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Primary structure, tissue distribution, and biological activity of chicken motilin receptor ☆

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ABSTRACT

Motilin is a peptide hormone involved in gastrointestinal motility. GPR38, initially cloned as an orphan receptor, is now considered a specific receptor for motilin. Previously, molecular characterization of the motilin receptor had only been performed in mammalian and fish species. In this study, we cloned cDNA for chicken motilin receptor from the duodenum and characterized its primary structure, tissue distribution, and biological activity. The cDNA encoded 349 amino acids showing significant overall sequence identity to mammalian motilin receptors. Chicken motilin increased intracellular Ca^{2+} concentration in human embryonic kidney (HEK) 293 cells transiently expressing the recombinant chicken motilin receptor. Comparison of the cDNA sequence with the genomic sequence of chicken motilin receptor revealed that the chicken motilin receptor gene consists of two exons separated by an intron. Real-time PCR analysis showed that chicken motilin receptor mRNA is expressed in a wide range of tissues in 21-day-old chickens, with markedly high levels in the proventriculus, duodenum, and oviduct. The expression levels of the mRNA in the proventriculus and duodenum were highest just before hatching and rapidly decreased during post-hatch development. These results suggest that chicken motilin receptor is largely involved in gastrointestinal functions at pre- and post-hatch periods through an intracellular signaling pathway accompanied by an increase in Ca^{2+} levels.

1. Introduction

Motilin is a 22 amino acid polypeptide that stimulates gastrointestinal motility (Poitras et al., 1994). This peptide has been identified in many mammalian species but not in rodents such as rats and mice. Motilin binding sites are present in the mammalian gastrointestinal tract and central nervous system, along with a G-protein-coupled receptor, GPR38, which has been identified as the motilin receptor (MTL-R) (Feighner et al., 1999; Dass et al., 2003). GPR38/MTL-R (referred to as MTL-R) was initially identified as an orphan receptor belonging to growth hormone secretagogue receptor (GHS-R) family that includes an orphan receptor GPR39 (McKee et al., 1997; Holst et al., 2004). GHS-R has recently been demonstrated to be a ghrelin receptor (Kojima et al., 1999). These receptors have a common seven transmembrane (TM) structure and mediate G-protein-activated intracellular signaling.

The GHS-R family genes are conserved in non-mammalian species. In pufferfish (*Spheroides nephelus*), three distinct genomic clones (75E7, 78B7, and 1H9) have been found to encode proteins of significant identity to the human GHS-R (Palyha et al., 2000), of which 75E7 and 78B7 are considered orthologous to human MTL-R and GHS-R, respectively. Genes encoding MTL-R and GHS-R have also been identified in zebrafish (Olsson et al., 2008). In chicken, we have identified GHS-R1a and its variant form (GHS-R1aV) by cDNA and genomic cloning (Tanaka et al., 2003; Geelissen et al., 2003). Recently, we cloned chicken GPR39 cDNA (Yamamoto et al., 2007). Although motilin has been purified from the small intestine of chickens (De Clercq et al., 1996) and its cDNA has been cloned (Huang et al., 1999), its receptor has not yet been identified in any avian species. It has been shown that ghrelin and motilin have contractile activities in the gastrointestinal tract and that their receptors, together with GPR39, are present in the gastrointestinal tract of chickens (Kitazawa et al., 1997; Tanaka et al.,

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2003; Geelissen et al., 2003; Yamamoto et al., 2007). To understand the regulatory mechanisms of gastrointestinal motility, comparative studies on the structure, tissue distribution, and function of GHS-R family members are important.

In the present study, we report the amino acid sequence and biological activity of recombinant chicken MTL-R and expression patterns of its mRNA in chicken tissues.

2. Materials and methods

2.1. Animals

Fertilized white leghorn eggs were purchased from a commercial source and incubated under standard conditions. After hatching, the chicks were grown for 3 weeks in an electric brooder and further in an unheated brooder. Food and water were given *ad libitum*. All procedures were performed in accordance with NIH guidelines regarding the principles of animal care. After decapitation, tissues were removed immediately and frozen in liquid nitrogen.

2.2. Cloning of cDNA for chicken motilin receptor

Total RNA was extracted from the duodenum of each 21-day-old chicken using TRIzol (Invitrogen, Carlsbad, CA). The amount of the RNA was measured by spectrophotometry. The RNA samples were subjected to electrophoresis in a 1% agarose gel containing 2.2 M formaldehyde, and the quality of the RNA were verified by visualization of ribosomal RNAs by ethidium bromide staining of the gel. Poly A^+ RNA was prepared from the total RNA using Oligo-Tex dT30 Super (Takara, Shiga, Japan) according to the manufacturer's instructions. A cDNA library was prepared from the poly A^+ RNA using the Marathon cDNA amplification kit (Clontech, Mountain View, CA) and a fragment of chicken MTL-R cDNA was amplified using primers 1 (sense) and 2 (antisense), designed from the predicted sequence for chicken MTL-R mRNA (GenBank Accession No. XM_425630). The 5′- and 3′-ends of the cDNA were amplified by PCR according to the manufacturer's instructions using chicken MTL-R-specific primers 3 (antisense) and 4 (sense), respectively, and an adapter primer supplied by the manufacturer. PCR was performed for 30 cycles of 98 °C for 10 s and 68 °C for 60 s with PrimeSTAR HS DNA polymerase with GC buffer (Takara), and 0.2 μM each of primers. The amplified fragment was cloned into pCR-Blunt II-TOPO vector (Invitrogen). Sequencing was carried out with the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) and the ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

2.3. Construction of the expression vector for chicken MTL-R

The coding region of chicken MTL-R cDNA was amplified from the duodenum cDNA library by PCR using PrimeSTAR HS DNA polymerase with GC buffer (Takara) and primers 1 (sense) and 2 (antisense), the primers of which included the cleavage sites for the restriction enzymes HindIII and XhoI, respectively. PCR was carried out as described above. The cDNA was cloned into pcDNA3.1/Hygro(+) expression vector (Invitrogen).

2.4. Functional analysis of chicken MTL-R

Human embryonic kidney 293 (HEK293) cells were cultured in DMEM containing 10% fetal bovine serum at a density of 1×10^5 cells in a collagen-coated 6 cm diameter dish for 24 h. The expression vector (1 μg) described above was transfected with FuGENE-6 (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. Twenty-four hours after transfection, the cells were plated onto a collagen-coated 96-well black plate at a density of 3×10^4 cells per well. Twenty hours after plating, cultured medium was aspirated, and 100 μl of a fluorescent dye solution containing 4.4 μM Fluo-4AM (Invitrogen), 1% fetal bovine serum, 0.045% pluronic acid (Sigma-Aldrich, St. Louis, MO) in a working buffer consisting of 1× Hank's BSS (Gibco-BRL), 20 mM HEPES buffer containing 250 μM probenecid (Sigma-Aldrich) was loaded onto each well. The plate was incubated for 1 h at 37 °C in a CO_2 incubator. After washing with working buffer three times, 100 μl of the designated concentration of synthetic chicken motilin (FVPFFTQSDIQKMQEKERNKGQ), human motilin (Peptide institute, Osaka, Japan), or chicken ghrelin-26-C8 (Kaiya et al., 2002) in working buffer containing 0.001% Triton X-100 was automatically added to the well using the $FLIPR^{tetra}$ system (Molecular Devices, Menlo Park, CA). Intracellular Ca^{2+} changes were measured by excitation at 488 nm and emission at 500–560 nm using $FLIPR^{tetra}$.

2.5. Reverse transcription (RT) and quantitative real-time PCR (Q-PCR) analysis

Total RNA (5 μg) extracted from various tissues of chicken was reverse-transcribed at 50 °C for 60 min in 20 μl of reaction mixture containing 200 units of Superscript III Reverse Transcriptase (Invitrogen), 0.5 mM dNTPs, 10 mM dithiothreitol, 50 μM random primers, and 1× first-Strand buffer, supplied by the manufacturer. After the inactivation of the reverse transcriptase by heating at 70 °C for 15 min, the cDNA product was subjected to Q-PCR performed according to the user instructions for the Real-time PCR system 7500 (Applied Biosystems). PCR was performed at 95 °C for 15 s and 60 °C for 35 s in 25 μl buffer containing 1× Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) and 0.2 μM each of primers 5 (sense) and 6 (antisense). Quantitative measurement was performed by establishing a linear amplification curve from serial dilutions of chicken MTL-R cDNA.

2.6. Statistical analysis

All data were analyzed by one-way ANOVA, and the significance of the F-value obtained was confirmed by Tukey's post hoc test. All analyses were performed using the GraphPad Prism (GraphPad Software, San Diego, CA). Statistical significance was achieved at $P < 0.05$.

3. Results

3.1. cDNA and genomic structure of chicken MTL-R

Chicken MTL-R cDNA was cloned from the duodenum cDNA library. The cDNA consisted of 44 bp of 5′-untranslated region (UTR), 1050 bp of coding region, and 580 bp of 3′-UTR (Fig. 1). A potential polyadenylation signal was present near the 3′ end. A deduced 349 amino acid sequence of chicken MTL-R displayed high overall sequence identity with MTL-R of rabbits (65%), humans (59%), pufferfish (55%) and chicken GHS-R1a (53%). The sequence displayed relatively low identity with chicken GPR39 (27%). On comparing the amino acid sequence of chicken MTL-R with that of mammalian MTL-R, the seven-TM region was found to be conserved in chicken MTL-R with relatively high identity to corresponding regions in rabbit and pufferfish MTL-R (Fig. 2). Chicken MTL-R had a large gap at the extracellular second loop region compared with other receptors. Comparison of the sequence of chicken MTL-R cDNA to its genomic DNA in the chicken genome sequence (NW_001471554) revealed the presence of an approximately 2.1 kb intron between positions 795 and 796 (Fig. 1). The exon–intron boundaries conformed to the GT-AG rule for splicing (data not shown). A phylogenetic tree analysis showed chicken MTL-R to be most similar to mammalian MTL-R (Fig. 3).

3.2. Functional analysis of chicken MTL-R

To examine whether the identified cMTL-R was functional, the effects of chicken motilin, human motilin, and chicken ghrelin-26-C8 on intracellular Ca^{2+} levels were examined using HEK293 cells transiently expressing chicken MTL-R (Fig. 4). Chicken motilin and human motilin increased intracellular Ca^{2+} concentrations in a dose-dependent manner in chicken MTL-R-expressing cells. The EC_{50} of chicken motilin and human motilin were 1.80 and 23.00 nM, respectively. No effect was observed in chicken ghrelin-28-C8.

3.3. Expression profile of chicken MTL-R mRNA in chicken tissues

Expression levels of chicken MTL-R mRNA in the tissues of chickens were examined by Q-PCR. In 21-day-old chickens, expression of the mRNA was observed in a wide range of tissues including pituitary, brain, thymus, bursa of fabricius, liver, kidney, bone marrow, proventriculus, duodenum, oviduct, ovary, and testis (Fig. 5). The highest level of expression was observed in the proventriculus and moderate levels of expression were found in the duodenum, oviduct and testis. Expression levels in other tissues were low. In 360-day-old hens, MTL-R mRNA was detected in all regions of the oviduct at the following levels (copies/ng ± SEM): infundibulum, 0.049 ± 0.034; magnum, 0.264 ± 0.178; isthmus, 0.116 ± 0.068, uterus, 0.062 ± 0.021; vagina, 0.037 ± 0.016. There was no significant difference among the expression levels of each region.