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Growth Hormone Secretagogue Receptor Family Members and Ligands

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We have previously reported the cloning and characterization of a new orphan G-protein-coupled receptor (GPC-R), the growth hormone secretagogue receptor (GHS-R), and shown that this receptor mediates the activity of the growth hormone–releasing peptides (GHRPs) and nonpeptide ligands such as L-692,429 and MK-0677. Because the GHS-R obviously does not belong to any of the known GPC-R subfamilies, we searched for GHS-R family members by screening a human genomic library using low-stringency hybridization and screening a Pufferfish genomic library. The Pufferfish was selected because of its compact genome. From the human genomic library, a homolog, GPR38, with 52% identity to the GHS-R was isolated. From the Pufferfish library, three family members were isolated. The Pufferfish gene having 58% identity to the GHS-R, on expression in HEK293 cells, was activated with GHRP-6 and MK-0677. These results indicate that the GHS-R has been conserved for at least 400 million years and that the Pufferfish genome is appropriate for isolation of GHS-R family members. In our search for endogenous ligands for the orphan receptors GHS-R and GPR38, we showed that adenosine is a partial agonist of the GHS-R and that motilin is the endogenous ligand for GPR38. We also confirmed that the endogenous ligand ghrelin is a full agonist of the GHS-R.

Key Words: Growth hormone secretagogue receptor; motilin; adenosine.

Introduction

There remain many important issues to be resolved concerning the biologic role of the growth hormone secretagogue receptors (GHS-Rs) and the identity of endogenous ligands for these receptors. Because the GHS-R appears to be the first of a new family of G-protein-coupled receptors (GPC-Rs) *(1)*, we wished to identify GHS-R family members and investigate the molecular evolution of the GHS-R. We initially focused on the isolation of GHS-R family members by searching expressed sequence tag (EST) databases and screening a human genomic library. It soon became evident that identification of GHS-R family members by the latter approach was an enormous task. We reported the isolation of three GHS-R homologs (GPR38, GPR39, and FM3) from a human genomic PAC library *(2,3)*. However, screening this library provided positive hybridization signals for 325 PAC clones *(2)*. The relatively low percentage of coding sequences in the human genome complicates characterization of GHS-R family members from this number of positive signals. To simplify the process, we evaluated the potential of using the Pufferfish, a vertebrate organism with a compact genome. The Pufferfish was selected because it is evolutionarily distant from human and homologous genes are likely conserved because of their functional importance. Our objectives were first to determine whether the orphan GHS-R had been evolutionarily conserved from Pufferfish to humans, and, second, to determine whether this vertebrate species would provide a shortcut for identifying new GHS-R family members. To identify natural ligands for the GHS-R and family members, we developed high-volume screening assays. The assays were developed such that they would be robust enough for identifying compounds in crude tissue extracts.

In this review we describe the identification of human GHS-R homologs in the Pufferfish. We also report the identification of adenosine as an endogenous ligand of the GHS-R and motilin (MTL) as the ligand for the closest GHS-R family member, GPR38.

Human GHS-R Homolog in Pufferfish

Teleost fish have the specialized functions of higher vertebrates; hence, genes necessary for complex functions are present in their genome. The genome of the Japanese Pufferfish (*Fugu rubripes*) had been proposed by Brenner et al. *(4)* to be a compact model genome for vertebrates. The Pufferfish genome (400 Mb) has approximately the same

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number of genes as the mammalian genome, but it has very little repetitive DNA and about 90% of the DNA is unique and lacks pseudogenes *(4)*. A comparison of the genomic structure of Pufferfish genes with their mammalian homologs indicates that the intron-exon organization has been conserved; but the intron size is generally smaller (60–120 bp) and intergenic sequences are greatly reduced *(5–7)*. Similarities between Pufferfish and mammalian genes have already been established *(8,9)*, but about 20% of the Pufferfish genome contains coding sequences compared with <3% in the human genome. This compactness facilitates gene isolation and characterization.

A Pufferfish (*Spheroides nephelus*) PAC genomic library (Genome Systems, St. Louis, MO) was hybridized with complete open reading frame (ORF) probes for GHS-R and GPR38. Three clones, 78B7, 75E7, and 1H9, were isolated and sequenced, and the predicted peptide sequences of the three clones are, respectively, 58, 47, and 41% identical to the human GHS-R *(10)*. The similarities in gene structure and close relationships of the deduced peptide sequences of the three Pufferfish clones to human GHS-R and the human MTL receptor (huMTL-R or GPR38) are illustrated in Fig. 1. Clones 78B7, 75E7, and 1H9 were FLAG tagged and engineered into the expression plasmid pcDNA-3.1. Following transfection into HEK-293/aeq17 cells, expression of each of the three proteins was confirmed by Western analysis taking advantage of the FLAG epitope. The cells were treated with coelenterazine and tested for a bioluminescent response following the addition of various ligands including MK-0677 and growth hormone–releasing peptide-6 (GHRP-6). MK-0677 and GHRP-6 caused an increase in bioluminescence in cells that expressed clone 78B7 but not in cells expressing 75E7 and 1H9. Higher concentrations of GHRP-6 and MK-0677 were needed to activate 78B7 compared to the human GHS-R (Fig. 2).

However, the different activities are not surprising considering that the ligands are synthetic and the receptors are separated by 400 million years of evolution. Figure 3 illustrates the similarities and differences between the deduced protein sequence of human GHS-R and Pufferfish 78B7. Remarkable conservation is evident in the transmembrane (TM) helices. Key amino acid residues, such as E124 in TM3, that have been shown by site-directed mutagenesis to be essential for activation by GHRP-6 and MK-0677 are conserved in the Pufferfish clone 78B7 *(10,11)*. Conservation of this receptor for at least 400 million years reinforces the notion that the GHS-R has potential importance in endocrinology and physiology and suggests that the endogenous ligand has also been evolutionarily conserved.

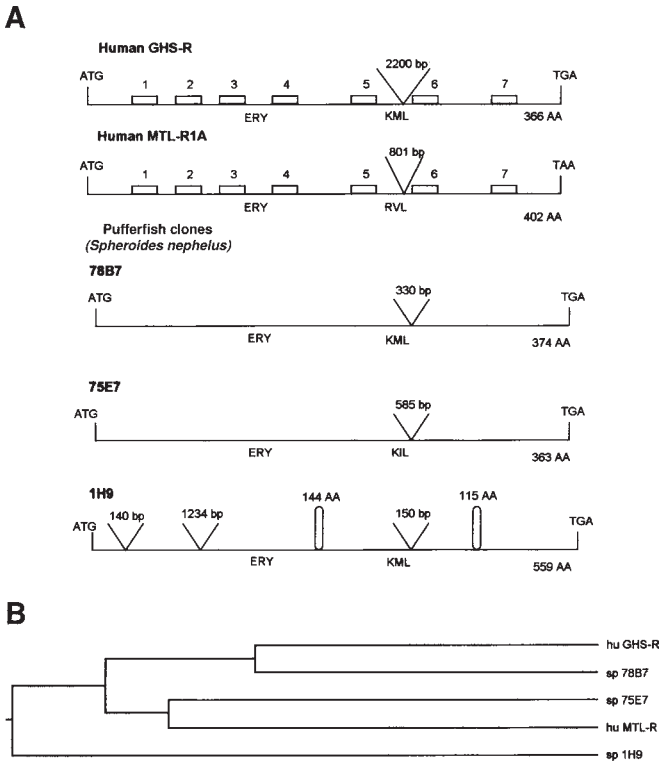

Fig. 1. Gene structure of *S. nephelus* clones and their relationship to human GHS-R and GPR38. (From ref. *10* with permission.) **(A)** Schematic representation of Pufferfish GHS-R related genes in comparison with GHS-R and GPR38 (FM-1) with several notable features highlighted. E/DRY motif responsible for G-protein binding is shown. Putative TM regions numbered from 1 to 7 are represented as open boxes. The intron location and amino acids present at exon/intron boundary following TM-5 is noted. For 1H9, predicted extended loop domains are shown by open ovals. **(B)** Schematic comparison of the relationships, which are based on alignments using the Pileup program (Wisconsin Package Version 9.1, Genetics Computer Group [GCG]), Madison, WI; gap extension 4, gap creation 12). The GAP alignment program (GCG) was utilized to determine amino acid identity for the complete protein or corresponding TM helices. Sequences utilized with GenBank, EMBL, or SWISSPROT database accession numbers were huGHS-R1a, human type 1a growth hormone secretagogue receptor (U60179); human GPR38 (lAF034632); Pufferfish clones 78B7 (AF082209), 75E7 (AF082210), and 1H9 (AF082211).

Identification of Endogenous Ligands for GHS-R in Hypothalamic Extracts

The GHS-R is abundantly expressed in the hypothalamus; therefore, this tissue was selected as a source of the GHS-R endogenous ligand. Pig hypothalami from a slaughter-house were frozen, lyophilized and extracted with boiling 1 *M* acetic acid. To assay for GHS-R ligand activity, we generated an HEK293 cell line stably expressing the GHS-R receptor and aequorin (GHS-R-HEK293/aeq17). In this cell line, binding of a GHS-R agonist induces inositol triphosphate–coupled mobilization of intracellular Ca^{2+} to produce a bioluminescent response in the presence of coelenterazine. To test specificity, positive fractions were assayed in the presence of the GHS-R antagonist L-765,867 *(12,13)*. Displacement of ^{35}S-MK-0677 from GHS-R was used as a secondary assay to determine whether active compounds occupied the same binding site as MK-0677.

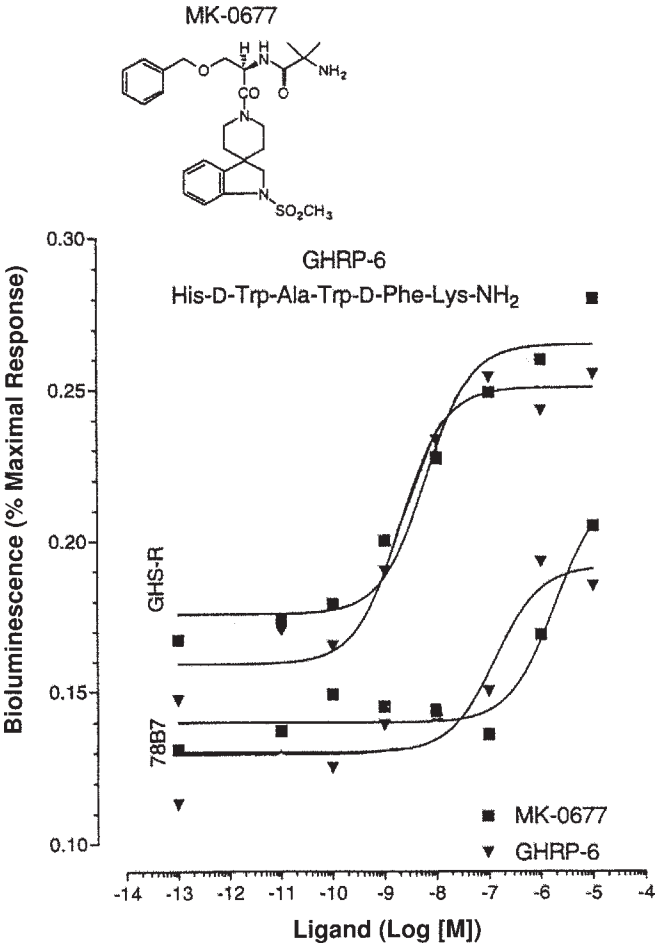

Fig. 2. Dose-response curves for the activation of 78B7 and the human GHS-R by GHRP-6 and MK-0677. (Adapted from ref. *10* with permission.) HEK-293-AEQ17 cells (8×10^5 cells) were transfected by the lipofectamine procedure with a contiguous ORF encoding 78B7 in the expression plasmid pcDNA-3. The apoaequorin in the cells was charged for 4 hours with coelenterazine ~40 h after transfection. In a 96-well plate assay format, 5×10^4 cells were then injected into the test plate containing MK-0677 and GHRP-6, and the integrated light emission was recorded over 30 s followed by an injection of lysis buffer (0.1% final Triton X-100 concentration) recorded over 10 s. The "fractional response" values for each well were calculated by taking the ratio of the integrated response to the initial challenge to the total integrated luminescence including the Triton X-100 lysis response.

The acid-soluble fraction of the hypothalamic extract was applied to a Sep-Pak chromatography column and eluted with 10, 40, and 90% acetonitrile (MeCN). The fraction eluted by 40% MeCN contained a compound(s) that behaved as a partial agonist on the GHS-R. This activity was inhibited by L-765,867. After purification by reverse-phase high-performance liquid chromatography, the active component was identified by time lag–focusing matrix-assisted laser desorption/ionization and mass spectroscopy (MS)/MS as adenosine. Another fraction contained activity that was not explained by adenosine; however, the concentration was too low to allow identification of the active component.

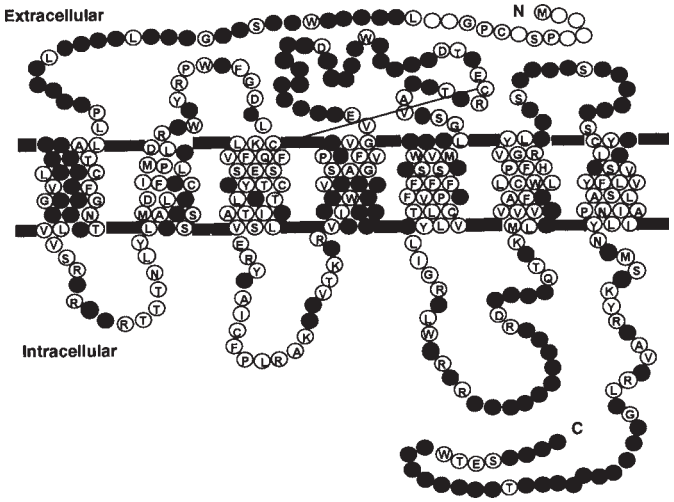

Fig. 3. Homology between human GHS-R and Pufferfish 78B7. (From ref. *10* with permission.) Conserved (white circles) and different (shaded circles) amino acid residues in the deduced protein sequences are illustrated by a schematic representation of receptor TM topology (N-terminus, extracellular; C-terminus, intracellular). To equalize spacing in the N-terminal domain, empty circles denote residues present in the human GHS-R and absent in the Pufferfish 78B7 protein.

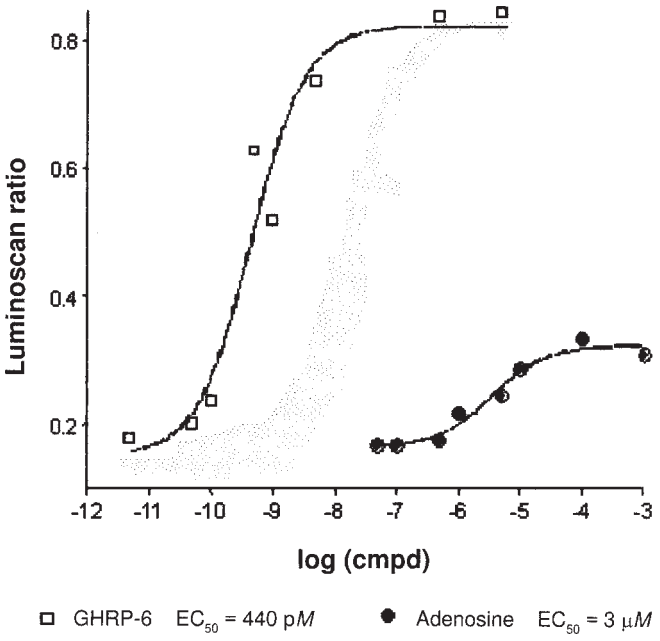

Fig. 4. Adenosine, in contrast to GHRP-6, is a partial agonist of the GHS-R. GHS-R-HEK-293-aeq17 cells were charged for 4 h with coelenterazine. In a 96-well plate assay format, approx 5×0^4 cells were then injected into the test plate containing GHRP-6 (0.05 n*M* to 10 μ*M*) and adenosine (50 μ*M* to 3 m*M*). The integrated light emission was recorded over 30 s followed by an injection of lysis buffer (0.1% final Triton X-100 concentration) recorded over 10 s. The fractional response values for each well were calculated by taking the ratio of the integrated response to the initial challenge to the total integrated luminescence including the Triton X-100 lysis response.

Experiments with authentic adenosine confirmed that it was indeed a GHS-R agonist. In contrast to GHRP-6 and MK-0677, adenosine behaved as a partial agonist on porcine and human GHS-R expressed in HEK293/aeq17 cells. Figure 4 illustrates the partial agonist activity of adenosine compared to GHRP-6. When GHS-R-expressing cells were pretreated with either adenosine or MK-0677, the cells could not be activated by either ligand illustrating cross-desensitization. These results provide additional evidence that both ligands are GHS-R agonists. However, in contrast to GHRP-6 and MK-0677, adenosine was ineffective in competing with ^{35}S-MK-0677 binding. Hence, adenosine activates the GHS-R through a site different from that occupied by MK-0677 and GHRP-6 *(39)*. Tullen et al. also identified adenosine as an agonist of the GHS-R *(40)*. However, in contrast to our findings, these investigators identified adenosine as a full rather than a partial agonist. This discrepancy might be related to the different cell lines used to express GHS-R stably.

Identification of the Natural Ligand for GHS-R Family Member 1 (GPR38)

We recently described the cloning of GPC-Rs related to the GHS-R from a human genomic DNA library *(2)*. The deduced amino acid sequence of one of these clones, GPR38, has 52% identity and 73% similarity to the human GHS-R. The TM domains show 73–89% similarity. GPR38 has a gene structure similar to that of the GHS-R and contains a single intron of ~1 kb. As with the GHS-R, Northern blot analysis was too insensitive to detect GPR38 expression in tissues. Using RNase protection assays, we demonstrated GPR38 expression in human stomach, thyroid, and bone marrow. Attempts to isolate cDNA clones by standard library screening proved unsuccessful. However, a combination of RACE and reverse transcriptase polymerase chain reaction (RT-PCR) techniques resulted in the identification of two spliced forms for GPR38 similar to that observed for the GHS-R *(1)*. GPR38a mRNA encodes a polypeptide of 412 amino acids with seven predicted alphahelical TM domains, typical of GPC-Rs, whereas GPR38b encodes a 363 amino acid polypeptide with the predicted TM1–5 domains but lacking TM6 and TM7.

To identify a ligand for this orphan GPC-R and to determine whether the full-length seven TM domain GPR38a is a functional GPC-R, a high-throughput assay was used in which GPR38a was expressed in HEK293/aeq17 cells. Expression of GPR38a in cell membranes was confirmed using epitope-tagged protein, which revealed a single protein species of *Mr* ~ 45,000. Three sets (>500 compounds/set) of known peptide and nonpeptide molecules were tested at a single concentration in transiently transfected HEK-293/aeq17 cells (100 n*M* peptide, 1 µ*M* non-peptide). Significant bioluminescent responses (greater than four-fold over background) were recorded for the peptide motilin (MTL) *(13)*. Titration of the activity showed that the half-maximal effective concentration (EC_{50}) for human/porcine MTL was 2.2 n*M*. Direct radioligand binding studies with ^{125}I-human MTL on cell membranes prepared from GPR38a cDNA transfected cells showed that expression of GPR38a confers high-affinity specific binding to two classes of binding sites (Kd_1=0.1 n*M*, Kd_2= 0.9 n*M*; B_{max1}=240 fmol/mg protein, B_{max2} = 520 fmol/mg; *see* Fig. 5). Both binding sites are strongly G-protein coupled (>80% inhibition of binding with 100 n*M* GTPγS). The rank order of potency of several MTL peptide analogs in the GPR38a functional and binding assays correlates with their reported potency measured by in vitro contractility assays *(13)*.

MTL was isolated from porcine intestine in 1972; since then the receptor has been sought in humans and other species. High-affinity MTL binding sites were detected in the gastrointestinal (GI) tract and in the central nervous system (CNS), but their molecular nature remained elusive *(14)*. MTL is highly conserved across species and is synthesized as part of a larger pre-prohormone. The gene structure illustrated in Fig. 6 shows that the mature 22 amino acid MTL is encoded by exons II and III and is generated by removal of its secretory leader peptide and cleavage at the first C-terminally located dibasic prohormone convertase recognition site *(15–18)*. Radioligand binding, autoradiography and in vitro biossays have shown high-affinity (KD ~ 2 n*M*) and low-density (B_{max} ~20 fmol/mg of protein) MTL binding in smooth muscle cells of the GI tract of humans, cats, and rabbits *(14,19–22)*. High-affinity MTL binding was also described in the cerebellum, supporting the notion that MTL may also act in the CNS *(14)*.

The distribution profile of GPR38 mRNA was examined in a panel of GI and non-GI human tissues by RNase protection assay. GPR38 mRNA expression is highly tissue specific, and using this sensitive assay could be detected only in whole stomach (most prominently), thyroid, and bone marrow *(13)*. Further analysis of human GPR38 expression using mRNA extracted from specific regions of the GI system revealed GPR38 transcripts in esophagus, ileum, and jejunum. *In situ* hybridization to the human GI tract demonstrated GPR38 expression in a subset of interstitial cells in the duodenum, jejunum, and colon. In the colon, most GPR38-positive cells were nitric oxide synthase positive, and a small number also stained positive for acetyl-choline transferase. Acetylcholine is a major stimulatory neurotransmitter of the gut. This expression pattern suggests that MTL is prokinetic in the colon as it is at other levels of the human GI tract. In the duodenum, a subset of cells having an elongate appearance consistent with smooth muscle was also GPR38 positive *(13)*.

A high amino acid sequence identity (52% overall) between the MTL-R and GHS-R implies that their natural ligands might also be related. There may also be functional overlap between the two neuroendocrine pathways suggesting the existence of an endogenous ligand other than adenosine. According to this premise, we continually moni-

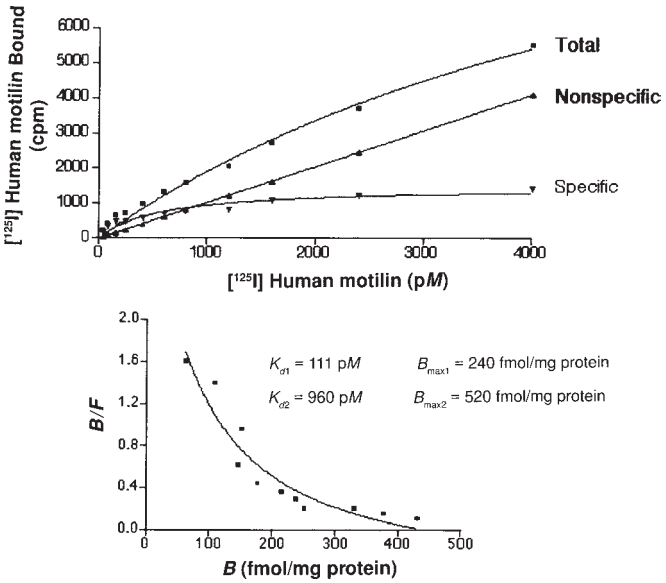

Fig. 5. Binding of [^{125}I] human motilin to crude membranes from HEK-293 cells transfected with the MTL-R1A cDNA. (Adapted from ref. *13* with permission.) **(Top)** [^{125}I]Motilin saturation isotherm: (■), total binding; (▲) nonspecific binding; (▼) specific binding. **(Bottom)** Scatchard analysis of [^{125}I]motilin binding (bound units are femtomoles per milligram of protein). Binding data were analyzed by a nonlinear curve-fitting program (Prism V, version 2.0; GraphPad Software, San Diego, CA). Results shown are the means (± SEM) of triplicate determinations.

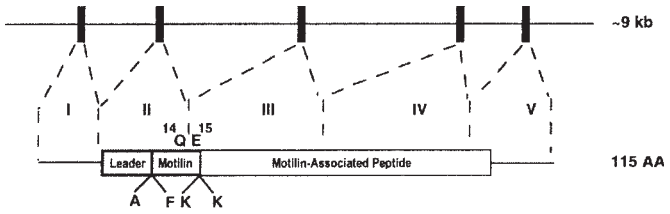

Fig. 6. Structural representation of the MTL gene illustrating processing of prepro-MTL to produce the mature peptide hormone *(15–18)*.

tored EST databases for MTL homologs. We identified an EST expressed in the stomach and pancreas that encoded a potential MTL homolog. Subsequently, the gene sequence was entered into GenBank. Like MTL, the small peptide had the unusual property that the few terminal amino acids of the predicted mature functional peptide were encoded in the relatively large second exon. The predicted MTL homologous peptide products were synthesized but were inactive when tested on the GHS-R-HEK293/aeq17 cells. Recently, Kojima et al. *(23)* identified an endogenous GHS-R ligand produced by the stomach. The most intriguing aspect of this exciting discovery is that the ligand is a 28–amino acid octanoylated peptide for which *o*-octanoylation on serine-3 is essential for biologic activity. These investigators cleverly named the ligand ghrelin *(23)*. Remarkably, ghrelin cDNA is identical to the MTL-like EST and gene just described. In contrast to adenosine, ghrelin stimulates GH release from pituitary cells in vitro and caused GH release when administered intravenously to anesthetized rats *(23)*. Ghrelin obviously is not related structurally to the synthetic GHRPs or to any of the nonpeptidyl ligands; therefore, it will be intriguing to map the binding pocket occupied by ghrelin.

Conclusions

The identification of three homologs of the human GHS-R in the Pufferfish establishes a new example of the value of the Tetraodontidae genome for the facile cloning of gene families. A fascinating aspect of the Pufferfish model is that gene regulatory elements are shared between *Fugu* and rat *(24)*. Our experiments have shown that the functional structure of the ligand-binding domain of the GHS-R has been conserved for at least 400 million years, implying that the endogenous ligand for the GHS-R has also been conserved. These observations strongly suggest that the GHS-R ligand plays a fundamentally important role in biology.

MTL has been identified as the endogenous ligand for GPR38, the closest GHS-R family member. MLT is produced in the stomach and is well known for its effect on gastric motility. Interestingly, GPR38 is also expressed in the bone marrow and thyroid. Its role in these tissues has yet to be defined. Intriguingly, the recently characterized endogenous ligand for the GHS-R, ghrelin, is also expressed in the stomach. Although the peptide sequences of MTL and ghrelin are not closely related, the gene structures of these ligands are remarkably similar. However, the site of production of ghrelin is surprising because the most clearly defined sites of expression of the GHS-R are in the anterior pituitary gland and brain *(25)*. Although expression of the GHS-R has been reported in a variety of peripheral tissues by RT-PCR, to date, its only demonstrated biologic function is stimulation of GH release from the anterior pituitary gland *(23)*.

Identification of adenosine as a partial agonist of the GHS-R is intriguing. This relationship might explain the reported beneficial roles of the peptide agonists on the heart *(26–31)*. For example, perhaps hexarelin action on the heart is mediated by an adenosine receptor. The EC_{50} for adenosine activation of the GHS-R (2 μ*M*) is similar to that required for activation of the adenosine receptor A2 subtype in the brain *(32,33)*; consequently, we cannot exclude the possibility that the GHS-R is a physiologic target for adenosine. Adenosine plays an important integrative effect on pathways regulated by dopamine and γ-aminobutyric acid *(32–34)*. During aging there is a decline in the capacity of hypothalamic neurons to secrete dopamine. A cytotrophic factor was isolated from the rat pituitary gland that was shown to increase the production of tyrosine hydroxylase in fetal hypothalamic cells and stimulate the secretion of catecholamines by dopaminergic neurons *(35,36)*. Subsequently, cytotrophic factor was identified as adenosine

(37). The GHS-R is expressed on dopaminergic neurons in the substantia nigra and ventrotegmental area *(25,38)*. Therefore, it is tempting to speculate that reductions in dopamine secretion by the aging brain might be a consequence of insufficient adenosine stimulation of dopaminergic neurons.

Acknowledgments

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