4]. The function of the resulting MAP remains unknown. In monkey it has the same length as in man, 68 amino acids, and there is considerable homology with only four amino acid differences between monkey and human motilin at 45, 48, 73 and 75, where Glu, Gly, Arg and Ala in monkey MAP are substituted by Arg, Glu, Pro, Thy in man. Comparison of all known MAP sequences (Fig. 2) confirms that there are three potential structural and functional regions in the MAP [5]. The first one is the Lys-Lys dibasic cleavage site where promotilin processing occurs. The second one is a PEST site, rich in proline, glutamic acid, serine and threonine. The third one consists of a 22 amino acid region, which is well conserved in human, monkey, porcine, rabbit, sheep dog and even in chicken [17]. This region may be responsible for the post-translational processing of motilin.

3.2. Motilin mRNA expression in monkey brain tissues

Daikh et al. [9] were unable to demonstrate motilin mRNA in extracts from the monkey brain, but it is unlikely that this was due to the fact that they used a cRNA probe derived from the sequence in man. First of all, they succeeded to demonstrate monkey mRNA in the intestine, secondly in the region they used to construct their probe the homology between monkey and man is 96%. Probably their negative results are due to the fact that the expression on motilin in the brain is low compared to the intestine, as is suggested by the recent data obtained by Depoortere et al. [13]. These authors, using total RNA from brain extracts, performed RT-PCR aimed at amplifying part of the motilin precursor and identified the product by subcloning and sequencing. We used a similar approach and isolated total RNA from three brain

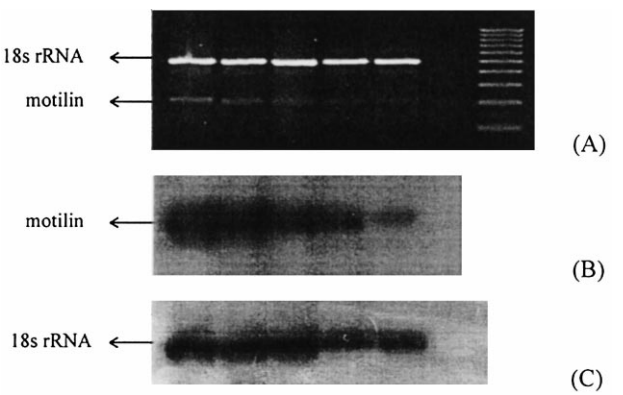

Fig. 3. Demonstration of the presence of motilin mRNA by RT-PCR. A: Agarose gel electrophoresis of amplified cDNA from duodenum (lane 1), antrum (lane 2), hypothalamus (lane 3), hippocampus (lane 4), cerebellum (lane 5), negative control (lane 6), size marker (100 bp ladder, Gibco BRL, New York, USA) (lane 7). 495 bp 18s rRNA served as internal standard. Motilin products correspond to the 197 bp band. B: PCR products from A were blotted to a nylon membrane and hybridized with a ^{32}P-labeled probe (monkey5′.rev3) that directed against part of the amplified sequence. C: Hybridization with a human 18s rRNA cDNA probe. Filter was exposed to x-film for 1 h.

regions and performed multiplex RT-PCR using primers based on the monkey motilin precursor nucleotide sequence and primers for part of 18s rRNA cDNA which served as an internal standard. Compared to other RT-PCR normalization standards such as GAPDH and β-actin, the expression of 18s rRNA is more stable in most tissues, including CNS and GI tract. The target sizes for motilin and 18s rRNA were 197 and 495 bp respectively. A series of RT-PCRs was performed with a different number of cycles (32, 35 and 38), in order to gather data within the exponential phase of the amplification (data not shown). Analysis of the PCR products on agarose gel revealed the desired band which hybridized with a monkey oligo probe at 50°C. The filter was exposed to x-film overnight at −80°C for autoradiography. The filter was then hybridized with 18s rRNA cDNA probe at 68°C and exposed to x-film for 1 h. Motilin expression was detected in monkey brain tissues, with the highest level in the hypothalamus, the lowest in the cerebellum (Fig. 3). However, compared with duodenum and antrum, motilin expression in brain tissues is very low, especially in cerebellum as was also the case in man and rabbit. Our data are also in agreement with evidence obtained by radioimmunoassay and immunohistochemistry showing the presence of motilin itself in the brain of several species.

In summary, we have identified prepromotilin from monkey, found that it has a high homology with human motilin, and demonstrated its presence in the monkey brain.

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