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Analysis of expression and structure of the rat *GH-secretagogue/ghrelin receptor* (*Ghsr*) gene: Roles of epigenetic modifications in transcriptional regulation

Hiroshi Inoue [a,b,*], Yukiko Sakamoto [b], Natsumi Kangawa [b], Chizuko Kimura [b], Tsutomu Ogata [c], Kenji Fujieda [d], Zhi Rong Qian [e], Toshiaki Sano [e], Mitsuo Itakura [b]

[a] *Diabetes Therapeutics and Research Center, The University of Tokushima, Tokushima, Japan*
[b] *Division of Genetic Information, Institute for Genome Research, The University of Tokushima, Tokushima, Japan*
[c] *Department of Endocrinology and Metabolism, National Research Institute for Child Health and Development, Tokyo, Japan*
[d] *Department of Pediatrics, Asahikawa Medical University, Asahikawa, Japan*
[e] *Department of Human Pathology, Institute of Health Biosciences, The University of Tokushima Graduate School, Tokushima, Japan*

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ABSTRACT

In the current study, to elucidate the molecular basis of cell type-specific expression of the *GH-secretagogue/ghrelin receptor type 1A* (*GHSR1A*), we characterized the structure and putative promoter region of the rat *Ghsr* gene. We identified an alternative 5′-untranslated first exon that contains multiple transcription start sites, and confirmed a 200-bp sequence proximal to this exon to be sufficient for basal promoter activity. A promoter-associated CpG island conserved across different species was found to be hypomethylated in *Ghsr1a*-expressing cell lines, while being heavily methylated in non-expressing cells. In cells with low or absent *Ghsr1a* expression, treatment with demethylating agents activated *Ghsr1a* transcription. Chromatin immunoprecipitation assays demonstrated *Ghsr1a*-expressing cells to display active histone modifications, whereas repressive modifications were present exclusively in other cell types. These results suggest epigenetic modifications at *GHSR* to play important roles in determining *GHSR1A* expression and abundance, and therefore the consequent sensitivity of cells to ghrelin.

1. Introduction

The growth hormone (*GH*)-*secretagogue/ghrelin receptor* (GHSR) is a member of the ghrelin receptor subfamily within class A of rhodopsin-like G protein-coupled receptors (GPCRs), originally discovered as the target of a family of small synthetic molecules, GH secretagogues (GHSs), that are capable of stimulating GH release from the pituitary (Howard et al., 1996; Smith et al., 1997). Later, ghrelin, an *n*-octanoylated gastric peptide of 28 amino acids, was identified as the natural ligand of this receptor (Kojima et al., 1999). There is now emerging evidence that ghrelin acts through GHSR to exert pleiotropic effects, which include not only the induction of GH secretion but also stimulation of appetite, regulation of energy metabolism, control of gastro-intestinal motility, cardiovascular and hemodynamic effects, as well as having anti-inflammatory and immunomodulating properties (van der Lely et al., 2004; Kojima and Kangawa, 2005; Davenport et al., 2005; Tritos and Kokkotou, 2006; Dixit and Taub, 2005). On the other hand, it has also been proposed that at least some of the effects of ghrelin are likely to be mediated through an as yet uncharacterized,GHSR-independent pathway (Baldanzi et al., 2002; Toshinai et al., 2006; Thielemans et al., 2007).

GHSR mRNA is predominantly expressed in the hypothalamus and pituitary (Howard et al., 1996), consistent with the important role of ghrelin in regulating GH release. On the other hand, lower endogenous expression has also been documented in other central and peripheral tissues (Gnanapavan et al., 2002; Zigman et al., 2006; Sun et al., 2007). The widespread distribution of *GHSR* may support and correlate with the diversity of ghrelin–GHSR system functions. However, it should be noted that several conflicting findings have been reported regarding *GHSR* expression and distribution in normal and tumor tissues, as well as in cell lines;

Abbreviations: 5-aza-dC, 5-aza-2-deoxycytidine; ChIP, chromatin immunoprecipitation; ERK1/2, extracellular signal-regulated kinase 1/2; DMEM, Dulbecco's modified Eagle's medium; DNMT, DNA methyltransferase; EST, expressed sequence tag; FBS, fetal bovine serum; GH, growth hormone; GHS, growth hormone secretagogue; GHSR, growth hormone secretagogue receptor; GPCR, G protein-coupled receptor; HDAC, histone deacetylase; IP1, inositol monophosphate; IP3, inositol triphosphate; MAPK, mitogen activated protein kinase; MSP, methylation-specific PCR; PMSF, phenylmethylsulfonyl fluoride; qRT-PCR, quantitative real-time PCR; RACE, rapid amplification of cDNA ends; RLM, RNA ligase-mediated; SP-analog, [D-Arg[1], D-Phe[5], D-Trp[7,9], Leu[11]]-Substance P; TM, transmembrane; TSA, Trichostatin A; TSS, transcription start site; UCSC, University of California, Santa Cruz; UT, untranslated.

* Corresponding author at: Division of Genetic Information, Institute for Genome Research, The University of Tokushima, Kuramoto 3-18-15, Tokushima 770-8503, Japan. Tel.: +81 88 633 9483; fax: +81 88 633 9484.
E-mail address: hinoue@genome.tokushima-u.ac.jp (H. Inoue).

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thus, caution is needed in their interpretation. These inconsistencies might be attributable to the use of different experimental techniques with variable sensitivities, but could also be linked to the presence of a variant *GHSR* transcript, designated *GHSR1B* (Howard et al., 1996; Gnanapavan et al., 2002; Jeffery et al., 2005). *GHSR1A*, the primary full-length gene transcript, produced by splicing of the two coding exons, encodes a typical GPCR with a seven-transmembrane (7TM) domain core. In contrast, the intron is not removed from *GHSR1B*; therefore, an alternative stop codon and a polyadenylation signal within the intron are used to produce a C-terminal truncated, non-functional GPCR form that is unable to bind to GHSs (Howard et al., 1996). Recent studies have, however, demonstrated that GHSR1B might modulate the function of GHSR1A or other 7TM GPCRs by interfering their cellular expressions and ligand binding properties (Chan and Cheng, 2004; Chu et al., 2007; Leung et al., 2007; Takahashi et al., 2006), yet the precise roles of GHSR1B under normal physiological condition are not well understood. In addition, several rodent and human cell lines reportedly express *GHSR* mRNA; however, in many of these cells, the level of *GHSR1A* expression is apparently too low to exert ligand-induced GHSR1A activation (Murata et al., 2002; Thielemans et al., 2007; Adams et al., 1998). Nevertheless, these cells have been shown to retain some ability to respond to ghrelin, providing evidence for the existence of an alternative ghrelin receptor.

For many plasma membrane receptors including GPCRs, their density on the cell-surface is finely controlled by various transcriptional, post-transcriptional and post-translational mechanisms, and is often a determinant of overall receptor function in a cell. To date, the transcriptional regulation and molecular basis for tissue and cell type-specific expression of *GHSR* have not yet been fully elucidated. There have been relatively few studies, in a limited number of species including human, chicken and fish (Kaji et al., 1998; Petersenn et al., 2001; Tanaka et al., 2003; Yeung et al., 2004), characterizing the 5′-flanking region of the *GHSR* gene. Previous studies of the human *GHSR* gene have shown that it contains a TATA-less promoter, similar to most other GPCR promoters, and suggested an alternative splicing in the 5′-untranslated (5′-UT) regions (Kaji et al., 1998; Petersenn et al., 2001). These studies also demonstrated that the minimal promoter is mapped to within a relatively small segment (~300-bp) of the 5′-flanking region of *GHSR*. In addition, sequence comparison of human and fish *GHSR* promoters identified putative binding sites for shared transcription factors, such as AP-1, NF-1, Oct-1 and USF (Yeung et al., 2004); however, the functional significance of these transcription factors in transcriptional regulation awaits further investigation.

Herein, we report data on the structural and functional characterization of the 5′-flanking region of the rat *Ghsr* gene. In addition, we demonstrated a highly restricted expression of *Ghsr1a* among endocrine cell lines of rodent origin, and replicated previous findings that original and purified RC-4B/C rat pituitary tumor cell lines express a high level of functional Ghsr1a (Falls et al., 2006; Perdonà et al., 2011). Furthermore, using newly established RC-4B/C subclones with either a high or a low level of *Ghsr1a* mRNA, we investigated cell type-specific expression and transcriptional regulation of *Ghsr1a*. Our results indicate that epigenetic changes through DNA methylation and chromatin/histone modifications make significant contributions to determining the level of *Ghsr1a* transcription.

2. Materials and methods

2.1. Cell culture

All cell lines used in this study are listed in Supplemental Table 1. Rat RC-4B/C (pluripotential), GH3 (somatotropic), MMQ (lactotropic), AR42J (pancreatic acinar), L6 (myoblastic), and murine AtT20 (corticotropic) cells were purchased from American Type Culture Collection (Rockville, MD, USA). Rat MtT/S (somatotropic), murine αT3-1 (gonadotropic) and MIN6 (insulinoma) cells were kindly provided by Professors Kinji Inoue (Saitama University), Pamela Mellon (University of California at San Diego) and Junichi Miyazaki (Osaka University), respectively. Human embryonic kidney (HEK) 293A cells were obtained from Invitrogen (Tokyo, Japan). GH3, MMQ and MtT/S cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/F-12 (1:1) supplemented with 10% horse serum, 2.5% fetal bovine serum (FBS) and antibiotic solution (100 U/ml penicillin and 100 μg/ml streptomycin). The remaining cell lines were cultured in high glucose (HG)-DMEM medium containing 10% FBS and antibiotic solution. Cell culture medium, serum and antibiotics were purchased from Invitrogen. All cell lines were certified to be mycoplasma-free by the suppliers, and maintained in a standard humidified incubator at 37 °C and 5% CO_2. To obtain RC-4B/C subclones, the parental cells were counted and plated in a limiting dilution titration in four 96-well cell culture plates (Iwaki, Tokyo, Japan). Clones established from single cells were transferred to new plates for expansion and used for gene expression analysis as described below.

2.2. Animal tissues

All animal experiments were approved by the Animal Research Ethics Committee at the University of Tokushima. Rat tissues (hypothalamus, pituitary, heart and liver) from male Fischer 344 (F344; F344/DuCrlCrlj) rats of three different ages (3, 6 and 18 months; $n = 4$) were purchased from Charles River Inc. (Yokohama, Japan). Tissues were frozen at −80 °C until needed. Each tissue was divided into two equal parts, one each for RNA and DNA extraction, before being processed separately.

2.3. RNA isolation, conventional and real-time RT-PCR

Isolation of total RNA from cell lines and tissues was performed using an RNeasy Mini Kit (QIAGEN, Tokyo, Japan) following the manufacturer's protocol including the on-column DNase digestion step. To remove residual genomic DNA, purified RNA was further treated with Turbo-DNase (Ambion, Austin, TX, USA), using a starting amount of up to 10 μg of RNA in a 50 μl reaction. Subsequently, first-strand cDNA was synthesized from 100 ng of total RNA using a SuperScript III First-Strand Synthesis System (Invitrogen). Negative control reactions without reverse transcriptase (RT-minus) were included to monitor genomic DNA contamination in the template RNA. The resulting 2 ng of cDNA (assuming 100% conversion of RNA to cDNA during the reverse transcription step) were amplified using AmpliTaq Gold 360 Master Mix (Applied Biosystems, Foster City, CA, USA) and standard buffer conditions. The reactions were performed in a 20 μl volume for each well on a MicroAmp 96-well reaction plate using the GeneAmp PE 9700 thermal cycler (Applied Biosystems). RT-PCR primers were designed to amplify the following rat target genes: *Ghsr1a*, *Ghsr1b*, *Gh1* (encoding growth hormone), *Prl* (prolactin), *Tshb* (thyroid-stimulating hormone-β subunit), *Lhb* (luteinizing hormone-β subunit), *Fshb* (follicle-stimulating hormone-β subunit), *Pomc2* (proopiomelanocortin-β or Pomc2), *Cga* (glycoprotein hormone [GSU]-α subunit), *Pou1f1* (Pit-1), *Aff4* (AF5q31) and *Gapdh* (a housekeeping gene). Primer sets were selected to span at least one intron of the genomic sequence whenever possible, to ensure that the RT-PCR products were from cDNA and not genomic DNA. PCR conditions were optimized and the linear amplification range was determined for each primer set by varying the annealing temperature and cycle number. Primer sequences and optimized conditions are summarized in Supplemental Table 2. All PCR experiments