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Goldfish Ghrelin: Molecular Characterization of the Complementary Deoxyribonucleic Acid, Partial Gene Structure and Evidence for Its Stimulatory Role in Food Intake

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Abstract Complementary deoxyribonucleic acid (cDNA) encoding goldfish preproghrelin was identified using rapid amplification of the cDNA ends (RACE) and reverse transcription (RT)-polymerase chain reaction (PCR). The 490 bp cDNA encodes a 103 amino acid preproghrelin which has a 26 amino acid signal peptide region, 19 amino acid mature peptide and a 55 amino acid C-terminal peptide region. The mature peptide region of goldfish ghrelin has two putative cleavage sites and amidation signals (GRR); one after 12 amino acids and the other after 19 amino acids. The serine (S) in the second amino acid position in the "active core" of ghrelin is substituted with threonine (T). The goldfish ghrelin gene has four exons and three short introns and resembles the human ghrelin gene. Ghrelin messenger RNA (mRNA) expression was detected in the brain, pituitary, intestine, liver, spleen and gill by RT-PCR followed by Southern blot analysis, and in the intestine by Northern blot. Intracerebroventricular (ICV) injection of n-octanoylated goldfish ghrelin (1-19) stimulates food intake in goldfish.

Introduction

Growth hormone secretagogues (GHS) are synthetic compounds that stimulate GH secretion (1) by binding to the growth hormone secretagogue receptor (GHS-R). Ghrelin, the first known endogenous ligand of GHS-R, is an acylated gut/brain peptide originally isolated from the rat stomach (2). The first four amino acids "GSSF", with the *n*-octanoyl group in the third residue (serine), is considered to be the "active core" of the peptide (3). Ghrelin cDNA has been identified from several species including human (2), rat (2), mouse (4), and the bullfrog (5). Ghrelin gene structure has been characterized in humans (6), rat and mouse (4). Ghrelin mRNA expression has been detected mainly in the brain and stomach of rat (1) and in the stomach of bullfrog (4). Recently, ghrelin and GHS-R mRNA distribution was demonstrated in human brain, intestine and several peripheral tissues including the liver and spleen (7). Ghrelin stimulates growth hormone (GH) secretion in mammals (2,5) and in bullfrog (4). Ghrelin stimulates food intake in rats (8,9).

GHS-R has been identified in pufferfish and it has a high similarity with the mammalian GHS-R (10). GHS, KP-102 stimulates GH secretion in tilapia (11). Goldfish is a well characterized model for studying the neuroendocrine regulation of GH secretion and food intake in non-mammalian vertebrates. The aim of this study was to identify the structure of ghrelin cDNA in the goldfish, to detect ghrelin mRNA expression in the brain and peripheral tissues, and to elucidate the role of native ghrelin peptide on food intake.

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Materials and Methods

Animals. Male and female goldfish (*Carassius auratus*) of the common or comet varieties and approximately 40 g body weight, were purchased from the Mount Parnell Fisheries (Marcersberg, PA, USA) and maintained under a simulated natural photoperiod of Edmonton, Alberta, Canada. Fish were anesthetized in 0.05% tricainemethane sulfonate (MS-222; Syndel Laboratories, Vancouver, BC, Canada) before decapitation and dissection of the brain and other tissues. All protocols were approved by the University of Alberta, Department of Biological Sciences Animal Care Committee.

Cloning and sequence analysis. Total RNA was extracted using the Trizol reagent (Invitrogen Life Technologies, Canada) from the goldfish intestine. RT and 3' RACE were carried out as described previously (12). To isolate the 3' end of the goldfish ghrelin cDNA, two degenerate primers, GRN1 (5'GTCAGYGCWGGYTCCAGCTTC3') and GRN2 (5'GGYTCCAGCTTCYTSAGCC3') were designed based on the sequences of mammalian ghrelin and trout ghrelin sequence (Kaiya H and Kangawa K, personal communication). The desired product was purified from the gel, subcloned and sequenced as described earlier (12). 5' RACE was conducted using gene specific primers GRN3 (AATCATTTAATGGTTTATA 3') and GRN4 (5'GCTGAATCTTGTAGAACTTT3') based on the 3' sequence. PCR for full-length cDNA was conducted with the gene specific primers GRLF (5'CTGTGCATTCTGCATACATATTTGAG3') and

GRLR (5'GTTTTGGAAGATTATTACATC3'). The conditions for PCR were 5 minutes of denaturation at 95^{0}C, followed by 35 cycles of denaturation at 95^{0}C, annealing at 45^{0}C and extension at 73^{0}C. To clone the ghrelin gene, PCR was conducted using 100 ng genomic DNA under the same conditions mentioned above

RT-PCR, Southern blot and Northern blot analyses. Ghrelin expression in different regions of the brain (olfactory bulbs and tracts, telencephalon, hypothalamus, midbrain and hindbrain), pituitary, intestine, liver, spleen, muscle, gill, heart, kidney, eye, testis and ovary was detected by RT-PCR using the same primers and PCR conditions used for cloning the full length ghrelin cDNA. The reactions were electrophoresed on a 1.5% agarose gel and transferred to a Hybond nylon membrane by capillary transfer. Hybridization with a [α-^{32}P]dCTP labeled probe covering the full length ghrelin cDNA was performed using methods described earlier (12). Membranes were exposed to a Phosphorscreen (Molecular Dynamics, Sunnyvale, CA) for 1 hour and the screen was scanned using a PhosphorImager 445 SI (Molecular Dynamics) and analyzed using the IMAGEQUANT software (Molecular Dynamics). PCR for β-actin was conducted as an internal control. For Northern blot, 40μg of total RNA was extracted from various brain regions described above and the intestine, electrophoresed on a denaturing agarose gel (1.5%) with formaldehyde, transferred to a membrane and hybridized as described earlier, except in this case the screen was exposed for 7 days. To check the integrity of the RNA used for the Northern blot, membranes were stripped and reprobed with an [α-^{32}P]dCTP labeled partial cDNA probe for goldfish β-actin (unpublished results, GenBank accession number AF079831).

ICV injections and goldfish ghrelin. Goldfish ghrelin (1-19) with an n-octanoyl modification in the third residue (serine) of the mature peptide was synthesized using the solid phase (9-fluorenylmethoxycarbonyl) strategy at the Salk Institute. ICV injections were conducted as described earlier (12). A 5 μl microsyringe was stereotaxically placed in the posterior preoptic region of the brain and 2 μl of the test solution [saline, 1 or 5 ng/g body weight (BW) of goldfish ghrelin (1-19)]administered. Following the ICV injection and closure of the head wound, fish were returned to their aquarium and recovered from anesthesia within 5 minutes. Food pellets were administered 15 minutes (including the recovery time) after the ICV injection and cumulative food intake for each fish measured for 1 h.

Statistical analysis. Statistical analysis was conducted using ANOVA followed by Student-Newman-Kuels multiple comparison test. Significance was considered at P<0.05. Data are expressed as mean ± SEM.

Results

A 490 bp preproghrelin cDNA was identified from the intestine and the brain (Figure 1; GenBank accession number AF454389). The deduced structure of preproghrelin peptide contains 103 amino acids. The signal peptide region is 26 amino acids in length, predicted by the SignalP server. The mature peptide region begins immediately after the signal peptide. There are two putative cleavage and amidation signals (GRR) after 12 amino acids and after 19 amino acids. The region after the mature peptide to the stop codon (C-terminal peptide) has 55 amino acids. Goldfish ghrelin gene has three short introns and four exons (sequence not shown, GenBank accession number AF454390, Figure 2). Exon 1 (205 bp) contains the 5' untranslated region (UTR) and the region encoding the first 12 amino acids of mature peptide region. Exon 2 (79 bp) encodes the remaining portions of the mature peptide and a part of the C- terminal peptide. Exon 3 (109 bp) encodes a part of the C-terminal peptide. Exon 4 (95 bp) encodes the terminal region of the C-terminal peptide and the 3' UTR. The introns are 129 bp (intron 1), 96 bp (intron 2) and 267 bp (intron 3) in length. RT-PCR followed by Southern blot analysis detected ghrelin mRNA

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 91        ctgtgcattctgcatacatatttgagacttttaaagtgcagccattcagagtgttgtcgtaaaacagaactaaaccaggtgacttcccagg -1

  1 ATG CCT CTG CGT CGT CGT GCC AGC CAC ATG TTT GTG CTC TTA TGT GCT CTT TCC TTG TGT GTT GAG TCT GTG AAA  75
  1 M   P   L   R   R   R   A   S   H   M   F   V   L   L   C   A   L   S   L   C   V   E   S   V   K    25

 76 GGT GGC ACC AGC TTC CTC AGC CCT GCT CAG AAA CCA CAG GGT CGA AGG CCA CCC CGG ATG GGC AGA AGA  GAT GTT 150
 26 G   G   T   S   F   L   S   P   A   Q   K   P   Q   G   R   R   P   P   R   M   G   R   R    D   V    50

151 GCA GAG CCA GAG ATC CCA GTG ATT AAA GAG GAT GAC CAG TTC ATG ATG AGT GCT CCG TTC GAA CTG TCT GTG TCT  225
 51 A   E   P   E   I   P   V   I   K   E   D   D   Q   F   M   M   S   A   P   F   E   L   S   V   S    75

226 CTG AGC GAG GCG GAG TAT GAG AAA TAT GGT CCT GTT CTG CAG AAG GTT TTG GTC AAT CTT CTT GGC GAT TCG CCA  300
 76 L   S   E   A   E   Y   E   K   Y   G   P   V   L   Q   K   V   L   V   N   L   L   G   D   S   P    100

301 CTT GAA TTC TGA caagagctaaaagttctacaagattcagctccttataaaccattaaaatgtttgtaaagtagaaatgatgtaataatcttccaa 396
101 L   E   F   *                                                                                        104

397 aac                                                                                                  399
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Figure 1. Nucleotide sequence of the goldfish preproghrelin cDNA. The mature peptide region is underlined. The two cleavage sites and amidation signals are boxed.

expression in the telencephalon, hypothalamus, intestine, spleen, and gill (Figure 3). Northern blot analysis detected ghrelin mRNA expression only in the intestine (Figure 4). ICV administration of goldfish ghrelin (1-19) stimulated food intake. A dose of 1ng/g bodyweight and 5 ng/g bodyweight ghrelin stimulated food intake significantly, compared to the unhandled and saline treated fish (Figure 5).

Discussion

The amino acid sequence of goldfish ghrelin (1-19) has 47%, 36% and 31% identity with the human, rat and bullfrog ghrelin, respectively. The "active core" (GSSF) of ghrelin is conserved in mammalian species whereas in the bullfrog it is "GLTF". In goldfish this region is "GTSF". The substitution of the second amino acid to threonine from serine is due to a single base substitution (AGC to ACC). Both serine and threonine are functionally similar and the substitution likely does not affect the structure and biological function of the goldfish ghrelin. The presence of two cleavage sites and amidation signals suggests the presence of two forms of the peptide, a short form which is 12 amino acids in length and a long form that is 19 amino acids in length. Both peptides retain the "active core" intact. Similarly, prepro-orexin contains two amidation signals and can be processed into two peptides, orexin-A and orexin-B, that are biologically active (13). We do not know the identity of the *n*-acyl modification in goldfish ghrelin. However, the goldfish ghrelin (1-19) we synthesized is n-octanoylated, the most commonly found modification of ghrelin. Bullfrog has both octanoylated and decanoylated ghrelin which are biologically active (5). Synthetic ghrelins with other acyl modifications were also found to be biologically active (14).

The intron:exon organization of the goldfish ghrelin gene resembles the human ghrelin gene organization. Goldfish ghrelin gene has short introns compared to the introns in mammalian ghrelin genes (4,6). All intron:exon junctions conform to the GT/AG rule of splicing. Recently, a non-coding 19 bp exon in the 5' UTR of the mouse and rat ghrelin genes was reported. In goldfish, we did not find this short exon.

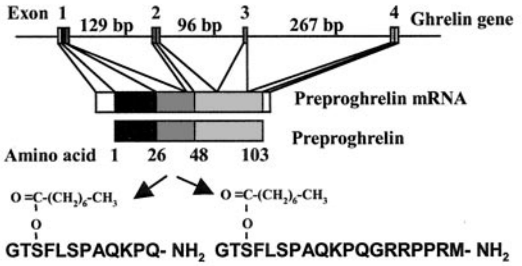

Figure 2. Proposed model of the formation of n-octanoylated ghrelin mature peptides from the ghrelin gene. The exons are represented by boxes and introns are represented by thin lines.

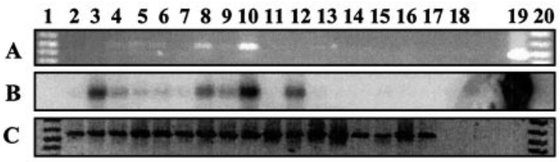

Figure 3. (A) RT-PCR analysis of the tissue distribution of ghrelin mRNA in the brain and peripheral tissues of goldfish. The different lanes represent the following: 1 and 20, molecular weight markers; 2, olfactory bulbs and tract; 3, telencephalon; 4, hypothalamus; 5, midbrain; 6, posterior brain; 7, pituitary; 8, intestine; 9, liver; 10, spleen; 11, muscle; 12, gill; 13, heart; 14, kidney; 15, eye; 16, testis; 17, ovary; 18, negative control; 19, positive control. **(B)** Southern blot on the same gel using a ^{32}P-labeled probe for full length ghrelin **(C)** β-actin PCR as an internal control for all cDNAs used for the distribution PCR.

Recently, wide distribution of ghrelin and GHS-R mRNAs in the brain and peripheral tissues has been demonstrated in humans, suggesting the involvement of ghrelin in vital physiological functions (7). From RT-PCR followed by Southern blot analysis, and Northern blot analysis the highest expression of goldfish preproghrelin mRNA was found in the intestine. Similar results have been obtained from rats (2) and the bullfrog (5). We repeated the Northern blot several times with total RNA and poly(A)$^+$ RNA from the brain regions, but no signal was found. The results of our mRNA expression studies suggest that intestine may be the primary site of ghrelin production in the goldfish. However, our RT-PCR studies show ghrelin expression in the brain. The physiological significance of strong ghrelin mRNA expression in intestine, spleen and liver is unknown. Brain ghrelin may be involved in the central regulation of food intake which is indicated by the present results.

A significant increase in food intake after the ICV administration of *n*-octanoylated ghrelin (1-19) suggests that the synthetic peptide is biologically active. It supports our hypothesis that native ghrelin is an orexigenic factor in goldfish. The increase in food intake after ghrelin administration is comparable to the increase in cumulative food intake for 1 hour after the ICV administration of

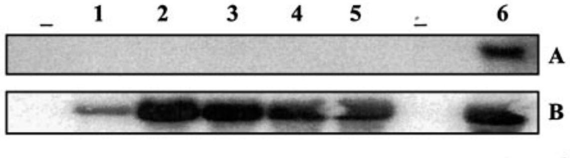

Figure 4. (A) Northern blot showing the distribution of ghrelin mRNA in the brain and intestine of goldfish. The different lanes represent the following: 1, olfactory bulbs and tracts; 2, telencephalon; 3, hypothalamus; 4, midbrain; 5, hindbrain; and 6, intestine. **(B)** The membrane was stripped and reprobed with the β - actin probe.

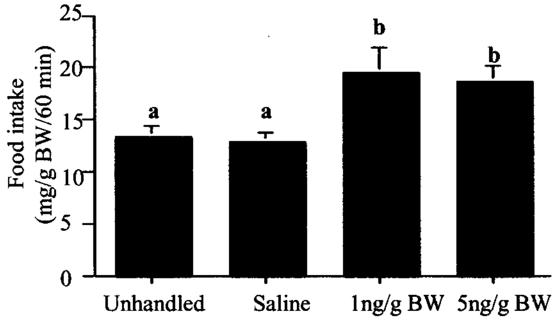

Figure 5. Effects of ICV injection of goldfish ghrelin (1-19) on food intake. Fish were unhandled (n = 12), saline injected (n = 8) or injected with 1ng/g BW (n = 8) or 5 ng/g BW (n = 8) goldfish ghrelin (1-19). Values with different letters are significantly different ($p<0.05$).

human ghrelin (unpublished observations from our lab). ICV administration of other orexigenic peptides including NPY, orexin, and galanin also stimulates food intake in goldfish (15). It has been demonstrated that an appetite regulatory mechanism similar to that in mammals exists in fish (16).

In summary, we report for the first time, the ghrelin gene structure and its orexigenic action in a non - mammalian vertebrate. This is the first report of a ghrelin peptide with "GTSF" in the active core and with two putative cleavage sites and amidation signals. Our results suggest that ghrelin structure and functions are highly conserved during evolution.

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