obestatin-like sequence is also found in avian ghrelin precursor protein, and it is possible that an obestatin-like peptide may be present in birds (Fig. 2). However, an avian obestatin-like peptide is predicted not to have the C-terminal amide structure that has been reported to be necessary for the putative biological actions of obestatin reported by Zhang et al. (2005). There is no evidence, at present, to suggest a biological action for obestatin in birds.

3. Changes in endogenous ghrelin

In mammals, plasma ghrelin levels increase in response to fasting and decrease with subsequent re-feeding (Toshinai et al., 2001). Associated with these changes, ghrelin mRNA expression in the stomach increases during fasting and decreases upon re-feeding, while ghrelin peptide content in the stomach decreases with fasting and increases after re-feeding (Toshinai et al., 2001). In humans, pre- and post-prandial changes in plasma ghrelin levels have also been reported (Shiiya et al., 2002). Together, these findings indicate that endogenous ghrelin acts to stimulate appetite in rodents and humans.

In adult Japanese quail, plasma ghrelin levels increase 5-fold after 24-h of fasting and decreases 3 h after re-feeding (Shousha et al., 2005). Similarly, in 6-day-old layer-type domestic hens, plasma ghrelin levels increase after 12-h fasting and return to control levels 6 h after re-feeding (Kaiya et al., 2007b). However, this observation was not confirmed in 3-week-old broiler chickens after 48-h of fasting (Richards et al., 2006). These differential responses may be due to different methodologies of plasma ghrelin measurement. However, in the chicken proventriculus, ghrelin mRNA levels increase in response to the food deprivation (Richards et al., 2006; Chen et al., 2007; Kaiya et al., 2007b), while ghrelin peptide content also increases in response to 12-h of fasting, returning to the control levels 6 h after re-feeding (Kaiya et al., 2007b). Similarly, hepatic ghrelin mRNA increases after fasting in a time-dependent manner, returning to control levels 12 h after re-feeding (Chen et al., 2007). In contrast, pancreatic ghrelin mRNA expression is depressed after 48-h of fasting and returns to control levels after re-feeding (Richards et al., 2006). In the chicken brain, ghrelin mRNA expression is not affected by fasting and re-feeding (Richards et al., 2006; Chen et al., 2007). These observations in Japanese quail and chickens at different ages indicate that endogenous ghrelin biosynthesis and release are stimulated in response to negative energy status caused by fasting. Furthermore, they suggest the possibility that peripheral ghrelin could act as a peripheral signal for stimulating food intake in birds. However, this prediction is not supported by reports of an anorectic effect of ghrelin administered peripherally to chickens (see below). Since the effects of ghrelin are mediated by GHS-R, it is possible that these apparently conflicting observations may be explained by differences in GHS-R expression.

4. The ghrelin receptor (GHS-R)

In mammals, two forms of GHS-R have been identified, a functional GHS-R1a and GHS-R1b, a truncated and inactive form of GHS-R1a (Soares and Leite-Moreira, 2008). Three forms of GHS-R have been reported in the chicken, GHS-R1a, GHS-R1aV (homologous to mammalian GHS-R1b) and GHS-Rtv, and are derived from alternative splicing of mRNA transcripts of the GHS-R gene (reviewed by Kaiya et al., 2007a, 2008). GHS-R1aV lacks a portion of trans-membrane domain-6 resulting from the deletion of 48 nucleotides (16 amino acids), and GHS-Rtv is a splice form of GHS-R. It has not known if GHS-R1aV and GHS-Rtv play a role in mediating any biological functions of ghrelin in birds.

In 3-week-old broiler chickens, a 48-h fast has been reported to have no significant effect on GHS-R1a mRNA expression in the proventriculus or brain, while pancreatic GHS-R1a mRNA tended to be depressed (Richards et al., 2006). Similarly, in 30-day-old broiler chickens, fasting has been found to have no effect on GHS-R1a mRNA expression in the hypothalamus (Chen et al., 2007). However, in these birds, GHS-R1a mRNA expression increased after fasting for 12 or 36 h, and decreased 12 h after re-feeding in the proventriculus and liver (Chen et al., 2007). Despite these conflicting observations, it is likely that fasting and re-feeding affect proventriculur and hepatic GHS-R1a mRNA expression and presumably the level of GHS-R1a activity. Of course, GHS-R1a mRNA expressed in the proventriculus, pancreas and liver, while not participating in feeding behavior may mediate other effects of ghrelin in peripheral tissues. To better understand how peripheral ghrelin affects food intake in birds, it will be necessary to investigate aspects of ghrelin signaling including presence of the ghrelin receptor in the vagus nerve as demonstrated in rodents (Soares and Leite-Moreira, 2008). It should be noted that in contrast to the chicken (Richards et al., 2006; Chen et al., 2007), in the rat, GHS-R mRNA expression is increased in the arcuate (although not in the ventromedial) nuclei in 48-h fasted rats (Nogueiras et al., 2004). This observation suggests that brain GHS-R1a transduces the elevated blood ghrelin signal in the fasted state to stimulate appetite in the rat, but not in the chicken.

5. Ghrelin and the regulation of food intake

The regulation of food intake in birds, especially domestic poultry, has been extensively investigated for over a half century with the goal of increasing production efficiency through understanding the neural and endocrine mechanisms involved. Recent findings indicate that many of the neuropeptides identified in mammals as playing a role in regulating food also have this function in birds (Furuse et al., 2008), although not all of these appetite-regulating peptides function the same way in birds and mammals (Table 1).

While the majority of peptides known to inhibit appetite in mammals have the same function in birds, less is known in birds

Table 1
Effects of feeding-regulatory peptides known in mammals on feeding regulation in chickens.

Stimulatory		Inhibitory	
AGRP	+, ne[a]	α-MSH	–
Galanin	ne	Amylin	–
Ghrelin	–	β-MSH	–
GHRH	–	Bombesin	–
MCH	ne	CART	–
Motilin	ne	CCK	–
NPY	+	CRF	–
Orexin A, B	ne	Gastrin	–
Visfatin[b]	+	GLP-1	–
		Leptin	–, ne[a]
		Neuropeptide FF	–
		Neuropeptide S	–
		Oxyntomodulin	–
		PACAP	–
		PrRP	+
		Urocortin	–
		Uroteinsin-I	–
		VIP	

Note: +, stimulation; –, inhibition; ne, no effect.
Abbreviations: AGRP, agouti-related peptide; GHRH, growth hormone-releasing hormone; MCH, melanophore-concentrating hormone; NPY, neuropeptide Y; MSH, melanophore-stimulating hormone; CART, cocaine- and amphetamine-regulated transcript peptide; CCK, cholecystokinin; CRF, corticotropin-releasing factor; GLP-1, glucagon-like peptide-1; PACAP, pituitary adenylate cyclase-activating polypeptide; PrRP, prolactin-releasing peptide; VIP, vasoactive intestinal peptide.
[a] Different effect is seen in broiler and layer chickens.
[b] Orexigenic effect of visfatin is only known in chickens.

about the peptides that stimulate appetite (Table 1). Some of the peptides that affect appetite in mammals either have no effect on regulating food intake in birds or produce the opposite effect. For example, prolactin-releasing peptide (PrRP) stimulates food intake in chicken but has an inhibitory effect in mammals (Tachibana et al., 2004) while ghrelin inhibits food intakes in chickens and stimulates it in mammals (Furuse et al., 2001).

5.1. Effects of exogenous ghrelin administered centrally

Ghrelin stimulates food intake when injected centrally in rodents (Wren et al., 2001) and goldfish (Miura et al., 2006, 2007). Subsequent studies revealed that the neuropeptides, NPY and orexin, mediate this effect in response to signaling through the ghrelin receptor (Toshinai et al., 2003; Mondal et al., 2005; Miura et al., 2006, 2007).

In birds, ICV injection of rat ghrelin in neonatal male broiler chicks inhibits food intake (Furuse et al., 2001). Further, ICV injection of a ghrelin mimetic, GHRP-6, also inhibits food intake in neonatal chicks (Khan et al., 2006). An inhibitory effect of chicken ghrelin on food intake in the chicken was first demonstrated by Saito et al. (2002a). Chicken ghrelin (0.1, 0.4, 1.6 and 3.2 nmol) administered by ICV injection suppresses food intake in 8-week-old adult broiler chickens in a dose-dependent manner, and this effect persists for 2–7 h depending on the dose (Chen et al., 2008). Similarly, in adult Japanese quail, food intake is inhibited for 12 h after an ICV injection (1 nmol) of rat ghrelin, and this effect is observed either during the dark (resting phase) or the light period (active phase) (Shousha et al., 2005).

Saito et al. (2002b) found that stepping and vocalization, which are characteristic of a hyperactive/agitated state in chicks, increased significantly following ICV injection of ghrelin. Such behavior is also observed after corticotropin-releasing factor (CRF) is injected ICV (Furuse et al., 1997a,b) and furthermore, ICV injection of CRF suppresses food intake (Zhang et al., 2001). It was therefore hypothesized that central ghrelin may exert its biological effects through the CRF system, which activates the hypothalamo-pituitary-adrenal (HPA) axis resulting in corticosterone (CORT) release from the adrenal glands. In support of this hypothesis, ICV injection of chicken ghrelin, in the neonatal chickens, increases plasma CORT levels, but CORT does not increase if a CRF receptor antagonist, astressin, was co-injected with ghrelin (Fig. 3A; Saito et al., 2005). Neuropeptide Y (NPY) plays a major role in mediating the orexigenic effect of ghrelin in rodents (Mondal et al., 2005). In bird, too, NPY is orexigenic (Ando et al., 2001) and it is therefore possible that ghrelin may affect hypothalamic NPY expression in birds, although exhibiting anorectic effects. However, this seems unlikely since, in neonatal chicks, hypothalamic NPY mRNA does not change 30 min after ICV injection of ghrelin (Saito et al., 2005). Furthermore, in the neonatal chick, hyperphagia induced in response to an ICV injection of NPY (50 pmol) is inhibited by co-injection of ghrelin (Fig. 3B; Saito et al., 2005). This strongly suggests that ghrelin does not activate NPY neurons in birds as it has been reported to do in rodents. Instead, in birds it appears that ghrelin activates CRF neurons and that CRF released from these neurons is involved in inhibiting food intake. However, it is also possible that other factors participate in decreasing food intake. For example, sleep-like behavior has been observed following ICV injection of ghrelin, and this effect may result in reduced food intake (Tachibana et al., 2001). Similar behavior is observed after ICV injection of glucagon-like peptide-1 (GLP-1) as well (Furuse et al., 1997a,b). Therefore, it is also possible that GLP-1 could play a role in mediating the inhibitory effect of ghrelin on food intake in birds. Clearly, further study is needed to clarify the central mechanism(s) of ghrelin action on food intake regulation in birds.

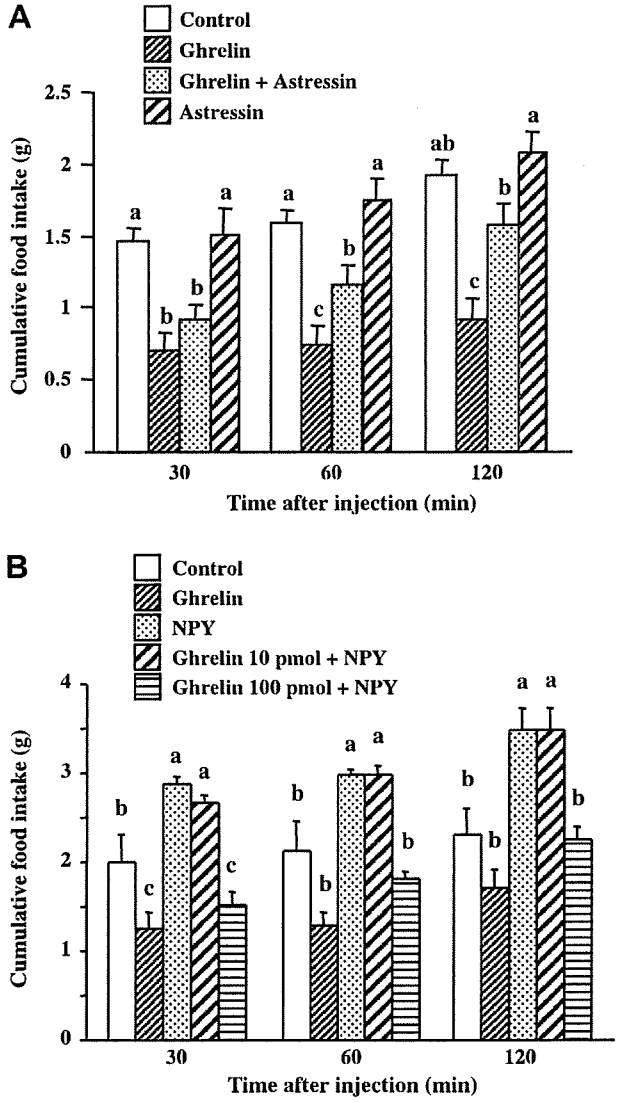

Fig. 3. Regulation of food intake by intracerebroventricular injection of chicken ghrelin in neonatal chicks. (A) Effects of a corticotropin-releasing factor (CRF) antagonist, astressin, on ghrelin-induced inhibition of food intake. (B) Effect of chicken ghrelin on NPY-induced hyperphagia. Reproduced from Saito et al. (2005). Inhibitory effect of ghrelin on food intake is mediated by the corticotropin-releasing factor system in neonatal chicks. Regul. Pept. 125, 201–208 with permission from Elsevier.

5.2. Effects of ghrelin administered peripherally

In mammals, ghrelin is the only known gut-derived hormone that stimulates food intake (Higgins et al., 2007); both intravenous (IV) and IP injection of ghrelin induces hyperphagia (Wren et al., 2001; Date et al., 2002).

In adult Japanese quail, IP injections of rat ghrelin at doses ranging from 0.5 to 1 nmol (0.4–0.9 nmol/100 g BW) increase food intake for 12 h (Shousha et al., 2005). However, injections of a very large dose of rat ghrelin (3 nmol, 2.4 nmol/100 g BW) suppress food intake for 12 h. These effects were observed during either the dark (resting phase) or the light period (active phase). In 7-day-old broiler chickens, IV injection of chicken ghrelin (1 nmol/100 g BW) weakly but significantly inhibits food intake for 2 h (Geelissen et al., 2006). On the other hand, no significant effect on food intake was observed after IV injection of chicken ghrelin (0.6 nmol/100 g BW) for 2 h in 8-day-old layer chickens (Kaiya

et al., 2007b). Thus, in birds, the effects of ghrelin administered peripherally on food intake observed to date are not consistent.

The mode of peripheral ghrelin action on food intake has been investigated in rodents (Date et al., 2002). Ghrelin-induced hyperphagia is negated by gastric or bilateral subdiaphragmatic vagotomy and peripheral injection of ghrelin decreases gastric vagal afferent activity. Ghrelin receptors are present in the vagal nodose ganglion and in afferent terminals (Date et al., 2002) and it appears that the peripheral ghrelin signals through these receptors to the CNS through vagal afferents. As a result, hypothalamic NPY and orexin neurons are activated through the noradrenergic pathway and hyperphagia is induced (Date et al., 2006).

It is yet unknown if a comparable signaling system is present in birds. The inhibitory effect of a high dose (3 nmol) of rat ghrelin on food intake, as observed in Japanese quail (Shousha et al., 2005), might be due to ghrelin passing through the blood–brain barrier and directly acting on NPY neurons located in arcuate nucleus of the hypothalamus (Banks et al., 2002). However, the mechanism through which lower doses of peripherally administered ghrelin stimulate food intake remains to be explained (Shousha et al., 2005; Geelissen et al., 2006; Kaiya et al., 2007b). Further study is required in birds to determine if activation of vagal afferents is involved in mediating the peripheral effects of ghrelin on regulating food intake.

6. Ghrelin and gastrointestinal (GI) motility

In rodents, ghrelin increases GI motility in conjunction with stimulating food intake (Peeters, 2005). It is thought that the increase in GI motility promotes the rapid transfer of food into the intestine thereby enhancing alimentary nutrient absorption. Since ghrelin inhibits food intake in chickens (Geelissen et al., 2006; Kaiya et al., 2007b), it is logical to predict that ghrelin inhibits GI motility in birds. However, there is no evidence for this action of ghrelin on the avian gut. Studies in vitro using guts from 2- to 4-week-old broilers chicks show that ghrelin (1 μM) stimulates contraction of the upper (esophagus and crop) and the lower (colon) parts of GI tract and has only a weak stimulatory effect on the middle part (proventriculus, duodenum and jejunum) of GI tract (Kitazawa et al., 2007). Khan et al. (2006) reported that ICV injection of a ghrelin mimetic, GHRP-6, does not affect food retention in the crop, proventriculus and gizzard of 5-day-old male layer chicks in vivo. These results suggest in birds, that ghrelin receptors are present in portions of the GI tract where they mediate stimulatory effects of ghrelin on gut motility.

7. Ghrelin and thermogenesis

In rodents, ghrelin plays a role in regulating energy balance by decreasing fat utilization without significantly changing energy expenditure or locomotary activity (Tschöp et al., 2000). There are only limited reports on involvement of ghrelin in the regulation of energy balance in birds. In adult Japanese quail, peripheral or central injection of ghrelin increases body temperature possibly by affecting energy expenditure (Shousha et al., 2005). In contrast, in 7-day-old broiler chickens, Geelissen et al. (2006) reported that 24 h after IV injection of chicken ghrelin there were no effects on thermogenesis, glucose, triglyceride, fatty acid, protein and tri-iodothyronine (T_3). However, they demonstrated that IV injections of chicken ghrelin (1 nmol/100 g BW) decreased the respiratory quotient (RQ) for 14 h (Geelissen et al., 2006). A reduction in RQ indicates lipolytic activity, suggesting that neonatal broiler chickens utilize lipid rather than glucose for energy production after ghrelin injection. This finding differs from observations in rodents where ghrelin injected subcutaneously increases the RQ and promotes lipid storage (Tschöp et al., 2000). The findings in quail and chickens suggest that ghrelin may affect energy expenditure in birds by promoting catabolic metabolism.

8. Conclusions

In this review, we summarized current knowledge of ghrelin and its potential roles in regulating different physiological functions in birds. Ghrelin plays an inhibitory role in the regulation of food intake in quail and chickens when injected centrally irrespective of age, sex, time of injection or doses tested. This contrasts with its stimulatory effect on food intake in mammals. As demonstrated in neonatal chickens, the CRF system may mediate the inhibitory action of ghrelin action on food intake in birds whereas in mammals it appears that the stimulatory effects of ghrelin on food intake are mediated by neuropeptides such as NPY and orexin. It is impossible to draw definitive conclusions about the effects of peripheral ghrelin in birds. Changes in endogenous ghrelin in the stomach and plasma suggest a possible action of ghrelin as an endogenous appetite stimulator in Japanese quail but not in chickens. Ghrelin receptor (GHS-R1a) expression in various peripheral organs is responsive to changes in feeding state, but it is not known if it is indirectly or directly associated with regulating feeding behavior. Portions of the GI tract contract in response to ghrelin administered in vitro, but it is not yet known if this response is related to the regulation of food intake or nutrient absorption. Ghrelin may regulate thermogenesis and energy balance in birds but the mechanism involved appears to differ from that in mammals.

Our knowledge of the functions and regulation of ghrelin in birds is still very limited and many fundamental questions remain to be answered:

1. Is endogenous ghrelin an anorexigenic hormone in birds? Why does ghrelin inhibit food intake when administered centrally to birds?
2. What site(s) within the brain are specifically targeted by ghrelin in birds?
3. What are responses to exogenous ghrelin administered peripherally?
4. What enzymes are involved in the post-translational processing of proghrelin involving proteolytic cleavage, acylation/deacylation, etc. and how do they regulate the function(s) of ghrelin in birds? What fatty acids are used to modify ghrelin and how do they affect ghrelin's function(s) in birds?
5. What is the function(s) of des-acyl ghrelin in birds?
6. Is obestatin produced from preproghrelin and what role, if any, does it have in birds?
7. What are the roles of the ghrelin receptors identified in birds? Are there other receptors that specifically bind ghrelin gene-derived peptides in birds?
8. How might ghrelin function in wild birds that exhibit behaviors retained in domesticated poultry such as seasonal changes in appetite or incubatoryor migratory behaviors?

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Ghrelin receptor (GHS-R)-like receptor and its genomic organisation in rainbow trout, *Oncorhynchus mykiss*

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ABSTRACT

Ghrelin, a GH-releasing and appetite-regulating peptide that is released from the stomach is an endogenous ligand for growth hormone secretagogue-receptor (GHS-R). Two types of GHS-R are accepted to be present, a functional GHS-R1a and GHS-R1b with unknown function. In this study, we identified cDNA that encodes protein with close sequence similarity to GHS-R and exon–intron organization of the GHS-R genes in rainbow trout, *Oncorhynchus mykiss*. Two variants of GHS-R1a proteins with 387-amino acids, namely DQTA/LN-type and ERAT/IS-type, were identified. In 3'-RACE PCR and genomic PCR, we also identified three GHS-R1b orthologs that are consisted of 297- or 300-amino acids with different amino acid sequence at the C-terminus, in addition to the DQTA/LN-type and ERAT/IS-type variations. Genomic PCR revealed that the genes are composed of two exons separated by an intron, and that two GHS-R1a and three GHS-R1b variants are generated by three distinct genes. GHS-R1a and GHSR-1b mRNA were predominantly expressed in the pituitary, followed by the brain. Identified DQTA/LN-type or ERAT/IS-type GHS-R1a cDNA was transfected into mammalian cells, and intracellular calcium ion mobilization assay was carried out. However, we did not find any response to rat ghrelin and a homologous ligand, des-VRQ trout ghrelin, of either receptor *in vitro*. We found that unexpected mRNA splicing had occurred in the transfected cells, suggesting that the full-length, functional receptor protein might not be generated in the cells. Gene structure and characterization of protein sequence identified in this study were closely similar to other GHS-R, but to conclude that it is a GHS-R for rainbow trout, further study is required to confirm activation of GHS-R1a by ghrelin or GHS. Thus we designated the identified receptor proteins in this study as GHS-R-like receptor (GHSR-LR).

1. Introduction

Growth hormone secretagogue (GHS)-receptor (GHS-R) was first identified in human and pig, as the receptor for artificial GHS compounds (Howard et al., 1996). In mammals, two types of GHS-R have been known thus far: a functional receptor with seven-transmembrane domains that responds to GHS, namely GHS-R1a, and a function-unknown receptor, which is a truncated form of GHS-R1a after transmembrane domain-5 and does not respond to GHS, namely GHS-R1b (Howard et al., 1996). McKee et al. (1997) and Yokote et al. (1998) subsequently reported rat GHS-R1a, and the number of GHS-R identified is increasing. GHS-R1a was an orphan receptor, whose endogenous ligand is unknown. However, it was deorphanized by the discovery of ghrelin, an endogenous ligand for the receptor (Kojima et al., 1999). Ghrelin, a 28-amino acid peptide with octanoyl-modification at the third serine residue (Ser-3) was purified from stomach extract. Ghrelin is a multifunctional peptide implicated in glucose and lipid metabolism, reproduction, gastrointestinal function, cardiovascular function, cellular proliferation, immunomodulation and bone physiology in addition to GH release and food intake (Kojima and Kangawa, 2005; Hosoda et al., 2006; Soares and Leite-Moreira, 2008).

In non-mammalian vertebrates, ghrelin peptide has been identified and its physiological actions have been demonstrated in various species (Unniappan and Peter, 2005; Kaiya et al., 2007, 2008). GHS-R has also been identified in a few non-mammalian vertebrates such as chicken and fish. Tanaka et al. (2003) and Geelissen et al. (2003) separately reported two types of GHS-R in chickens: GHS-R1a which is predicted to be functional, and GHS-R1aV (truncated variant of GHS-R1a, and identical to GHS-R1c) in which 16-amino acids (48 bp) in a part of transmembrane-6 are deleted. The chicken *GHS-R* gene is composed of two exons that are separated by an intron, like in mammals (Tanaka et al., 2003). Chicken GHS-R mRNA predominantly expresses in the pituitary, followed by the brain (Tanaka et al., 2003; Geelissen et al., 2003). Expression of GHS-R1a and 1c mRNAs varies in chicken pituitary by treatments of ghrelin, GRP 1-29, GH or corticosterone (Geelissen et al., 2003). In fish, Palyha et al. (2000) identified a pufferfish GHS-R1a, namely 78B7, which responds to several GHSs. Chan and Cheng (2004) reported two types of GHS-R, namely sbGHS-R1a and sbGHS-R1b, in the black seabream. The seabream GHS-R gene has a two exons-one intron structure, as seen in mammals and chickens. Expression of GHS-R1a mRNA in the seabream is predominantly detected in the pituitary,

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whereas GHS-R1b mRNA mainly expresses in the telencephalon. Functional analyses demonstrated that GHRP-6 or human ghrelin increase intracellular calcium ion [Ca^{2+}] concentrations and extracellular acidification rates in sbGHS-R1a cDNA-transfected human embryonic kidney (HEK) 293 cells. On the other hand, sbGHS-R1b did not show such responses, but acted as a dominant negative mutant for sbGHS-R1a (Leung et al., 2007). Recently, homologous seabream ghrelin has been identified (Yeung et al., 2006). Further analyses using the homologous system would provide more reliable information about interaction between ghrelin and its receptor in fish.

We have isolated ghrelin peptide from the stomach of rainbow trout, *Oncorhynchus mykiss* (Kaiya et al., 2003). Two types of ghrelin peptide are present in rainbow trout: trout ghrelin, which is a 23-amino acid peptide and des-VRQ trout ghrelin which is a 20-amino acid splice variant of trout ghrelin. Trout ghrelin stimulates GH release from the pituitary of juvenile rainbow trout *in vivo* and *in vitro* (Kaiya et al., 2003). Yada et al. (2006) demonstrated involvement in immune function of trout ghrelin through the ghrelin-GH interaction in trout leukocytes derived from the head kidney. Recently, Jönsson et al. (2007) reported that trout ghrelin does not stimulate food intake when injected intraperitoneally. For further understanding how these physiological actions occur, it is necessary to identify and characterize ghrelin receptor in rainbow trout.

A partial sequence of rainbow trout GHS-R was previously reported by Yada et al. (2006) in determining mRNA expression of GHS-R in leucocytes of rainbow trout. The aim of the present study was to determine the full-length cDNA and the gene encoding ghrelin receptor in rainbow trout, and to examine tissue distribution of receptor mRNA in rainbow trout. We further examined whether the identified receptor protein was functional using mammalian cells.

2. Materials and methods

2.1. cDNA cloning of tout GHS-R1a

We first tried to amplify a short 230-bp fragment of the ghrelin receptor. Total RNA was extracted from the pituitary of immature male rainbow trout (*Oncorhynchus mykiss*, Salmonidae). First-strand cDNAs were synthesized from 5 μg total RNA using oligo-dT_{12-18} primer and Superscript II reverse transcriptase (Invitrogen, Grand Island, NY, USA), and purified using Wizard PCR preps DNA purification system (Promega, Madison, WI, USA) to remove interferences. PCR primers used throughout this study are summarized in Table 1. To amplify a 230-bp fragment of GHS-R by RT–PCR, a degenerate sense primer (Ghr-R-s1 (primer 1) corresponding to the RTTTNLY sequence of GHS-R), and anti-sense primer (Ghr-R-as3 (primer 2) corresponding to the CFPLRAK sequence of GHS-R) were designed based on the alignment of GHS-R sequences of human (U60179), rat (U94321), pig (U60178), chicken (AB095995) and pufferfish (AF082209). RT–PCR was performed using purified cDNA (100 ng total RNA equivalent), a 100 pmol/μL degenerated sense and anti-sense primer and *ExTaq* DNA polymerase (TaKaRa, Shiga, Japan). The PCR conditions were 94 °C for 1 min, and subsequent 40 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, and a final extension for 3 min at 72 °C. Amplified products were electrophoresed by 1.5% agarose gel containing ethidium bromide. Candidate products were excised from the gel and purified using Wizard PCR preps, and then subcloned into the pCRII-TOPO vector (Invitrogen). The nucleotide sequence of the inserted cDNA was determined by automated sequencing (ABI3100, Applied Biosystems, Foster City, CA, USA) according to the protocol for BigDye terminator cycle sequencing kit (Applied Biosystems).

Next, we carried out 3'- rapid amplification of cDNA ends (RACE) PCR based on the sequence information of a 230-bp fragment. First-strand cDNAs were synthesized from pituitary poly $(A)^+$RNA (1 μg) with an adaptor primer (T_{17} adaptor primer: 5'-GAG TCG ACT CGA GAA TGG T_{17}-3'), mixed DNA polymerase of ReverTra Ace (TOYOBO, Osaka, Japan) and Superscript II (Invitrogen). The PCR was performed with the template (125 ng poly $(A)^+$ RNA equivalent), a sense primer (rtGHSR-3RACE-s (primer 3), corresponding to the position of AVSDLL) and *KOD plus* DNA polymerase (TOYOBO) using touch-down amplification conditions (94 °C for 2 min, 1 cycle at 94 °C for 1 min, 50 °C for 1 min and 72 °C for 3 min, followed by 37 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 3 min). An amplified product, approximately 1350 bp, was subcloned and sequenced.

Table 1
Primers used in this study.

No.	Name	Sequence (5'-3')	Purposes
1	Ghr-R-s1	CAC CAC CAC CAA CTT STA C	RACE
2	Ghr-R-as3	CTT RGC SCG SAG YGG GAA GC	RACE
3	rtGHSR-3RACE-s	GGC TGT CTC AGA CTT GTT G	RACE
4	GSP-as7	GTA CCG CTC CAC GGA CAG AGC	RACE
5	GSP-as6	AAC GAA CTG AAA CAG CTT ACA	RACE
6	rtGHSR-full-s1	GAG GAC ATG CGG AGA CAA GAG GAG	cDNA, genomic PCR
7	rtGHSR-full-as1	CCA TTT CAA AAG CGG TTT AAT AGT	cDNA, genomic PCR
8	rtGHSR-full-s2	AGC ATG CGC TCC TGG CCG AAC	cDNA, genomic PCR
9	rtGHSR-full-as2	TCA CAG GCT AAC AGT GGA CTC	cDNA, genomic PUR
10	ERAT-lb-s	TTG CTC ATA TTC AAA ACC ATG TTT TAA	cDNA, genomic PCR
11	ERAT-lb-as	TTA AAA CAT GGT TTT GAA TAT GAG	cDNA, genomic PCR
12	DQTA-lb-s	TTC AAA ACT ATT TTA ACC ACT GCA TAA	cDNA, genomic PCR
13	rtGHSR-s2	GCG GTT GAG TCT GGG CTT CTA	Realt-time PCR
14	rtGHSR-as2	CAA AAT GGC TAC AGT CAC AGG	Realt-time PCR
15	rtGHSR-1b-as2	TTA TGC AGT GGT TAA AAT AGT	Realt-time PCR
16	rtGHSR-gnm-TMF-as	CTT TAC ACG ACT GGC TGT TAC CAT	Genomic PCR
17	rtGHSR-1b-as3	AAT GTC TCC AAT TCT GTG CCT	Genomic PCR

Then, we carried out 5'-RACE PCR using the Gene Racer™ kit (Invitrogen). First-strand cDNA was synthesized from 5 μg total RNA according to manufacturer's instructions. Primary PCR was performed using the template (250 ng total RNA equivalent), a 10 pmol/μl anti-sense primer (GSP-as7 (primer 4), corresponding to the position of ALSVERY), 5'-primer supplied with the Gene Racer ™ kit, *FastStar* DNA polymerase (Roche Applied Science, Mannheim, Germany) and GC-solution supplied with the DNA polymerase kit. Reaction conditions were 95 °C for 10 min, 35 cycles at 95 °C for 1 min, 65 °C for 1 min and 72 °C for 1.5 min, and a final extension for 10 min at 72 °C. The reacted product was purified by Wizard PCR preps, and 1/50 (1 μL) of the purified product was used as the template for the second-round nested PCR. The nested PCR was performed with a 10 pmol/μL anti-sense primer (GSP-as6 (primer 5), corresponding to the position of CKLFQFV), 5'-nested primer supplied with the Gene Racer ™ kit and *FastStar* DNA polymerase. Reaction conditions were 95 °C for 10 min, 30 cycles at 95 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min, and a final extension for 10 min at 72 °C. An approximately 450-bp product was subcloned, and the nucleotide sequence was determined.

To confirm the correct nucleotide sequence of the identified cDNA, full-length cDNA was amplified from a template synthesized from pituitary total RNA using 10 pmol/μL sense primer (rtGHSR-full-s1, primer 6), anti-sense primer (rtGHSR-full-as1, primer 7) and a proof-reading *Pyrobest* DNA polymerase (TaKaRa). Reaction conditions were 98 °C for 30 s, 30 cycles at 98 °C for 10 s, 57 °C for 30 s and 72 °C for 2 min, and a final extension for 3 min at 72 °C. For TA-cloning, 1 μL 10 mM dNTP and 0.2 μl *ExTaq* DNA polymerase were added to the 20-μL-reaction mixture, and it was incubated for 2 min at 94 °C followed by 72 °C for 10 min (adenine-overhang reaction). The product was subcloned into pCRII-TOPO vector and sequenced.

2.2. Genomic PCR for the rainbow trout GHS-R

Genomic DNA was extracted from rainbow trout stomach using the GenomicPrep Cells and Tissue DNA isolation Kit (Amersham Pharmacia Biotech). PCR was performed with genomic DNA template (100 ng), a primer set (primers 6 and 7, 10 pmol/μL each) and *Pyrobest* DNA polymerase. The primer set used was designed at 5'-

A

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-78  GAGGACATGCGGAGACAAGAGGAGCGCGCCAGCCGCCCCAAGGAGAACCTCCAGCCGCGTGAATGACCCGCTGGCAGCATGCGCTCCTGG  12
                                                                                  M  R  S  W

 13  CCCAACCGAACTGACTGTCTCTCTCCCGTTAACTGCAGCTGGGAGGATAACTACTGGAACTACTACTTTAACGGGAGCTACCAGGGTCCC  102
     P  N  R  T  D  C  L  S  P  V  N  C  S  W  E  D  N  Y  W  N  Y  Y  F  N  G  S  Y  Q  G  P

103  GTGCCCCCCGAAAACCTATTCCCCATCCCCGTTCTAATGGGAATCACCATCACCTGTACTCTCCTGTTCCTAGCGGGAGTGGCCGGGAAT  192
     V  P  P  E  N  L  F  P  I  P  V  L  M  G  I  T  I  T  C  T  L  L  F  L  A  G  V  A  G  N

193  GTAATGACTATACTGGTCGTGTCTAAATATAGAGACATGAGAACAACCACTAACCTCTACTTATGTAGCATGGCTGTCTCAGACTTGTTG  282
     V  M  T  I  L  V  V  S  K  Y  R  D  M  R  T  T  T  N  L  Y  L  C  S  M  A  V  S  D  L  L

283  ATATTCCTCTGTATGCCCCCTGATGTGTATCGGTTATGGAAGTACAGGCCATGGATATTTGGAGATACATTCTGTAAGCTGTTTCAGTTC  372
     I  F  L  C  M  P  P  D  V  Y  R  L  W  K  Y  R  P  W  I  F  G  D  T  F  C  K  L  F  Q  F

373  GTTAGTGAGTGCTGTACTTATTCAACGATTTTGAATATAACCGCTCTGTCCGTGGAGCGGTACCTGGCCATCTGCTTCCCACTTCGCGCC  462
     V  S  E  C  C  T  Y  S  T  I  L  N  I  T  A  L  S  V  E  R  Y  L  A  I  C  F  P  L  R  A

463  AAACGCCTGGTCACCAAGCGACGCGTTCGTGCGCTCATCCTCTTTCTCTGGCTCGTGTCGCTGTTGAGCGCGGGACCTGTCTTCGTTCTG  552
     K  R  L  V  T  K  R  R  V  R  A  L  I  L  F  L  W  L  V  S  L  L  S  A  G  P  V  F  V  L

553  GTGGGAGTGGAGCACGAGACTCGCCCAGCGGCGGGAAACTCTGTGACTGCTGGTGGGGCGGAAGGACAGACAGAGATAGACACTAGCGAG  642
     V  G  V  E  H  E  T  R  P  A  A  G  N  S  V  T  A  G  G  A  E  G  Q  T  E  I  D  T  S  E

643  TGTAAACCCACCCAGTACGCGGTTGAGTCTGGGCTTCTAGCCGCCATGGCGTTGGTTAGCTCTGTGTTTTTCTTCCTGCCGGTGTTTTGT  732
     C  K  P  T  Q  Y  A  V  E  S  G  L  L  A  A  M  A  L  V  S  S  V  F  F  F  L  P  V  F  C

733  TTGACTGTGGTGTACAGTTTGATTGGACGAAGGTTGTGGAAGAGACGGAGAGAGAACAATATTGGAGCTAATGTAGCACACAGGGACAAG  822
     L  T  V  V  Y  S  L  I  G  R  R  L  W  K  R  R  R  E  N  N  I  G  A  N  V  A  H  R  D  K

823  AGCAACAGACAGACCGTCAAGATGTTAGCTGTAGTGGTCTTTGCCTTCGTCCTGTGCTGGCTGCCTTTCCACTTGCATCGTTACCTTATG  912
     S  N  R  Q  T  V  K  M  L  A  V  V  V  F  A  F  V  L  C  W  L  P  F  H  L  H  R  Y  L  M

913  TCCCATTCCTCCGAGGGCTCCTCTCCTCTCTGGTCTCTCTTCACCCAGTACTGCTCCCTTGTTTCCACTGTCCTCTTCTATCTCTCGGCT  1002
     S  H  S  S  E  G  S  S  P  L  W  S  L  F  T  Q  Y  C  S  L  V  S  T  V  L  F  Y  L  S  A

1003 GCCATCAACCCTGTCCTCTATAACACCATGTCTAGGAAGTACCGGTCAGCTGCTGCCCAACTGTTTGGTCTACAGGAGACTCAACCACCC  1092
     A  I  N  P  V  L  Y  N  T  M  S  R  K  Y  R  S  A  A  A  Q  L  F  G  L  Q  E  T  Q  P  P

1093 AGAGGGAGAACAGCAAGCACTGTCAAAGGAGAGAGTTCACCTGCCTGGACAGAGTCCACTGTTAGCCTGTGACTGTAGCCAATTTGGAGG  1182
     R  G  R  T  A  S  T  V  K  G  E  S  S  P  A  W  T  E  S  T  V  S  L  *

1183 GGGAATGGGCGCTAGATATATACTTCTCTGATTTTGATATGTAGTTGTTGTGATCAACGTCTTCTTGTGTGTTTTTCATCAGTGACCACG  1272

1273 GCTTTCCCTGGTTGATCAAACTCAAACAGGCACCATTATGGACCATCAAGTCTTTAATCCAAGAGAGCACCGGCTTACGCATAACGTCCA  1362

1363 TGAACGTTGATTATTGATTGAAATTTGATCAGTCCGCCCTGGCCTTGCGCTGAACATATTAATTATATTAGCCTCAAGGACTCTGAACGA  1452

1453 GACTGTTGCTTTGATGTCACAACCCGCCCGGCAAAGGCGTTTGAGAAGATGTTTTATAAAATATTTATAAAACAAGGCGTTTCTGTCACC  1542

1543 CAAAAAGAAGCACACCATTAGCAACAAAAACTATTAAACCGCTTTTGAAATGG  1595
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B

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rtGHS-R1a(DQTA/LN)     1 MRSWPNRTDCLSPVNCSWEDNYWNYYFNGSYQGPVPPENLFPIPVLMGITITCTLLFLAG   60
rtGHS-R1a(ERAT/IS)     1 MRSWPNRTDCLSPVNCSWEENYWNYYFNGSYRGPVPPENLFPIPVLMGITITCALLFLAG   60
                         ******************** *********** ********************** ******

rtGHS-R1a(DQTA/LN)    61 VAGNVMTILVVSKYRDMRTTTNLYLCSMAVSDLLIFLCMPPDVYRLWKYRPWIFGDTFCK   120
rtGHS-R1a(ERAT/IS)    61 VTGNVMTILVVSKYRDMRTTTNLYLCSMAVSDLLIFLCMPPDVYRLWKYRPWIFGDTFCK   120
                         * ************************************************************

rtGHS-R1a(DQTA/LN)   121 LFQFVSECCTYSTILNITALSVERYLAICFPLRAKRLVTKRRVRALILFLWLVSLLSAGP   180
rtGHS-R1a(ERAT/IS)   121 LFQFVSECCTYSTILNITALSVERYLAICFPLRAKRLVTKRRVRALIIFLWLVSLLSAGP   180
                         ********************************************** ************

rtGHS-R1a(DQTA/LN)   181 VFVLVGVEHETRPAAGNSVTAGGAEGQTEIDTSECKPTQYAVESGLLAAMALVSSVFFFL   240
rtGHS-R1a(ERAT/IS)   181 VFVLVGVEHETRPAAGNSVTAGGAEGQTEIDTSECKPTQYAVESGLLAAMALVSSVFFFL   240
                         ************************************************************

rtGHS-R1a(DQTA/LN)   241 PVFCLTVVYSLIGRRLWKRRRENNIGANVAHRDKSNRQTVKMLAVVVFAFVLCWLPFHLH   300
rtGHS-R1a(ERAT/IS)   241 PVFCLTVVYSLIGRRLWKRRRENSIGANVAHRDKSNRQTVKMLAVVVFAFVLCWLPFHLH   300
                         ************************ ************************************

rtGHS-R1a(DQTA/LN)   301 RYLMSHSSEGSSPLWSLFTQYCSLVSTVLFYLSAAINPVLYNTMSRKYRSAAAQLFGLQE   360
rtGHS-R1a(ERAT/IS)   301 RYLMSHSSEGSSPLWSLFTQYCSLVSTVLFYLSAAINPVLYNTMSRKYRSAAAQLFGLQE   360
                         ************************************************************

rtGHS-R1a(DQTA/LN)   361 TQPPRGRTASTVKGESSPAWTESTVSL                                  387
rtGHS-R1a(ERAT/IS)   361 TQPPRGRTASTVKGESSPAWTESTVSL                                  387
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and 3'-ends of trout GHS-R cDNA. Another primer set (primers 8 and 9, 10 pmol/μL each) was designed at the open reading frame (ORF) of the trout GHS-R1a cDNA. Reaction conditions were 98 °C for 2 min, 40 cycles of 98 °C for 30 s, 57 °C for 15 s and 72 °C for 2 min, and a final extension for 3 min at 72 °C. In some cases, *PrimeSTAR Max* master mix (TaKaRa) was used for long PCR of trout GHS-R gene amplification. Reaction conditions were 98 °C for 10 s, and subsequent 30 cycles of 98 °C for 10 s, 55 °C for 5 s and 72 °C for 30 s.

In genomic PCR, two intron sequences were found. We designed two primers at 3'-end of ERAT/IS-type GHS-R1b (ERAT-1b-s and ERAT-1b-as, primers 10 and 11), and a sense primer at 3'-end of DQTA/LN-type GHS-R1b (DQTA-1b-s, primer 12). To examine whether DQTA/LN-type and ERAT/IS-type GHS-R gene locate tandem, genomic PCR with primer sets of primers 12 and 11, primers 12 and 7, primers 10 and 7, primers 6 and 17 and primers 6 and 11 were performed (Fig. 6).

2.3. Quantitative real-time PCR for trout GHS-R1a and GHS-R1b

Quantitative real-time PCR was performed using the LightCycler system (Roche Applied Science). Total RNA of the pituitary, brain area except the hypothalamus, hypothalamus, heart, stomach, pyloric appendage, intestines, liver, spleen, kidney, head kidney and gill was extracted separately from four individuals using Trizol regent. First-strand cDNA was synthesized from 5 μg of DNase I (Invitrogen)-treated total RNA except pituitary, and from 500 ng of DNase I-treated total RNA of the pituitary using Superscript II reverse-transcriptase (Invitrogen) with oligo-dT_{12-18} primers. The reacted mixture was cleaned up with phenol-chloroform treatment and followed by ethanol precipitation for removing interferences. PCR was performed using the QuantiTect SYBR Green PCR kit (QIAGEN GmbH, Hilden, Germany). Expression of GHS-R1a mRNA was analyzed with a sense primer (rtGHSR-s2, primer 13) and an anti-sense primer (rtGHSR-as2, primer 14). An expected amplicon size was 518 bp. This primer set is not able to recognize separately DQTA/LN-type and ERAT/IS-type GHS-R1a (see Results). The reaction mixture consisted of 1 × master mix and 250 nM each of primer and template (20 ng for the pituitary or 200 ng for other tissues total RNA equivalent). The analytical amplification conditions were 95 °C for 15 min, and subsequent 38 cycles at 94 °C for 15 s, 56 °C for 30 s and 72 °C for 30 s.

Two different C-terminal amino acid sequences of GHS-R1b protein were identified in 3'-RACE PCR and genomic PCR. In this experiment, we quantified expression of DQTA/LN-type GHS-R1b mRNA with a TTA sequence at the C-terminus because the transcript was identified in 3'-RACE PCR. A sense primer (primer 13) and an anti-sense primer (rtGHSR-1b-as2, primer 15) were used for the analysis. An expected amplicon size was 243 bp. The reaction mixture consisted of 1 × master mix and 250 nM each of primer and template (40 ng for the pituitary or 400 ng for other tissues total RNA equivalent). Reaction conditions were 95 °C for 15 min, 38 cycles at 94 °C for 15 s, 53 °C for 30 s and 72 °C for 20 s.

For quantification of RNA number, a linear regression line was generated by serial dilution (from 10^3 to 10^6 copies) of full-length target cDNA clone into pCRII-TOPO vector and linearized with *Xba-I* restriction. For visualizing PCR products, RT–PCR was performed using *HotStarTaq* master mix kit (QIAGEN GmbH) with a primer set (primers 13 and 14) for GHS-R1a (518 bp), and another primer set (primers 13 and 15) for GHS-R1b (243 bp). Template cDNA was synthesized from 1 μg total RNA using QuantiTect RT kit (QIAGENGmbH) and oligo-dT_{12-18} primers. Amplification conditions were 95 °C for 5 min, subsequent 27 and 30 cycles at 95 °C for 30 s, 56 °C for 30 s and 72 °C for 30 s for GHS-R1a, and subsequent 33 and 36 cycles at 95 °C for 30 s, 53 °C for 30 s and 72 °C for 30 s for GHS-R1b. The products were electrophoresed by 1.5% agarose gel containing ethidium bromide. PCR products were visualized using LAS-4000 mini EPUV (FUJIFILM corporation, Tokyo, JAPAN).

2.4. Functional analysis of trout GHS-R1a

The coding region for the two types of trout GHS-R1a cDNAs (DQTA/LN-type and ERAT/IS-type, see Results) was amplified separately from each plasmid containing full-length cDNA using *Pyrobest* DNA polymerase and a primer set (primers 8 and 9). Reaction conditions were 98 °C for 2 min, 30 cycles of 98 °C for 30 s, 57 °C for 15 s and 72 °C for 2 min, and a final extension for 3 min at 72 °C. After the adenine-overhang reaction, the product was cloned into pcDNA3.1-V5-His-TOPO mammalian cell expression vector (Invitrogen).

GHS-R agonists such as ghrelin increase intracellular Ca^{2+} concentrations in cells expressing GHS-R1a (Kojima et al., 1999). Functional assay of trout GHS-R1a was conducted using HEK293 or Chinese hamster ovary (CHO) cells with a transient expression of the cDNA. FLIPR^{tetra} (Molecular Devices, Menlo Park, CA, USA) was used for the measurement of intracellular Ca^{2+} mobilization. HEK293 cells were cultured in DMEM (Gibco BRL) containing 10% fetal calf serum (FCS) at a density of 1×10^6 cells in a 10-cm collagen-coated dish for 24 h. CHO cells were cultured in alpha-MEM (Gibco BRL) containing 10% FCS at a density of 0.5×10^6 cells in a 10-cm dish for 24 h. Expression vector (2.5 μg) containing the coding region of trout GHS-R1a or rat GHS-R1a was transfected using FuGENE6 (Roche Diagnostics) according to the manufacturer's protocol. Twenty-four hours after the transfection, HEK293 cells were plated onto a collagen-coated 96-well black plate (Corning Inc., Corning, NY, USA) at a density of 3×10^4 cells *per* well. CHO cells were plated onto a non-coated 96-well black plate at a density of 5×10^4 cells *per* well. Twenty hours after the plating, culture medium was aspirated, and 100 μL fluorescent dye solution containing 4.4 μM Fluo-4AM (Invitrogen), 1% FCS, 0.045% pluronic acid (Sigma-Aldrich Chemical, St. Louis, MO, USA) in a working buffer (1 × Hanks BSS (Gibco BRL), 20 mM HEPES buffer containing 250 μM probenecid (Sigma) were loaded onto each well. The plate was incubated for 1 h at room temperature. After washing with working buffer three times, 100 μL designated concentrations (10^{-11} M to 10^{-6} M) of synthetic des-VRQ trout ghrelin (Kaiya et al., 2003), rat ghrelin, 10^{-7} M human motilin and 10^{-7} M rat neuromedin U that dissolved in working buffer containing 0.001% Triton X-100 were automatically added to each well by the FLIPR system. Changes in intracellular Ca^{2+} concentrations were measured by excitation at 488 nm and emission at 500–560 nm.

2.5. Expression of trout GHS-R1a mRNA in transfected cells

To confirm the expression of full-length trout GHS-R1a mRNA in transfected cells, residual cells after plating were collected, and total RNA was extracted using the RNeasy RNA extraction kit (QIAGEN GmbH) according to the manufacturer's protocols. First-strand cDNA was synthesized from 1 μg total RNA using QuantiTect RT kit (QIAGENGmbH) with oligo-dT_{12-18} primer. RT–PCR was performed by *HotStarTaq* master mix kit (QIAGENGmbH) with a primer set (primers 8 and 9) that amplifies the ORF of trout GHS-R1a. The

Fig. 1. Rainbow trout GHSR1a-like receptor cDNA and protein sequence of its variants. (A) Nucleotide sequence and deduced amino acid sequence of rainbow trout DQTA/LN-type GHS-R1a-like receptor cDNA. Potential polyadenylation signal (ATTAAA) is *boxed*. Number indicates location from adenine of the initiation codon (ATG). *An asterisk* in the line of amino acid sequence indicates stop codon (TGA). (B) Comparison of amino acid sequence of DQTA/LN-type and ERAT/IS-type GHSR1a-like receptor. *Arrowheads* indicate amino acid substitutions: D20E, Q32R, T54A, A62T, L168I and N264S. The nucleotide sequences for DQTA/LN-type and ERAT/IS-type GHSR1a-like receptor were deposited in the DDBJ/EMBL/GenBank databases with the accession numbers AB362479 and AB362480, respectively.

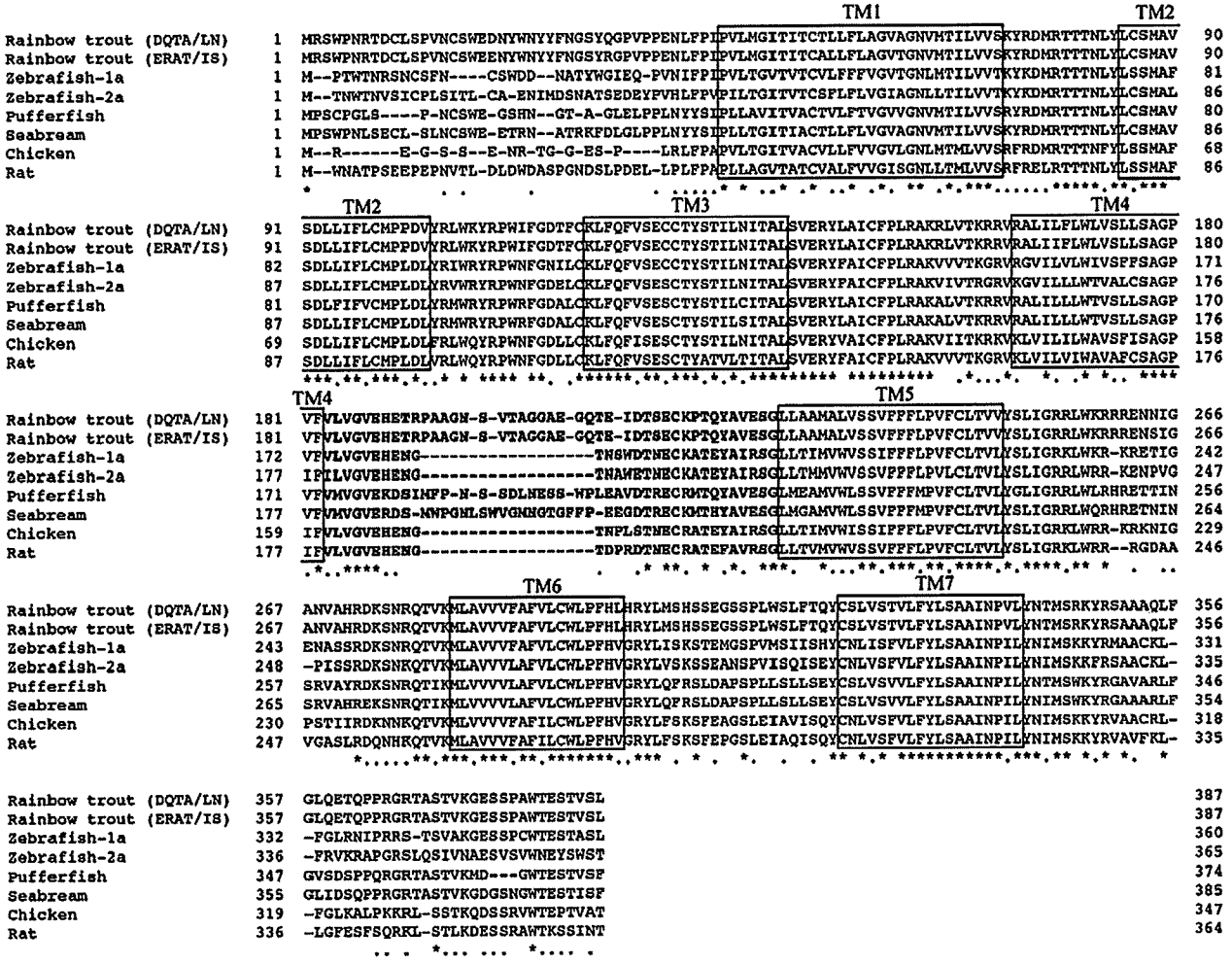

Fig. 2. Comparison of amino acid sequences of trout GHSR1a-like receptor and GHS-R1a. *Asterisks* indicate identical amino acids across all species. *Dots* indicate more than half of identical amino acids across all species. Amino acid sequences are available from the DDBJ/EMBL/GenBank databases: rainbow trout (AB362479 (DQTA/LN-type) and AB362480 (ERAT/IS-type)), zebrafish-1a (XM001335981), zebrafish-2a (XM001340372), pufferfish (AF082209), seabream (AY151040), chicken (AB095995) and rat (U94321). Predicted transmembrane domains are *boxed*. Region of the second extracellular loop indicated by bold letters.

product was electrophoresed by 1.5% agarose gel containing ethidium bromide.

3. Results

3.1. Two variants of trout GHS-R1a

We isolated a 232-bp fragment, which showed high similarity with rat GHS-R1a. In RACE PCR, approximately 1370-bp 3' fragment and 480-bp 5' fragment, whose nucleotide sequence and encoded protein sequence show high similarity with other GHS-R1a, were obtained. Assembled full-length trout GHS-R1a-like receptor cDNA was to be 1673 bp, which is composed of a 78-bp 5'-untranslated region (UTR), an open reading frame (ORF) of 1164 bp, which encodes a 387-amino acid protein, and a 431-bp of 3'-UTR (Fig. 1A). In RT–PCR using a proofreading DNA polymerase and a primer set designed at the 5'- and 3'-ends of the UTR of expected cDNA, we confirmed that the nucleotide sequence was correct, but also found some nucleotide substitutions accompanied with amino acid substitutions of D20E (GAT to GAG), Q32R (CAG to CGG), T54A (ACT to GCT), A62T (GCC to ACC), L168I (CTC to ACT) and N264S (AAT to AGT) (Fig. 1B). We noticed that all clones obtained in 5'-RACE PCR had the substituted amino acid sequences of ERAT/IS, and all clones obtained by RT–PCR using a proofreading DNA polymerase had the DQTA/LN sequence (Fig. 1A). To confirm whether DQTA/LN-type and ERAT/IS-type cDNA are present in the same specimen, we performed RT–PCR with a primer set for the ORF in each template obtained from four different trout pituitaries. As a result, both types of transcript were found in the same specimen, suggesting that rainbow trout pituitary expresses DQTA/LN-type and ERAT/IS-type GHS-R1a-like receptor cDNA. Appearance rate of DQTA/LN-type and ERAT/IS-type clone in a RT-PCR was 7:1, suggesting that each receptor gene separately controls the expression.

Comparison of protein sequences of DQTA/LN-type and ERAT/IS-type trout GHS-R1a-like protein revealed presence of many consensus sequences with other GHS-R1a (Fig. 2). The two trout proteins showed

Table 2
Amino acid sequence identity of rainbow trout GHSR1 La-like receptor with GHS-R1 in other vertebrates.

Species and type	Identity (%)	Accession no.	References
Pufferfish 1a	67	AFO82209	Palyha et al. (2000)
Black seabream 1a	71	AY151040	Chan and Cheng (2004)
Zebrafish 1a	67	XM001335981	Olsson et al. (2008)
Zebrafish 2a	61	XM001340372	Kaiya et al. (2008)
Chicken 1a	60	NM_204394	Geelissen et al. (2003); Tanaka et al. (2003)
Rat 1a	58	NM_032075	McKee et al. (1997); Yokote et al. (1998)

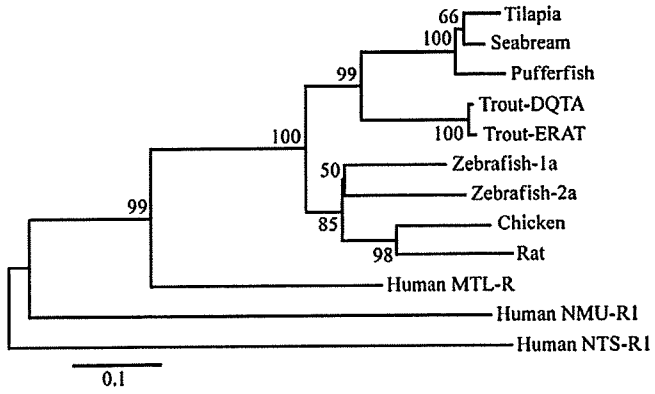

Fig. 3. Phylogenetic tree of the amino acid sequences of trout GHSR1a-like receptor, GHS-R1a and related receptor. Phylogenetic tree was made by the Neighbor-joining method of MEGA4 (*http://www.megasoftware.net/*)(Tamura et al., 2007). Human motilin, neuromedin U and neurotensin receptors were included as GHS-R family receptors. Amino acid sequences are available from the DDBJ/EMBL/GenBank databases: tilapia (AB361053), rainbow trout (DQTA/LN-type, AB362479 and ERAT/IS-type, AB362480), zebrafish-1a (XM001335981), zebrafish-2a (XM001340372), pufferfish (AF082209), Seabream (AY151040), chicken (AB095995), rat (U94321), human neuromedin U receptor1 (NMU-R1) (NM_006056), human motilin receptor (MTL-R) (NM_001507) and human neurotensin receptor1 (NMU-R1) (NM_002531). Scale bar indicates relative measure of evolutionary distance.

the highest identity with seabream GHS-R1a (71%) (Table 2). A unique feature of the trout proteins was that second extracellular domain between transmembrane (TM)-4 and TM-5 is longer than those of zebrafish, chicken and rat GHS-R1a, and is similar in length to pufferfish, seabream and tilapia GHS-R1a (Fig. 2). Phylogenetic analysis supported the sequence identity and feature (Fig. 3): the trout proteins were clustered within a clade of a GHS-R group for pufferfish, seabream and tilapia GHS-R1a, and were categorized as another GHS-R group from zebrafish, chicken and rat. From these results, we called these proteins as DQTA/LN-type and ERAT/IS-type trout GHS-R1a, respectively (accession numbers, AB362479 (DQTA/LN-type) and AB362480 (ERAT/IS-type)).

3.2. Identification of trout GHS-R1b

In 3'-RACE PCR, we isolated another cDNA, which was 1033 bp in length. The cDNA was composed of a 78-bp 5'-UTR, a 903-bp ORF that encodes a 300-amino acid protein, and a 52-bp 3'-UTR (Fig. 4, accession number AB362481). The deduced amino acid sequence was identical to that of DQTA/LN-type GHS-R1a until 283-amino acid residues, then differed by 17-amino acids extended at the C-terminal portion. We assumed that this protein might be a GHS-R1b ortholog for rainbow trout GHS-R.

3.3. Identification of the trout GHS-R gene

A part of GHS-R1b sequence has been encoded in the intron of the *GHS-R* gene. Genomic PCR was performed using two primer sets: rtGHS-R-full-s1 and rtGHS-R-full-as1, which covers full-length cDNA of trout GHS-R1a; rtGHS-R-full-s2 and rtGHS-R-full-as2, which covers trout GHS-R1a ORF. As a result, we could not obtain any product in the PCR using the former primer set, but could obtain a fragment in the PCR used the latter primer set. The identified DNA was 1688 bp in length and contained identified GHS-R1a sequence (Fig. 5, accession number AB362482). We found an exon-intron boundary according to the GT–AG rule for splicing, and identified a 524-bp intron (Fig. 5). Here, we noticed that the GHS-R protein deduced from genomic DNA shows ERAT/IS-type character, and there was no clone having DQTA/LN-type sequence. Furthermore, deduced amino acid sequence of GHS-R1b protein was different from that identified in 3'-RACE PCR: the protein was terminated by a stop codon, TAA, and the C-terminal

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-78  GAGGACATGCGGAGACAAGAGGAGCGCGCCAGCCGCCCCAAGGAGAACCTCCAGCCGCGTGAATGACCCGCTGGCAGCATGCGCTCCTGG  12
                                                                                  M  R  S  W

 13  CCCAACCGAACTGACTGTCTCTCTCCCGTTAACTGCAGCTGGGAGGATAACTACTGGAACTACTACTTTAACGGGAGCTACCAGGGTCCC  102
      P  N  R  T  D  C  L  S  P  V  N  C  S  W  E  D  N  Y  W  N  Y  Y  F  N  G  S  Y  Q  G  P

103  GTGCCCCCCGAAAACCTATTCCCCATCCCCGTTCTAATGGGAATCACCATCACCTGTACTCTCCTGTTCCTAGCGGGAGTGGCCGGGAAT  192
      V  P  P  E  N  L  F  P  I  P  V  L  M  G  I  T  I  T  C  T  L  L  F  L  A  G  V  A  G  N

193  GTAATGACTATACTGGTCGTGTCTAAATATAGAGACATGAGAACAACCACTAACCTCTACTTATGTAGCATGGCTGTCTCAGACTTGTTG  282
      V  M  T  I  L  V  V  S  K  Y  R  D  M  R  T  T  T  N  L  Y  L  C  S  M  A  V  S  D  L  L

283  ATATTCCTCTGTATGCCCCCTGATGTGTATCGGTTATGGAAGTACAGGCCATGGATATTTGGAGATACATTCTGTAAGCTGTTTCAGTTC  372
      I  F  L  C  M  P  P  D  V  Y  R  L  W  K  Y  R  P  W  I  F  G  D  T  F  C  K  L  F  Q  F

373  GTTAGTGAGTGCTGTACTTATTCAACGATTTTGAATATAACCGCTCTGTCCGTGGAGCGGTACCTGGCCATCTGCTTCCCACTTCGCGCC  462
      V  S  E  C  C  T  Y  S  T  I  L  N  I  T  A  L  S  V  E  R  Y  L  A  I  C  F  P  L  R  A

463  AAACGCCTGGTCACCAAGCGACGCGTTCGTGCGCTCATCCTCTTTCTCTGGCTCGTGTCGCTGTTGAGCGCGGGACCTGTCTTCGTTCTG  552
      K  R  L  V  T  K  R  R  V  R  A  L  I  L  F  L  W  L  V  S  L  L  S  A  G  P  V  F  V  L

553  GTGGGAGTGGAGCACGAGACTCGCCCAGCGGCGGGAAACTCTGTGACTGCTGGTGGGGCGGAAGGACAGACAGAGATAGACACTAGCGAG  642
      V  G  V  E  H  E  T  R  P  A  A  G  N  S  V  T  A  G  G  A  E  G  Q  T  E  I  D  T  S  E

643  TGTAAACCCACTCAGTACGCGGTTGAGTCTGGGCTTCTAGCCGCCATGGCGTTGGTTAGCTCTGTGTTTTTCTTCCTGCCGGTGTTTTGT  732
      C  K  P  T  Q  Y  A  V  E  S  G  L  L  A  A  M  A  L  V  S  S  V  F  F  F  L  P  V  F  C

733  TTGACTGTGGTGTACAGTTTGATTGGACGAAGGTTGTGGAAGAGACGGAGAGAGAACAATATTGGAGCTAATGTAGCACACAGGGACAAG  822
      L  T  V  V  Y  S  L  I  G  R  R  L  W  K  R  R  R  E  N  N  I  G  A  N  V  A  H  R  D  K

823  AGCAACAGACAGACCGTCAAGATGTTAGGTAGGGTGCAATACATGTTGCTCATATTCAAAACTATTTTAACCACTGCATAATTGAGATAT  912
      S  N  R  Q  T  V  K  M  L  G  R  V  Q  Y  M  L  L  I  F  K  T  I  L  T  T  A  *
                                 ----------------------------------------------------------

913  GGATGTTTATAATATTTTTAAAAGGCACAGAATTGGAGACATT  955
```

Fig. 4. Nucleotide and deduced amino acid sequence of the rainbow trout GHSR1b-like receptor cDNA. This GHSR1b-like receptor cDNA, DQTA/LN-type as indicated by *circles*, was identified by 3'-RACE PCR, and the polyadenylation was present at the 3'-end. Another GHSR1b-like receptor, ERAT/IS-type with a different C-terminal amino acid sequence, is shown in Fig. 5. Extended amino acid sequence derived from the intron is indicated by the *dashed line*. Number indicates location from adenine of the initiation codon (ATG). Potential polyadenylation signal (TTTATA or TTTAAA) is *boxed*. The nucleotide sequence has been deposited in the DDBJ/EMBL/GenBankdatabases (accession number AB362481).

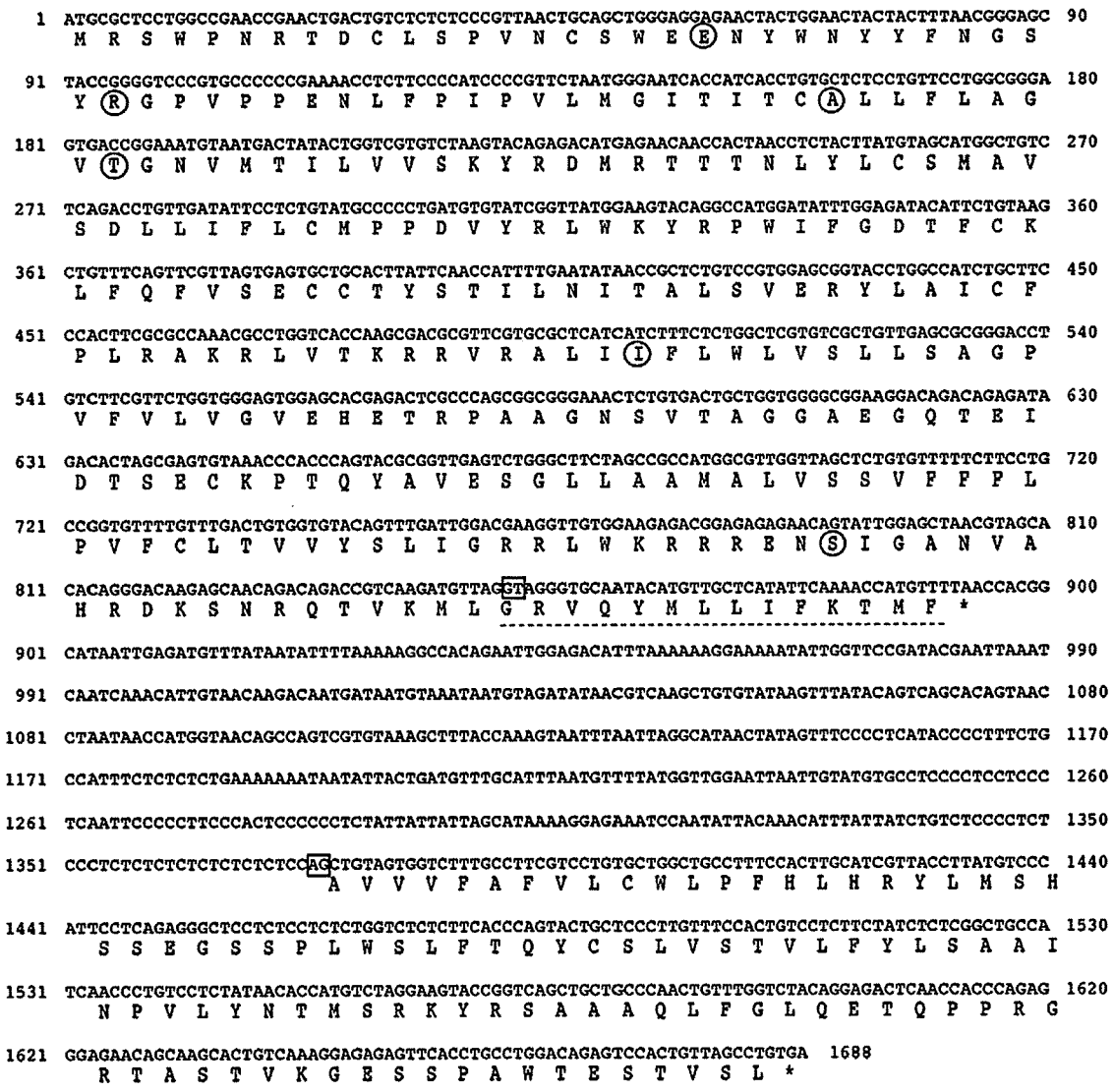

Fig. 5. Partial nucleotide sequence and deduced amino acid sequence of rainbow trout GHSR-like receptor gene (ERAT/IS-type with TMF sequence at the C-terminus of GHSR1b-like receptor). There are three *GHS-R* genes in rainbow trout, and this is one of them. Substituted amino acids are *circled*. This gene generates a 297-amino acid GHSR1b-like receptor with TMF sequence at the C-terminus. Nucleotide sequence of the 5'- and 3'-untranslated region was unidentified. Extended amino acid sequence of GHSR1b-like receptor derived from the intron is indicated by the *dashed line*. The splicing sites, GT for the 5'-side and AG for the 3'-side of the intron, are *boxed*. The nucleotide sequence of the gene is available from the DDBJ/EMBL/GenBank databases (accession number AB362482).

sequence shortened from previous 17 residues (GRVQYMLLIFK*TILTTA*) (Fig. 4) to 14 residues (GRVQYMLLIFK*TMF*) (Fig. 5).

3.4. Possible presence of two distinct genes for trout GHS-R

We hypothesized that there are two distinct *GHS-R* genes in rainbow trout (Fig. 6A): one is the gene encoding DQTA/LN-type GHS-R1a and the 17-amino acid extension (TTA sequence at the C-terminal end) in the intron for *GHS-R1b* (Fig. 6A, Gene-1), and the other is the gene encoding ERAT/IS-type GHS-R1a and the 14-amino acid extension (TMF sequence at the C-terminal end) in the intron for *GHS-R1b* (Fig. 6A, Gene-2). To confirm whether the DQTA/LN-type and ERAT/IS-type *GHS-R* genes have different introns, two anti-sense primers were used: rtGHSR-gnm-TMF-as (primer 16, Table 1) that was designed in the intron identified by genomic PCR, and rtGHSR-1b-as3 (primer 17, Table 1) that was designed at the 3'-end of DQTA/LN-type GHS-R1b identified in 3'-RACE PCR. Genomic PCR was performed with each anti-sense primer and a sense primer, rtGHSR-full-s2 (primer 8) that was designed at 5'-end of the GHS-R1a ORF. All clones obtained by the former primer set (primers 8 and 16) encoded ERAT/IS-type sequence, and the C-terminus of GHS-R1b was terminated by amino acid sequences TMF as shown in the genomic PCR (Figs. 5 and 6A). On the other hand, all clones obtained by the latter primer set (primers 8 and 17) encoded DQTA/LN-type sequence, and the C-terminus of GHS-R1b was terminated by amino acid sequences TTA. These results indicate that there are two *GHS-R* genes, whose first exon encodes DQTA/LN-type or ERAT/IS-type GHS-R sequence with the same 5'-end ORF nucleotide sequences and different introns (Fig. 6A).

3.5. Do DQTA/LN-type and ERAT/IS-type GHS-R genes have a common second exon?

Next, we hypothesized that DQTA/LN-type and ERAT/IS-type GHS-R might share a common second exon because amino acid sequences of the bounded region are identical between the two types of GHS-R1a, although there was a case that the PCR product did not yield identical sequence when rtGHSR-full-as1 (primer 7), which was designed at the 3'-end UTR of the GHS-R1a cDNA, was used. To examine the possibility that the first exon and the intron for each DQTA/LN-type or ERAT/IS-type GHS-R may locate tandem and a single second exon follows the sequences (then each type of whole receptor could be generated by an alternative splicing of each first

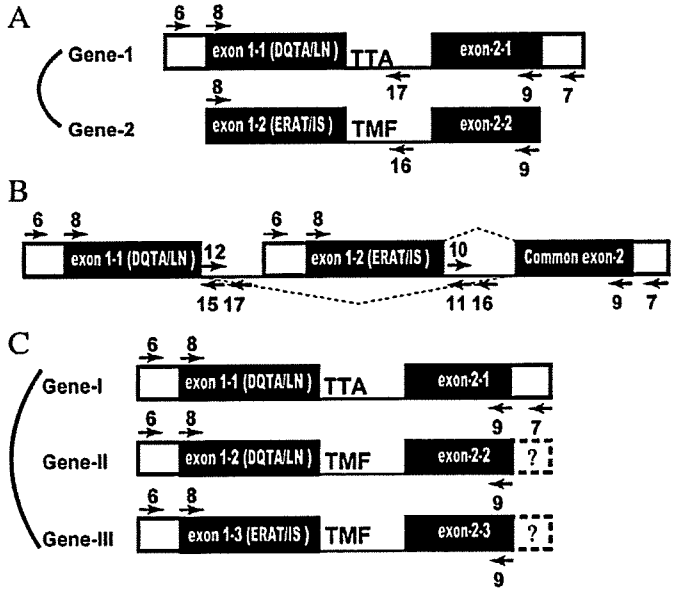

Fig. 6. Schematic drawing of predicted trout *GHSR-like receptor* gene composition. Primers for experiments were listed in Table 1 and are indicated by *arrows and numbers*. (A) The first model of the trout *GHSR-like receptor* gene was based on the results of first genomic PCR with primers 6 and 7, which covered the full-length of the cDNA including 5'- and 3'-UTR regions, and primers 8 and 9, which covered the ORF region of trout GHSR1a-like receptor. (B) The second model of the trout *GHSR-like receptor* gene with tandem arrangement of DQTA/LN-type and ERAT-type first exons and the common second exon. (C) The third model of the trout *GHSR-like receptor* gene. Three distinct genes were assumed to generate two GHSR1a-like receptors and three GHSR1b-like receptors.

exon and a common second exon (Fig. 6B), we performed long genomic PCR using two primer sets, primers 6 and 7 or primers 6 and 9. As a result, we could get a single band in each PCR. All clones obtained by the former primer set encoded DQTA/LN-type GHS-R1a (Fig. 7). On the other hand, clones obtained by the latter primer set encoded both DQTA/LN-type and ERAT/IS-type GHS-R1a. Notably, the gene that encoded DQTA/LN-type GHS-R amplified by the latter primer set had a different intron sequence from that amplified by the former primer set: the encoded GHS-R1b protein was terminated by the TMF sequence that we thought to be the sequence for ERAT/IS-type GHS-R1b, at the C-terminal end. Furthermore, we could not find ERAT/IS-type GHS-R1b with the TTA sequence. These results indicate that three distinct exon–intron combinations are present: DQTA/LN-type first exon and the intron with the TTA sequence; DQTA-type first exon and the intron with TMF sequence; and ERAT/IS-type first exon and the intron with the TMF sequence (Fig. 6C). Furthermore, since primers 6 and 7 together could only amplify the DQTA/LN-type GHS-R with TTA sequence (Gene-I), it is possible that different second exons encode a common GHS-R1a protein sequence in the coding region with different 3'-UTR sequences (Fig. 6C, Gene-II and Gene-III). We tried to identify the novel 3'-UTR sequence by 3'-RACE PCR, but unfortunately could not get any different sequence.

We could not get a product showing that both receptors locate tandem in long genomic PCR. To obtain much shorter products that contain both receptor sequences, genomic PCR with primer sets of DQTA-1b-s/ERAT-1b-as (primer 12 and 11, Table 1, Fig. 6B), DQTA-1b-s/rtGHSR-full-as1 (primers 12 and 7), ERAT-1b-s/rtGHSR-full-as1 (primers 10 and 7), rtGHSR-full-s1/rtGHSR-1b-as3 (primers 6 and 17) and rtGHSR-full-s1/ERAT-1b-as (primers 6 and 11) were conducted. A single band was observed using two primer sets, primers 6 and 17 and primers 12 and 7. The product (approximately 1.1 kbp) obtained from the former primer set contained the first exon that encodes DQTA/LN-type GHS-R1a sequence. The product (approximately 1.3 kbp) obtained by the latter primer contained TTA sequence at the intron and is followed by the GHS-R1a sequence that is encoded at the second exon. These results indicate that the first exon for DQTA/LN-type GHS-R connects the single intron with TTA sequence and followed by the second exon for DQTA/LN-type (Fig. 6C, Gene-I). On the other hand, PCR with primers 12 and 11 together did not yield any product. Thus, we concluded that the first exon for each DQTA/LN-type or ERAT/IS-type GHS-R does not locate tandem (Fig. 6B).

3.6. Expression of trout GHS-R1a and 1b mRNA

As the sequences in Figs. 4, 5 and 7 illustrate, due to a few differences in nucleotide sequence between DQTA/LN-type and ERAT/IS-type GHS-R1a, the number of GHS-R1a mRNA was estimated as the total number of these variants. Furthermore, GHS-R1b mRNA estimated the number of mRNA encoding TTA sequence at the C-terminus of DQTA/LN-type GHS-R1b because the transcript was isolated by 3'-RACE PCR.

Quantitative real-time PCR revealed that GHS-R1a mRNA was predominantly expressed in the pituitary (Fig. 8A, closed column). GHS-R1a mRNA expression in the brain, hypothalamus and intestines were 16, 14 and 11% relative to the pituitary, respectively. Expression of other tissues ranged from 0.08 to 9% relative to the pituitary. Semi-quantitative RT–PCR supported the real-time PCR result (Figs. 8B and C).

Quantitative real-time PCR revealed that GHS-R1b mRNA was predominantly expressed in the pituitary, and followed by the brain without the hypothalamus (13% relative to the pituitary) (Fig. 8A, open column). GHS-R1b mRNA expression in other tissues ranged from 0.3 to 6.9%. Semi-quantitative RT–PCR supported the real-time PCR results (Figs. 8D and E).

3.7. Functional analyses of trout GHS-R1a in mammalian cells

To confirm whether the identified trout GHS-R1a is functional or not, DQTA/LN-type or ERAT/IS-type trout GHS-R1a cDNA was transfected into CHO or HEK293 cells. The cells transfected with rat GHS-R1a cDNA used as a positive control responded well to 10^{-8} to 10^{-10} M rat ghrelin and to des-VRQ trout ghrelin with a similar degree of magnitude (Fig. 9, columns B and C). On the other hand, the cells transfected with DQTA/LN-type or ERAT/IS-type trout GHS-R1a cDNA did not show any response to either rat ghrelin or des-VRQ trout ghrelin at doses of 10^{-8} to 10^{-9} M (Fig. 9, columns D to G). No responses were observed even when an increased dose of des-VRQ trout ghrelin (10^{-6} M) or other related peptides, 10^{-7} M human motilin and rat neuromedin U, were added to the cells.

To known why the cells transfected with trout GHS-R1a cDNAs did not respond even when homologous ligand was applied, we examined expression of trout GHS-R1a mRNA in the transfected cells. We could not find the complete length of mRNA corresponding to trout GHS-R1a in trout GHS-R1a-transfected cells, whereas the complete length of rat GHS-R1a mRNA was detected in rat GHS-R1a-transfected cells (Fig. 10). Instead, several unexpected bands of low molecular sizes were observed (Fig. 10). The products were fragments that were unexpectedly spliced within the trout GHS-R1a cDNA sequence according to the GT-AG rule (data now shown).

4. Discussion

In non-mammalian vertebrates, GHS-R had been identified only in four species: three teleosts, pufferfish (Palyha et al., 2000), black seabream (Chan and Cheng, 2004) and zebrafish (Olsson et al., 2008; Kaiya et al., 2008), and one bird, chicken (Geelissen et al., 2003; Tanaka et al., 2003). Recently, we also published the amino acid sequences of rainbow trout and tilapia GHS-R (Kaiya et al., 2008). Detailed information about tilapia GHS-R identification has been reported by Kaiya et al. (in press). Here, we report the identification of a fifth member of fish GHS-R, rainbow trout GHS-R.

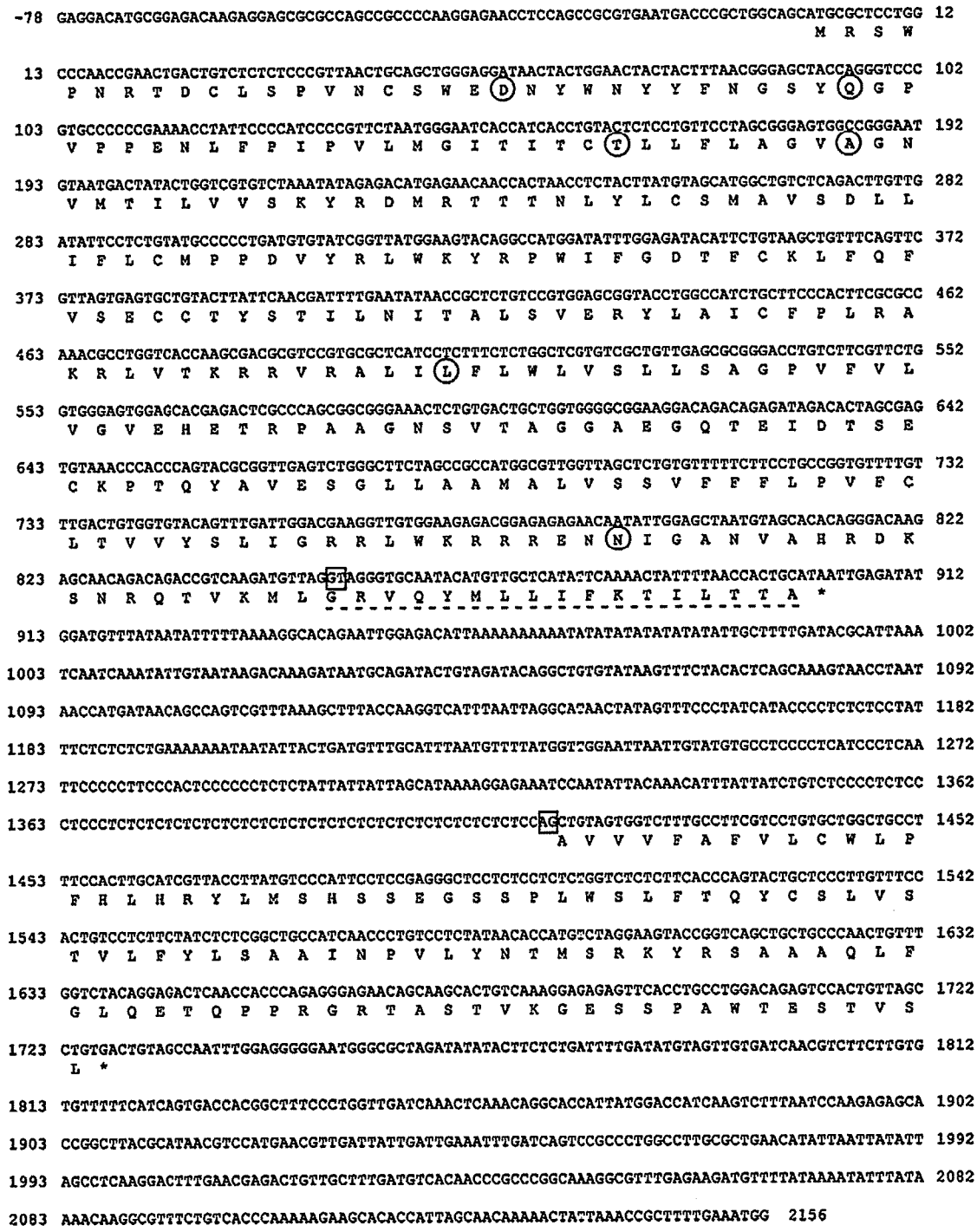

Fig. 7. Nucleotide sequence and deduced amino acid sequence of the rainbow trout *GHSR*-like receptor gene (DQTA/LN-type with TTA sequence at the C-terminus of GHSR1b-like receptor). Substituted amino acids are *circled*. This gene generates a 300-amino acid GHSR1b-like receptor with TTA sequence at the C-terminus, and was amplified with primers 6 and 7 (Table 1). Extended amino acid sequence for GHSR1b-like receptor derived from the intron is indicated by the *dashed line*. The splicing sites, GT for the 5'-side and AG for the 3'-side of intron, are *boxed*. The sequence of the gene is available from the DDBJ/EMBL/GenBank databases (accession number AB479381).

4.1. Nomenclature of identified protein

In this study, we identified cDNAs that encode proteins closely similar with GHS-R1a in rainbow rout, from viewpoints of the amino acid sequence, gene organization and tissue distribution, as described below. However, to conclude that the identified protein is the receptor for trout ghrelin, it is essential to confirm the receptor activation by the expected ligand ghrelin or GHS. Unfortunately we could not confirm activation of the identified receptor protein by ghrelin. For this purpose, we used mammalian cells, but found they may be unsuitable for the evaluation of receptor function. It may be necessary to establish an evaluation system using fish cells or trout itself. Conclusively, we propose to designate this identified receptor protein as GHS-R-like receptor (GHSR-LR) at present.

4.2. Two variants of GHSR1a-LR in rainbow trout

Two GHSR1a-LR proteins with different amino acid sequences, DQTA/LN-type and ERAT/IS-type (named based on the substituted amino acids) were identified (Fig. 1B). This indicates that rainbow

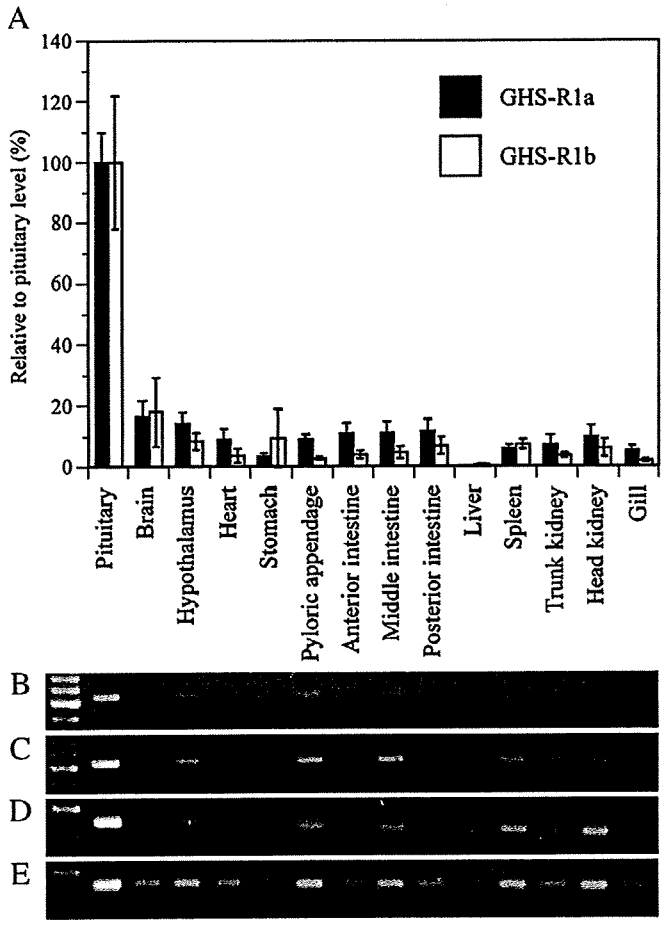

Fig. 8. Tissue expression of rainbow trout GHSR1a-like receptor and GHS1b-like receptor mRNA. (A) Gene expression was analyzed by quantitative real-time PCR. Values represent the mean ± SE ($n = 4$–5 individuals) as a ratio to expression level in the pituitary (100% = 24 copies/ng total RNA for GHS-R1a; 3 copies/ng total RNA for GHS-R1b). (B–E) Gel view of RT-PCR of trout tissues. Samples were prepared from 27 (B) and 30 (C) cycles for GHSR1a-like receptor, and from 33 (D) and 36 (E) cycles for GHSR1b-like receptor.

trout is the second animal possessing two GHS-R1a. It has been known so far that zebrafish has two potential functional GHS-R1a, namely GHS-R1a and GHS-R2a (Olsson et al., 2008; Kaiya et al., 2008), whereas no report is present in mammals. Identity between the two zebrafish receptors is 74% in the amino acid level and 71% in the nucleotide level. Each receptor locates at different chromosomes; GHS-R1a and GHS-R2a locate at chromosome 2 and 24, respectively, indicating that each zebrafish GHS-R is generated from a distinct gene. Detailed distribution and function of each receptor are yet unknown. The two trout GHSR1a-LR shared 98% identity at the amino acid level and 99% at the nucleotide level, suggesting that these proteins are paralogs. As described later, trout GHSR-LR is generated from three distinct genes, and it is assumed that the genes are derived by tandem duplication of the ancestral gene. We do not know whether amino acid substitutions affect receptor function, although some key amino acids that are important for receptor function are located in the amino acid sequence (Feighner et al., 1998).

4.3. Tissue expression of rainbow trout GHSR1a-LR mRNA

Expression levels between DQTA/LN-type and ERAT/IS-type GHSR1a-LR mRNA (appearance rate of each receptor in RT-PCR) were different in individuals, suggesting that expression of each gene is differently regulated. In the present study, we could not quantify expression of each gene because the nucleotide substitutions of the two receptors were too few, although tissue-specific expression of each receptor may occur.

GHSR1a-LR mRNA was predominantly detected in the pituitary of rainbow trout. Similar expression of GHS-R1a mRNA in the pituitary has been observed in fish (Chan and Cheng, 2004), birds (Geelissen et al., 2003; Tanaka et al., 2003; Richards et al., 2006) and mammals (Yokote et al., 1998). This result is consistent with the previous results reported for rainbow trout showing ghrelin and GHS stimulate GH secretion from the pituitary *in vivo* and *in vitro* (Kaiya et al., 2003; Shepherd et al., 2007). Trout GHSR1a-LR may mediate the GH-releasing activity of ghrelin as has been seen in mammals (Sun et al., 2004).

Moderate expression of GHSR1a-LR mRNA was observed in the hypothalamus (14% relative to the pituitary). In the black seabream and chicken, hypothalamic expression levels of GHS-R1a mRNA were 50–60% relative to the pituitary (Chan and Cheng, 2004; Geelissen et al., 2003) and substantial amount of hypothalamic GHS-R gene expression was also observed in rats (Yokote et al., 1998). This is consistent with the fact that the hypothalamus is the regulatory center of feeding, and is a target organ of ghrelin in rat (Nakazato et al., 2001; Lawrence et al., 2002), chicken (Saito et al., 2002) and goldfish

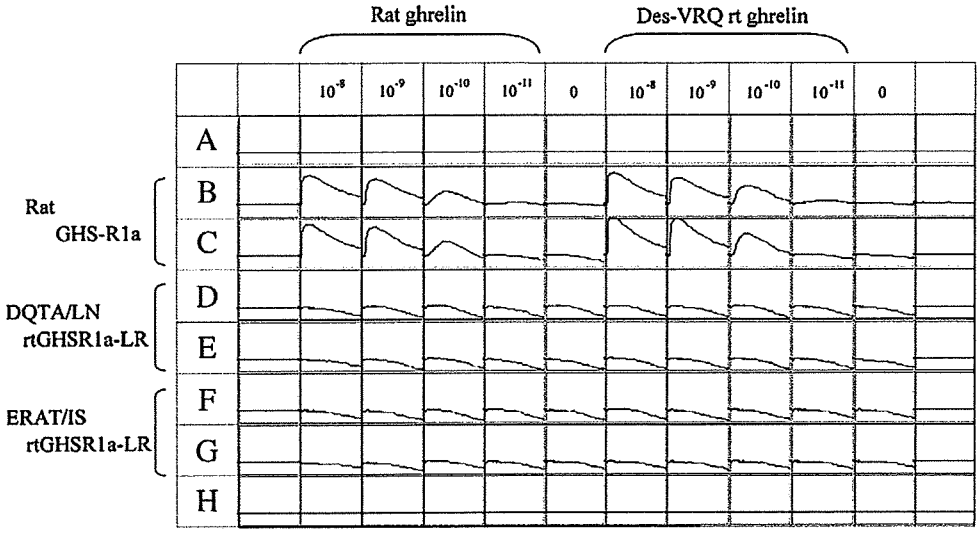

Fig. 9. Changes in intracellular calcium ion concentrations in HEK293 cells transfected with rat GHS-R1a or rainbow trout GHSR1a-like receptor cDNA. Rat ghrelin and des-VRQ trout ghrelin (10^{-8} to 10^{-11} M) were added to cells transfected with rat GHS-R1a cDNA, DQTA/LN-type or ERAT/IS-type trout GHSR1a-like receptor. Each dose was tested in duplicate. The cells that were transfected with trout GHSR1a-like receptor cDNAs did not respond to 10^{-6} M des-VRQ trout ghrelin.

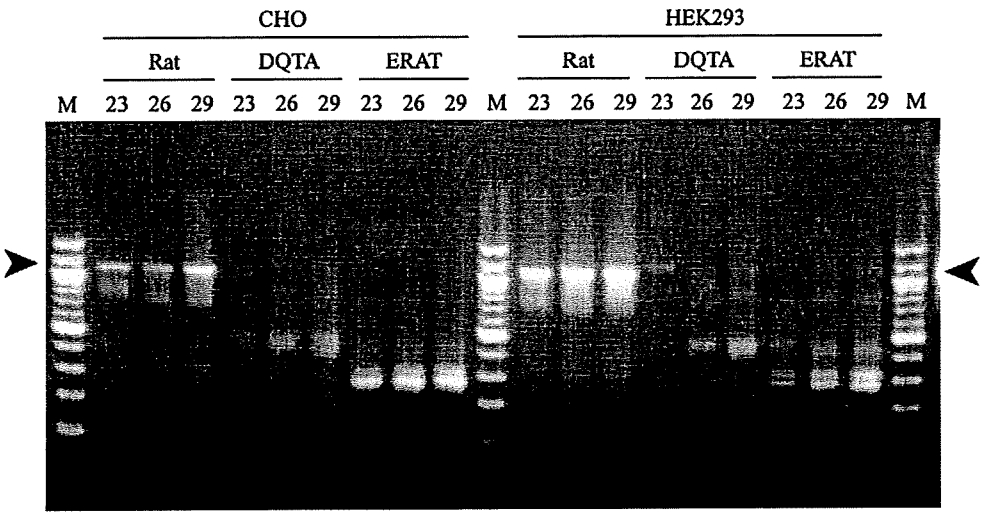

Fig. 10. Expression of GHSR1a-like receptor mRNA in transfected mammalian cells. Expression of full-length GHS-R1a mRNA in CHO and HEK293 cells that transfected rat GHS-R1a, DQTA/LN-type (DQTA) or ERAT/IS-type (ERAT) trout GHSR1a-like receptor cDNA was examined by semi-quantitative RT-PCR (23, 26 and 29 cycles). Expected full-length mRNA showing the size with *arrowheads* was detected in rat GHS-R1a (1.1 kbp), and the receptor was functional as shown in Fig. 9. On the other hand, expected full-length mRNA was not detected for either DQTA/LN-type or ERAT/IS-type trout GHSR1a-like receptor. Instead, a few bands with small size were detected. M: 100-bp molecular ladder.

(Matsuda et al., 2006). Presence of GHS-R1a mRNA in trout hypothalamus would suggest physiological functions such as appetite regulation. In rat and goldfish, central ghrelin led to increased food intake by mediation of hypothalamic orexigenic neuropeptide Y and orexin (Nakazato et al., 2001; Lawrence et al., 2002; Toshinai et al., 2003; Miura et al., 2006, 2007). In chicken, however, the corticotropin-releasing factor (CRF) system mediates the effect of ghrelin, and food intake is oppositely inhibited (Saito et al., 2005). Although a central effect of ghrelin on food intake remains unclear in rainbow trout, trout ghrelin did not stimulate acute food intake when injected peripherally (Jönsson et al., 2007). On the contrary, Shepherd et al. (2007) reported promotion of food intake by peripheral injections of ghrelin and GHS. In rodents, the effect of peripheral ghrelin is mediated though the vagal nerve afferent (Asakawa et al., 2001; Date et al., 2002). Further study is required to clarify the relationship between the effect of ghrelin on appetite regulation and the receptor distribution in rainbow trout.

GHSR1a-LR mRNA expression was low in trout tissues other than the pituitary and brain. Discrepancy of the quantitative relationship between receptor expression level and function of ghrelin has been found. For example, in chickens, expression level of GHS-R1a mRNA in the intestine is 20-times lower than that in chicken pituitary (Geelissen et al., 2003), but ghrelin potently stimulates contraction of the gastrointestinal tract (Kitazawa et al., 2007). In the case of human, GHS-R mRNA and protein are expressed in the gastrointestinal tract, but ghrelin is ineffective in mechanical responses and neural contraction (Dass et al., 2003; Takeshita et al., 2006). We recently observed that ghrelin does not affect intestinal motility in rainbow trout (T. Kitazawa and H. Kaiya, unpublished observations). An interesting issue to solve would be the relationship between the quantity of receptor mRNA expression and the function of ghrelin.

4.4. Three GHSR1b-LR variants in rainbow trout

In general, GHS-R1b is a truncated form of GHS-R1a, which lacks the structure after TM-5, as has been seen in human, pig and black seabream (Chan and Cheng, 2004; Howard et al., 1996). Chicken GHS-R1aV (or 1c) is structurally different from GHS-R1b, and partially lacks TM-6 domain of GHS-R1a (Tanaka et al., 2003; Geelissen et al., 2003). In chickens, Sirotkin et al. (2006) has reported another GHS-R variant, GHS-R1tv, which contains a part of the intron sequences by different splicing pattern from GHS-R1b. Our identified GHSR1b-LR was structurally similar to GHS-R1b, and three GHSR1b-LR proteins were identified: which were composed of 297- or 300-amino acids with DQTA/LN-type and ERAT/IS-type variations in addition to two different C-terminal amino acid sequences. There is no animal having three GHS-R1b proteins. GHS-R1b is not activated by ghrelin or GHS, and physiological importance has not been fully elucidated. Chan and Cheng (2004) reported in sbGHS-R that GHS-R1b attenuates GHS-R1a activity when co-expressed with GHS-R1a in HEK293 cells. Subsequent study revealed that sbGHS-R1b acts as dominant negative mutant for sbGHS-R1a (Leung et al., 2007). Trout GHSR1b-LR may exhibit a similar function in rainbow trout because the structures are similar to sbGHS-R1b.

4.5. Tissue expression of trout GHS-R1b mRNA

In the present study, the tissue expression pattern of GHSR1b-LR mRNA is similar to that of GHSR1a-LR mRNA: predominant expression was seen in the pituitary, and followed by the brain. This is inconsistent with the results observed in human and seabream, where GHS-R1b mRNA is predominantly expressed in the skin (Gnanapavan et al., 2002) and telencephalon (Chan and Cheng, 2004), respectively. Expression level of GHS-R1b was much greater in human skin than that of GHS-R1a (Gnanapavan et al., 2002). In rainbow trout, overall expression level of trout GHSR1b-LR was about 1/8 that of GHSR1a-LR. It is necessary to investigate physiological importance of different expression level in each organ in addition to its function.

4.6. Composition of the trout GHS-R gene

The trout *GHSR-LR* gene was composed of two exons separated by an intron. This is identical to that of the *GHS-R* gene in other animals such as human (Petersenn et al., 2001), chicken (Tanaka et al., 2003), black seabream (Chan and Cheng, 2004) and pufferfish (Palyha et al., 2000). The *GHS-R* gene encodes two receptor proteins, GHS-R1a and GHS-R1b. The former protein consists of the first and second exons, but the latter GHS-R1b protein consists of the first exon and a part of the intron. Especially, the C-terminal amino acid sequence of the GHS-R1b that extends from coding region of the first exon originates from the intron sequence (Chan and Cheng, 2004; Howard et al., 1996). In the present study, we identified DQTA/LN-type and ERAT/IS-type GHSR1a-LR, and presumed that two trout *GHSR-LR* genes would be present. However, the fact that three GHS-R1b orthologs were identified in 3'-RACE PCR and genomic PCR led us to conclude that rainbow trout has three distinct *GHSR-LR* genes that encode GHSR1b-

LR proteins having (1) 300-amino acids with the DQTA/LN-type first exon and an intron exhibiting the TTA sequence (Fig. 6C, Gene-I); (2) 297-amino acids with the DQTA/LN -type first exon and an intron exhibiting the TMF sequence (Fig. 6C, Gene-II); and (3) 297-amino acids with the ERAT/IS-type first exon and an intron containing the TMF sequence (Fig. 6C, Gene-III). It is interesting to note that two *GHSR-LRs* are generated from three genes. A similar gene diversity in salmonids has been reported in the case of the calcitonin (*CT*)/calcitonin-gene related peptide (*CGRP*) gene (Niall et al., 1969; Jansz et al., 1996); three or four CT molecules are identified, and the existence of multiple CT isoforms in salmonids may be explained by the genomic tetraploidization event they underwent, leading to the presence of extensive gene duplication (Vandepoele et al., 2004).

There was the possibility that another second exon, which encodes a common GHS-R1a protein sequence in the coding region and has different 3'-UTR sequence, is present. Un fortunately, we could not identify a novel 3'-UTR sequence by extensive survey of 3'-RACE PCR clones. Further detailed exploration is required to determine the complex composition of the rainbow trout *GHSR-LR* gene.

4.7. Are the identified receptor proteins specific for ghrelin?

Ghrelin led to increased intracellular Ca^{2+} concentrations through GHS-R1a (Howard et al., 1996; Kojima et al., 1999). In the present study, we could not observe such a response in DQTA/LN-type or ERAT/IS-type trout GHS-R1a cDNA-transfected mammalian cells, even when an expected homologous ligand, des-VRQ trout ghrelin or rat ghrelin was treated at doses from 10^{-6} M to 10^{-11} M. On the other hand, rat GHS-R1a cDNA-transfected cells that performed as a positive control clearly responded to ghrelin at a dose of 10^{-9} M. Previously, increases in intracellular Ca^{2+} concentrations or extracellular acidification rates was observed in seabream or pufferfish GHS-R1a-expressed mammalian cells when 10^{-5} M ghrelin or GHS compound was applied (Chan and Cheng, 2004; Palyha et al., 2000). The applied dose was extremely high, but these results suggest that the identified GHS-R1a is functional in these species. We examined the expression of trout GHSR1a-LR mRNA in the transfected cells and found the desired full-length mRNA was not expressed, whereas rat GHS-R1a mRNA was expressed (Fig. 10). Presence of short products that were unexpectedly spliced suggests that the functional trout GHSR1a-LR protein was not produced accurately in mammalian cells used in this study. It may be necessary to use intact cells, e.g., pituitary cells or a cell line from rainbow trout for looking at functional activity of trout GHSR1a-LR to ghrelin.

From another point of view, our identified trout GHSR1a-LR has a longer second extracellular loop, which connects TM-4 and TM-5, in the receptor structure, compared to those of tetrapod GHS-R1a such as chicken, rat and human (Fig. 2). Similar structure has been seen in other fish species such as tilapia, pufferfish and black seabream (Chan and Cheng, 2004; Kaiya et al., in press; Palyha et al., 2000). Interestingly, doses required to activate the pufferfish and seabream receptors are relatively high: Palyha et al. (2000) observed activation of HEK 293-AEQ17 cells expressing pufferfish GHS-R1a with sub-nanomolar range using three GHS (GHRP-6, MK-0677 and L-163,540). Chan and Cheng (2004) observed sbGHS-R1a activation by indication of increased intracellular Ca^{2+} concentrations at a dose of 10 μM human ghrelin and GHSs or increased extracellular acidification rate at a dose of 10^{-5} M GHRP-6. In tilapia GHS-R1a, no response was observed even when a homologous ligand tilapia ghrelin was treated at a dose of 10^{-6} M (Kaiya et al., in press). On the other hand, zebrafish GHS-R1a and 2a do not have such a long second extracellular loop, with the loop structure being similar to those of tetrapod animals (Olsson et al., 2008; Kaiya et al., 2008). This led us to expect activation of the receptor with a similar manner to GHS-R1a for tetrapod animals. In fact, we have isolated GHS-R1a cDNA in goldfish in our laboratory. The receptor is closely similar to zebrafish GHS-R1a and 2a, and goldfish GHS-R1a-transfected HEK293 cells responded well to homologous goldfish ghrelin, rat ghrelin or GHSs at a dose of 10^{-9} M (H. Kaiya, unpublished data). This result strongly suggests that the second extracellular loop plays a crucial role in ligand binding and eliciting subsequent functional activity of GHS-R1a, as has been reported in other GPCRs (Avlani et al., 2007; Banères et al., 2005; Conner et al., 2007; Scarselli et al., 2007; Shi and Javitch, 2004; Matsuura et al., 2006). To conclude that the identified GHSR1a-LR is the receptor for ghrelin, we have to demonstrate ghrelin binding to the receptor and cell activation mediated through the receptor by ghrelin and GHS.

5. Conclusions

In this study, we identified trout GHSR1a-LR, as determined from the deduced amino acid sequence, gene organization and expression pattern of the mRNA. Three distinct genes generate two GHSR1a-LR and three GHSR1a-LR. Unfortunately, we could not confirm functional activity of the trout GHSR1a-LR in mammalian cells. Further study is required to conclude this identified receptor protein is the ghrelin receptor in rainbow trout. For this purpose, it appears necessary to establish an evaluation system using fish cells or trout itself. Conclusively, we propose to designate this identified receptor protein as GHS-R-like receptor (GHSR-LR).

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Ghrelin-like peptide with fatty acid modification and O-glycosylation in the red stingray, *Dasyatis akajei*

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Abstract

Background: Ghrelin (GRLN) is now known to be an appetite-stimulating and growth hormone (GH)-releasing peptide that is predominantly synthesized and secreted from the stomachs of various vertebrate species from fish to mammals. Here, we report a GRLN-like peptide (GRLN-LP) in a cartilaginous fish, the red stingray, *Dasyatis akajei*.

Results: The purified peptide contains 16 amino acids (GVSFHPQPRS[10]TSKPSA), and the serine residue at position 3 is modified by *n*-octanoic acid. The modification is the characteristic of GRLN. The six N-terminal amino acid residues (GVSFHP) were identical to another elasmobranch shark GRLN-LP that was recently identified although it had low identity with other GRLN peptides. Therefore, we designated this peptide stingray GRLN-LP. Uniquely, stingray GRLN-LP was *O*-glycosylated with mucin-type glycan chains [*N*-acetyl hexosamine $(HexNAc)_3$ hexose$(Hex)_2$] at threonine at position 11 (Thr-11) or both serine at position 10 (Ser-10) and Thr-11. Removal of the glycan structure by *O*-glycanase made the *in vitro* activity of stingray GRLN-LP decreased when it was evaluated by the increase in intracellular Ca^{2+} concentrations using a rat GHS-R1a-expressing cell line, suggesting that the glycan structure plays an important role for maintaining the activity of stingray GRLN-LP.

Conclusions: This study reveals the structural diversity of GRLN and GRLN-LP in vertebrates.

Background

Ghrelin (GRLN), which generally consists of 28 amino acids, was first identified in the stomachs of rats and humans as an endogenous ligand for the growth hormone secretagogue-receptor 1a (GHS-R1a)[1]. The serine residue at position 3 of this peptide (Ser-3) contains a unique octanoyl modification, and the acylation is necessary for the peptide to bind and activate GHS-R1a [1,2]. In mammals, GRLN is an important hormone involves in various physiological events such as pituitary, cardiovascular, steroidogenic, and developmental functions and energy homeostasis [3-6].

In non-mammals, GRLN has been identified in species from fish to birds (reviewed by [7-11]). Very recently, endogenous GRLN form was determined in a bony fish, goldfish [12]. Non-mammalian mature GRLNs are composed of 17 to 28 amino acids, and are known to involve in regulating pituitary functions in teleosts [6], amphibians [13] and birds [14], and feeding in teleosts [15-19] and birds [7,20-23]. An inhibitory effect of GRLN on drinking in birds (chicken) was also recently reported [24].

We identified a GRLN-like peptide (GRLN-LP) in the stomach of primitive vertebrates, cartilaginous fish, the hammerhead shark (*Sphyrna lewini*) and blacktip reef shark (*Carcharhinus melanopterus*) [25]. The GRLN-like peptide from both sharks consisted of 25 amino acids, and only three amino acids differed between the two species. Like other vertebrate GRLN, Ser-3 of GRLN-LP from sharks had been modified by *n*-octanoic or *n*-decanoic acid. However, the first seven N-terminal residues, which are generally highly conservative region of GRLN including the active core, had low identity between sharks (GVSFHPR) and those of other species (GSSFLSP, GTSFLSP and GSTFLSP). This difference is one of the reasons why this peptide was designated GRLN-LP though there is Ser-3 acylation. In addition to this, the C-terminal end of the shark GRLN-LP has not been amidated, which is a specific feature of teleost GRLN. However, we have proposed that shark GRLN-LP exhibits ancestral features of GRLN molecule that are present in higher vertebrate.

Here we report the structure of GRLN-LP from the stomachs of another elasmobranch, the red stingray, *Dasyatis akajei*, and identification of the cDNA that encodes the precursor protein. Interestingly, we revealed that the stingray GRLN-LP is not only octanoylated at Ser-3 but also possesses a mucin-type glycan structure at threonine (Thr)-11 or both Ser-10 and Thr-11.

Methods

Purification of GRLN-LP from stingray stomachs

Stingrays, *Dasyatis akajei*, were collected at Toyama bay (Toyama, Japan). Frozen stomachs (approximately 40 g) were used as the starting material. All animal experiments were conducted in accordance with Guidelines for the Care and Use of Animals of the University of Toyama and of National Cardiovascular Center (ref. no. 8053). GRLN was purified as previously described [26] with slight modifications. During the purification process, GRLN activity was monitored by measuring changes in the intracellular calcium ion (Ca^{2+}) concentrations in a cell line that stably expressing rat GHS-R1a (CHO-GHSR62).

Stomach tissues were boiled in five volumes of Milli-Q level water, minced, acidified with concentrated acetic acid (AcOH) to 1 M, and homogenized. The supernatant was obtained by centrifugation. Next, cold acetone was added to the AcOH-extracts at a final concentration of 66%, after which the mixture was stirred over night and then centrifuged. The resulting supernatant was evaporated and purified with a Sep-Pak Vac 35 cc C18 cartridge (Waters, Milford, MA) to enrich the peptide components. The cartridge was successively eluted with 25% acetonitlile containing 0.1% trifluoroacetic acid (TFA) and 60% acetonitlile containing 0.1% TFA. The lyophilized Sep-Pak fraction that was eluted with 60% acetonitlile containing 0.1% TFA was dissolved in 1 M AcOH, and then subjected to cation-exchange chromatography using a SP-Sephadex C-25 (GE Healthcare UK Ltd., Buckinghamshire, England). Successive elution with 1 M AcOH, 2 M pyridine and 2 M pyridine-AcOH (pH 5.0) yielded three fractions: SP-I, SP-II and SP-III, respectively.

The basic peptide-enriched SP-III fraction was subjected to carboxymethyl (CM)-ion exchange HPLC (TSKgel CM-2SW, 4.6 × 250 mm, Tosoh, Tokyo, Japan) at a flow rate of 1 ml/min. A two-step gradient was made from solvent A (10 mM ammonium formate (pH 4.8) in 10% acetonitlile) to 25% solvent B (1 M ammonium formate (pH 4.8) in 10% acetonitlile) for 10 min and then to 55% solvent B for 90 min. The eluate was collected in 1-ml fractions from the start of the gradient program. Based on the assay results, GRLN activities were eluted in clusters. Thus, we performed secondary CM-HPLC on the active fractions with a more shallower two-step gradient profile from solvent A to 15% solvent B for 10 min and then to 35% solvent B for 80 min. The eluate was collected in 1-ml fractions every 1 min, 20 min after injection into the HPLC system.

Active CM-HPLC fractions were desalted by Sep-Pak treatment, lyophilized, and then subjected to an anti-rat GRLN1-11 immunoglobulin G (IgG) immuno-affinity column to purify GRLN-immuno-cross reactive substances [26]. This immuno-affinity column effectively absorbed the shark GRLN-LP [25]. The adsorbed substances were eluted with 60% acetonitlile containing 0.1% TFA and then separated by two different reverse-phase (RP)-HPLC methods. The samples were first applied to a preparative RP-HPLC with a Symmetry C18 column (3.9 × 150 mm, Waters) at a flow rate of 1 ml/min under a linear gradient from 10% to 60% acetonitlile containing 0.1% TFA for 40 min. Active fractions were further purified on a Symmetry C18 column (2.1 × 150 mm, Waters) or a diphenyl column (2.1 × 150 mm, 219TP5215, Vydac, Hesperia, CA) at a flow rate of 0.2 ml/min under a gradient from 10% to 60% acetonitlile containing 0.1% TFA for 40 min. The eluate that corresponded to each absorbance peak was collected. For peptide sequencing, approximately 5 pmol of the purified

peptide, which was estimated based on the absorbance peak height, was subjected to protein sequencing (model 494, Applied Biosystems, Foster City, CA).

Mass spectrometry

The molecular weights of the purified peptides were determined using matrix-assisted laser desorption ionization-time of flight-time of flight (MALDI-TOF-TOF) mass spectrometry (4700 proteomics analyzer, Applied Biosystems, Foster City, CA) with α-Cyano-4-hydroxycinnamic acid (CHCA) (Sigma-Aldrich Chem. Corp., WI) as a matrix.

Prediction of O-glycosylation sites

The detected peptide mass and its profile indicated that the peptide was possibly glycosylated. The NetOGlyc program, which is available at the Center for Biological Sequences CBS Prediction Server http://www.cbs.dtu.dk, was used to predict the potential *O*-glycosylation sites.

Cloning of stingray GRLN-LP cDNA

The nucleotide sequence of stingray GRLN-LP cDNA was determined using the Rapid Amplification of cDNA Ends (RACE) PCR Kit (Invitrogen, Carlsbad, CA). Total RNA (2 μg) was extracted from a stingray stomach and then reverse-transcribed using Omniscript RT (QIAGEN GmbH, Hilden, Germany). For 3'-RACE PCR, four degenerate primers were designed based on the sequence of the seven N-terminal amino acids in stingray GRLN-LP ($G^1VSFHPQ^7$) that was identified by protein sequencing. Among these primers, the rayGRL-s2 primer (5'-GGN GTN AGY TTY CAY CCN CA-3') effectively amplified expected cDNA fragment. PCR was performed using 50 pmol/reaction of rayGRL-s2, an adaptor primer supplied in the kit, *ExTaq* DNA polymerase (TaKaRa, Shiga, Japan) under the following amplification conditions: 94°C for 1 min, 35 cycles at 94°C for 30 sec, 53°C for 30 sec, and 72°C for 1 min; followed by a final extension for 3 min at 72°C. The amplified products were purified by the Wizard PCR Preps (Promega, Madison, WI). Second-round nested PCR was performed on the purified cDNA with four degenerate sense primers (50 pmol/reaction) that were based on the amino acid sequence of stingray GRLN-LP ($F^4HPQPRS^{10}$). Among these primers, two primers, rayGRL-s6 (5'-TTY CAY CCN CAR CCN CGN AG-3') and rayGRL-s8 (5'-TTY CAY CCN CAR CCN AGR AG-3') effectively amplified expected cDNA fragment under the following conditions: 94°C for 1 min, 35 cycles at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min; and a final extension for 3 min at 72°C. The approximately 400-bp amplified products were subcloned into the pCRII-TOPO vector (Invitrogen). The nucleotide sequence of the insert was determined by a DNA sequencer (ABI PRISM 3100 Genetic analyzer, Applied Biosystems), according to the BigDye® Terminator Cycle Sequencing Kit protocol (Applied Biosystems) using the M13 forward or reverse primer.

For 5'-RACE PCR, two gene-specific primers were designed based on the partial sequence of the stingray GRLN-LP cDNA that was obtained by 3'-RACE PCR: rayGRL-as1, 5'-GGA CGA TGC ATT GAT CTG CGG-3' and rayGRL-as2, 5'-CCC GTT CAG GTC GGA CGA TGC-3'. Primary PCR was performed using rayGRL-as1, an anchor primer supplied in the 5'-RACE Kit, and *ExTaq* DNA polymerase under the following reaction conditions: 94°C for 2 min, 35 cycles at 94°C for 30 sec, 56°C for 30 sec, and 72°C for 1 min; and a final extension for 3 min at 72°C. The second-round nested PCR was performed with 5 pmol/reaction of the rayGRL-as2, an abridged universal amplification primer (AUAP) supplied in the 5'-RACE Kit, and *ExTaq* DNA polymerase under the same conditions described above. The rayGRL-as2 primer was designed inside the rayGRL-as1 primer, but nine nucleotides on the 3'-side of the rayGRL-as2 were identical to the primer sequence of rayGRL-as1. Thus, the target cDNA could be amplified effectively by the second-round nested PCR. The amplified products, which were approximately 480 bp, were subcloned into the pCRII-TOPO vector and sequenced.

Amplification of the full-length stingray GRLN-LP cDNA

To confirm the precise nucleotide sequence of the full-length stingray GRLN-LP cDNA, we performed PCR using a proofreading, *Pyrobest* DNA polymerase (TaKaRa). The template cDNA for the 3'-RACE PCR was used because the Tm of the antisense primer for the 3'end of the stingray GRLN-LP cDNA was too low to amplify the full-length cDNA. Thus, PCR was conducted using a sense primer from the 5'end of the stingray GRLN-LP cDNA (5'-ACG ACC ACA GAT CCA ACT CGA-3') and AUAP under the following conditions: 98°C for 30 sec, 30 cycles at 98°C for 15 sec, 60°C for 30 sec, and 72°C for 1 min. For TA cloning, an additional 10-min cycle at 72°C was performed using *ExTaq* DNA polymerase (overhang reaction). The amplified product was subcloned into the pCRII-TOPO vector and sequenced.

The putative stingray GRLN-LP sequence was analyzed by BLAST against the NCBI database, and amino acid sequence alignment and identity analysis were performed by multiple comparison and maximum matching program using GENETYX-Mac ver. 15.0.1 (gap penalty, insert: -1; extend: -1 for multiple comparison, and default condition for maximum matching). Phylogenetic tree was made using Mega 4 software http://www.megasoftware.net/.

Deglycosylation with O-glycanase

Stingray GRLN-LP was *O*-glycosylated with mucin-type sugar chains. To examine the role of this sugar chain modification in the GRLN-like activity of stingray GRLN-LP, a high-yield preparation of purified stingray GRLN-LP (peak 2, Table 1) was treated with the *O*-glycanase, end-α-