Table 1: Summary of stingray GRLN-LP purification

Group	Peak	Yields (pmol)	Mass-1 $[M+H]^+$	Mass-2 $[M+H]^+$
A	1	10	2580.15	
B	2	24	3351.68	3513.74
	3	4	3375.42	3537.46
C	4	21	2580.26	2742.33
	5	4	2604.15	2766.20
	6	3	2604.17	2766.26
D	7	2	2594.15	2756.21

N-acetylgalactosaminidase (Calbiochem, Darmstadt, Germany). This enzyme catalyzes the hydrolysis of the unsubstituted Galβ1,3GalNAc core dissaccharide attached to a Ser or Thr of glycopeptides to generate free oligosaccharides. Approximately 5 pmol of native stingray GRLN-LP (peak 2), synthesized stingray des-acyl GRLN-LP, and octanoylated stingray GRLN-LP were incubated separately with 1.25 mU *O*-glycanase in 100 μl 50 mM sodium phosphate (NaH_2PO_3) buffer (pH 5.0) for 16 h at 37°C, followed by an incubation for 15 min at 70°C to inactivate the enzyme. The reaction mixture was subjected to RP-HPLC on a Symmetry C18 column (2.1 × 150 mm, Waters) at a flow rate of 0.2 ml/min under a linear gradient from 10% to 60% acetonitlile containing 0.1% TFA for 40 min. The absorbance peaks that corresponded to each catalyzed peptide were collected. Two peaks appeared from this reaction: one was stingray GRLN-LP without sugar chains, and the other was the original native peptide. We did not observe any changes in the elution pattern of synthetic stingray des-acyl GRLN-LP and octanoylated GRLN-LP after the catalytic treatment.

Quantitative real-time PCR of stingray GRLN-LP in stingray tissues

We performed quantitative real-time PCR (qPCR) to examine the GRLN-LP cDNA expression levels in various stingray tissues. Total RNA was extracted from 21 tissues from two separate stingrays to examine the variation in expression levels. First-strand cDNAs were synthesized from 1 μg of DNase-I- (Invitrogen) treated total RNA using the QuantiTect RT Kit (QIAGEN GmbH) and an oligo-dT_{12-18} primer. PCR amplification was performed using the LightCycler system (Roche Applied Science, Mannheim, Germany) and the QuantiFast SYBR Green PCR Kit (QIAGEN GmbH). To generate a standard curve, full-length stingray GRLN-LP cDNA or partial stingray β-actin cDNA fragment in the pCRII-TOPO vector was linearized by *Xba*-I digestion. The linearized plasmid was serially diluted from 5×10^6 to 5×10^3 molecules to generate a standard curve that was used to determine the cDNA copy number. All qPCR amplifications were performed in duplicate. All specific quantities were normalized as the copy number relative to the total RNA [27] and to stingray β-actin. For the GRLN-LP analysis, sense (5'-TCC CTC ACC CTC AAG GCA GAG-3') and antisense primers (5'-TCAT CTC CCA CTG GCA ACT GG-3') were designed to amplify a 170-bp product. For the β-actin analysis, sense (5'-GAT CTG TAT GCC AAC AAC GTC-3') and antisense primers (5'-CAG AGA TGC CAG AAT AGA GCC-3') were designed to amplify a 194-bp product. The PCR reaction mixture (20 μl) contained 100 ng of cDNA, 1× QuantiFast SYBR Green PCR mix, and 5 pmol of each primer was prepared according to the manufacturer's protocol. The PCR conditions were 95°C for 5 min, 35 cycles at 95°C for 10 sec, 60°C for 30 sec. Amplified products were electrophoresed on a 1.5% agarose gel to determine the product size and quantity.

Results

Purification of stingray GRLN-like peptide

The SP-III fraction from the SP-Sephadex C-25 cation-exchange chromatography contained GRLN-like activity. Next, we subjected the basic peptide-enriched SP-III fraction to CM-HPLC (pH 4.8) with a gradient program. However, the GRLN-like activities are present in positions where a large number of peptides eluted (fractions 17-29, data not shown). To reduce the peptide content, active fractions were subjected again to CM-HPLC with a much shallower two-step gradient profile. As a result, the GRLN-like activity was divided into four groups from A to D (Fig. 1A). Each group was purified by anti-rat GRLN1-11 IgG immuno-affinity column chromatography, followed by RP-HPLC until a single peak was isolated. Figs. 1B and 1C show representative preparative RP-HPLC and final RP-HPLC profiles after immuno-affinity chromatography of group B (Fig. 1A).

The estimated peptide yield from each peak height is summarized in Table 1. Overall, seven species of peptides were isolated. The amino acid sequences of peptides in high yield peaks 2 and 4 were analyzed. The amino acid sequences of 16 residues were determined: GVXFHPQPRXXSKPSA for peak 2 and GVXFHPQPRSXSKPSA for peak 4 (X, unidentified). The amino acid at position 3 was not detected, probably due to the characteristic acyl modification at this position of GRLN. However, the reason why amino acid at positions 10 and 11 could not be identified was uncertain.

Cloning of cDNA encoding the stingray peptide precursor

To determine the complete amino acid sequence of the stingray peptide, we isolated cDNA encoding the peptide precursor from stingray stomach mRNA based on the identified amino acid sequence. The identified nucleotide sequence of the stingray peptide was 527 bp in length, which consisted of a 90-bp 5' untranslated region (UTR), a 297-bp coding region, and a 140-bp 3'UTR (accession number AB480033, Fig. 2A). A polyadenylation signal

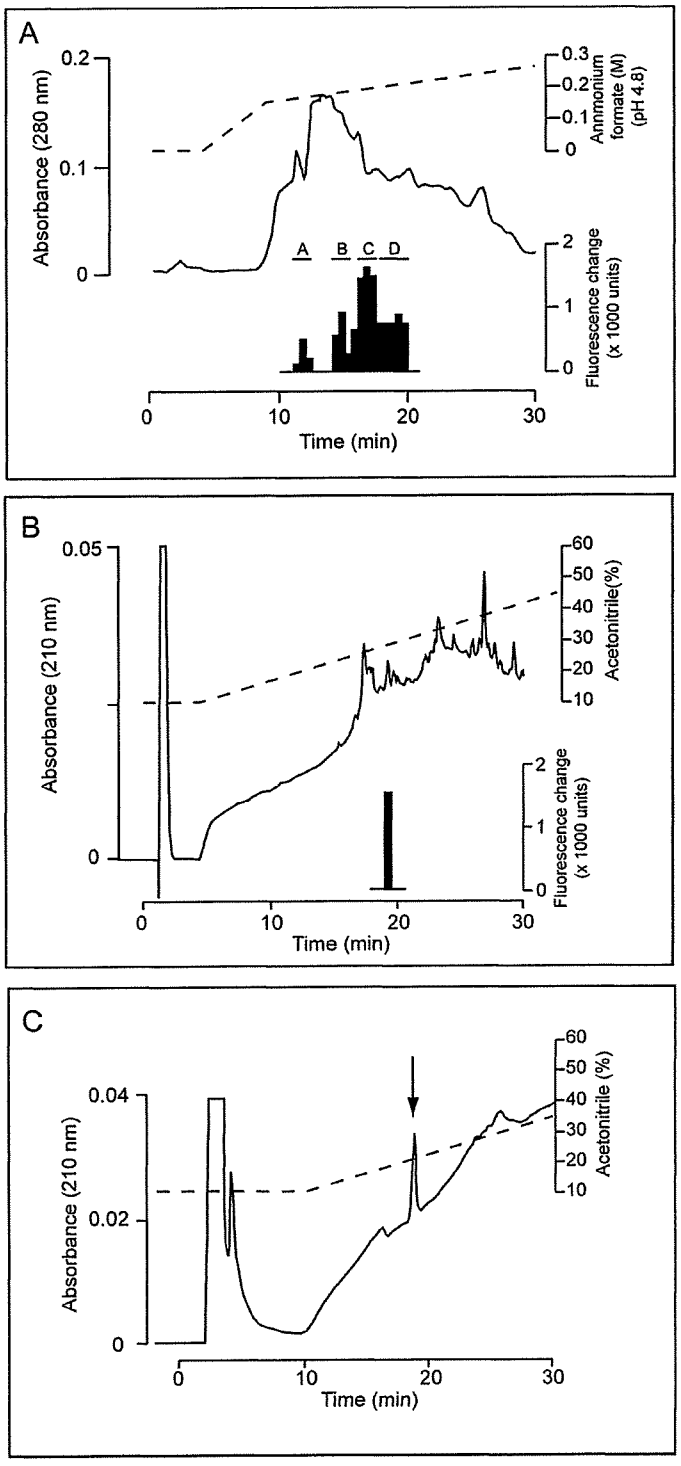

Figure 1

Purification of stingray ghrelin-like peptide (GRLN-LP) from stomach extracts. *Black bars* indicate the measured fluorescence changes in intracellular calcium ion concentrations in CHO cells expressing rat GHS-R1a (CHO-GHSR62). (A) Carboxymethyl (CM)-cation ion-exchange HPLC (pH 4.8) of the SP-III fraction of stomach extracts. The GRLN-like activity was divided into four groups (A-D). (B) Preparative reverse-phase (RP)-HPLC (Symmetry C18, 3.9 × 150 mm) of group B after purification with an anti-rat GRLN1-11 immuno-affinity column. (C) Final purification of the active fraction indicated in (B) by another RP-HPLC (Vydac diphenyl, 219TP5215, 2.1 × 150 mm).

was present in the 3'UTR (positions 506-511). The deduced amino acid sequence showed that the stingray peptide precursor consisted of 98 amino acids. The unidentified amino acids at positions 3, 10 and 11 were identified as Ser-3, Ser-10 and Thr-11, respectively. Therefore, this newly identified stingray peptide contains 16 amino acids, GVSFHPQPRSTSKPSA. A typical dibasic processing signal (arginine-arginine, RR) followed the peptide, and processing at this site generated a peptide that was identical to the purified peptide. In addition, we identified another dibasic processing signal 14-amino acids downstream of the first processing sequence (Fig. 2A). Based on this motif, it is expected that a 31-amino acid peptide could be generated. However, this 31-amino acid peptide was not identified during this purification process. Deduced stingray peptide precursor showed relatively high sequence identities compared with shark GRLN-LP precursors (Fig. 2B, Table 2). Multiple comparisons of the precursor protein revealed that the identity of the stingray peptide was low compared to GRLNs from teleosts to mammals (Fig. 3, Table 2). Fig. 4 shows a phylogenetic tree of GRLN, and the stingray peptide was categorized in the same clade as shark GRLN-LP.

Mass spectrometric analyses of the purified stingray peptide

The protein sequencing results strongly indicated that Ser-3 was acylated. The theoretical mono-isotopic mass of a 16-residue peptide with *n*-octanoic acid modification is 1808.87 $[M+H]^+$. During the analysis, we observed two types of mass peak profiles; one profile showed a single peak, while the other exhibited two peaks that had a constant mass difference of 162.1 (Fig. 5). The pattern of this spectrum is characteristic of glycosylated proteins or peptides, and the mass difference corresponded to the mass of hexose (Hex). The mass values measured for the peptides were greater from 795.3 to 957.3 than the predicted mass for an octanoylated 16-amino acid peptide (Table 1). Therefore, we assumed that all purified peptides were glycosylated.

Table 2: Amino acid sequence identity of stingray preproGRLN-LP with other preproGRLNs

Species	Identity (%)	Accession No.
Arctic char	21.1	AB490668
Atlantic halibut	23.3	EF493849
Blacktip-reef shark	34.5	AB254129
Broiler chicken	26.9	AB075215
Bullfrog	26.3	AB058510
Channel catfish-1	26.8	AB196449
Goldfish	25.9	AF454389
Hammerhead shark	32.7	AB254128
Human	25.8	AB029434
Japanese eel	19.8	AB062427
Large yellow croaker	27.0	FJ560488
Mozambique tilapia	22.9	AB077764
Orange-spotted grouper	23.6	DQ343147
Rainbow trout-1	20.7	AB096919
Sea bass	22.4	DQ665912
Seabream (Black porgy)	20.6	AY643808
Turtle-2	21.4	AB161458
Zebrafish	25.2	NM_001083872

We analyzed the potential glycosylation sites in the identified peptide sequence using the NetOGlyc program, and found that Thr-11 is a potential *O*-glycosylation site (G-score 0.728, I-score 0.521). Interestingly, Thr-11 corresponded to one of the unidentified amino acids in the protein sequencing. The most common type of *O*-glycosidic linkage is an attachment through the *N*-acetyl hexosamine (HexNAc) to the side chain of Ser or Thr. Thus, we analyzed peak 3 (parent masses of m/z 2580.26 and 2742.33) using MALDI-TOF MS/MS spectrometry. As a result, we were able to assign a mass to the octanoylated stingray peptide (1808.8 $[M+H]^+$) from each parent mass (data not shown), indicating that the peak 3 stingray peptide is octanoylated. In addition, glycoside and glycochain-specific masses, Hex (162), HexNAc (203) and HexNAc-Hex (365) were assigned based on an MS/MS analysis of the parent mass of m/z 2580.26, and HexNAc3-Hex1 (771) and HexNAc3-Hex2 (933) were identified from an MS/MS analysis of the parent mass of m/z 2742.33 (data not shown). Since one unit of HexNAc-Hex was assigned, it was predicted that this glycochain might have a mucin-type core-1 or core-2 structure. Based on this information, we next attempted to identify the composition of the mucin-type glycan chains using a purified peptide from peak 5 (parent mass of m/z 2766.20). The fragment masses that were estimated based on the expected glycan chains are shown in Table 3. In this analysis, the peak observed at m/z 1832.8 was m/z 24 greater than the mass of octanoylated 16-amino acid peptide. We could not be identified what is the origin of m/z 24. Fig. 6 shows the fragment mass spectra obtained from an MS/MS analysis of the parent mass. Each fragment mass shown in Table 3 was assigned as shown in Figs. 6A to 6E. Finally, the structure of the mucin-type glycan chains was predicted as shown in Fig. 6F. Representative predicted primary structures of the stingray peptide are shown in Fig. 7. A mass difference of 162.1 is considered to occur when one Hex is deleted from the glycan chain, suggesting that the mass-2 shown in Table 1 is the molecular mass of the original form. Table 4 summarizes the predicted compositions of the glycan chains in the isolated stingray peptide. The mass spectrometric analysis showed that all purified peptides would be modified by *n*-octanoic acid. Based on the high identity with shark GRLN-LP and the octanoyl modification of this peptide, we named this peptide stingray GRLN-LP.

A

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  1  ACGACCACAGATCCAACTCGACTGGTCAGAAGAGGCAGACAGGTGACTGGGCATAGACTG   60
 61  GAGACTGAAGCAGCCAGGCCTGGTCTCGGAATGGAGGGTGCTCGTCTGCTCGTCGTCCTG  120
                                      M  E  G  A  R  L  L  V  V  L
121  CTGTCCGCGGGTCTTCTCGCCTCCCTCACCCTCAAGGCAGAGGCGGGCGTCAGCTTCCAC  180
      L  S  A  G  L  L  A  S  L  T  L  K  A  E  A  G  V  S  F  H
181  CCCCAACCCCGGAGCACTTCGAAACCAAGTGCAAGGCGGGAGGTGTACGACAACATGTTC  240
      P  Q  P  R  S  T  S  K  P  S  A  R  R  E  V  Y  D  N  M  F
241  TTTCAGGTGGAGGGAGATCGACGTGACCCTGCGGCGCAGCGGGTGCCCTCCCAGTTGCCA  300
      F  Q  V  E  G  D  R  R  D  P  A  A  Q  R  V  P  S  Q  L  P
301  GTGGGAGATGATGACGCTCAGCAGTACCGAGATCTGCTGTTGCAGTTGTTTGACAGCCTG  360
      V  G  D  D  D  A  Q  Q  Y  R  D  L  L  L  Q  L  F  D  S  L
361  CTGGGATCGGGAGGACAGGGAAACTGACTGCCTTCCCCTTCGGCCTCGCCGCAGATCAAT  420
      L  G  S  G  G  Q  G  N  *
421  GCATCGTCCGACGTGAACGGGGCATGTCGAGGGTTGTTTAACTCCGCGAGCCCCGCTCTC  480
481  TTCAACCTTTGTAGAAGTAGTGACGAATAAAGCAACAATTGTCAGCG  527
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B

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Stingray                1 MEGARLLVVL-L-SAGLLASLT-LKAEAGVSFHP---QPR--STS-----K---PSARREV   45
Blacktip-reef shark     1 MK-P-L-I-LAMFAVALLATLAA-EAEAGVSFHPRLKE-KDDNSSGNTRKFS-P--KRQL   51
Hammerhead shark        1 MK-P-L-I-LAMFAVALLVSLTA-ETEAGVSFHPRLKE-KDDNSSGNSRK-SNP--KRQL   51

Stingray               46 YDNM-FFQV-EGDRRDPA-AQ--RVPSQLPV--GDDDAQQYRD-LLLQLF-DSLLGSGG-   95
Blacktip-reef shark    52 YEDVAI-QMEEGQS-DSALAQAG-IPSQLGLRKSKADSMQYAEQML-QMLSD-LLDSDDS   106
Hammerhead shark       52 YEDVSL-QMKEGQS-DSAVAQAG-IPSQLGLRKSKADSAQYTEQML-QMLSA-LLGSDDS   106

Stingray               96 QGN                                                          98
Blacktip-reef shark   107 QS-                                                         108
Hammerhead shark      107 QN-                                                         108
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Figure 2

Nucleotide sequences and deduced amino acid sequence of stingray GRLN-LP. (A) Nucleotide sequence and deduced amino acid sequence of stingray GRLN-LP. The cDNAs have been deposited in the DDBJ/EMBL/GenBank™ databases (AB480033). *Bolded letters* indicate isolated peptide. A typical processing signal (RR) is *boxed*, and the polyadenylation signal (AATAAA) is *underlined*. (B) Comparison of the amino acid sequences of GRLN-LP in elasmobranch. Sequence alignment was performed using GENETYX-Mac ver 15.0.1. The sequences that are identical to all species are densely shadowed, and the sequences conserved in more than two species are thinly shadowed.

Tissue expression of GRLN-LP mRNA in stingray

Stingray GRLN-LP mRNA is expressed in almost of all of the examined tissues. The highest expression levels were in the stomach, with moderate levels in the pituitary, esophagus and duodenum (Fig. 8, top). Expression level of the β-actin gene varied among tissues (Fig. 8, middle): muscle tissues highly express the β-actin gene compared to soft tissues such as brain. Ratio of GRLN-LP mRNA to the β-actin gene was the highest in the stomach, followed by the rectal gland (Fig. 8, bottom).

Biological activity of stingray GRLN-LP

The unglycosylated synthetic stingray GRLN-LP dose-dependently increased intracellular Ca^{2+} levels in CHO-GHSR62 cells stably expressing rat GHS-R1a (Fig. 9A). Compared to the homologous ligand, rat GRLN, the dose-response curve for the stingray peptide was shifted to the right, suggesting that the stingray peptide has a lower affinity for rat GHS-R1a than rat GRLN. Catalysis of the *O*-glycosylation of the native (glycosylated) stingray GHRL-LP reduced this activity (Fig. 9B, bottom).

Discussion

In the present study, we identified a 16-amino acid peptide that exhibits GRLN-like activity in the stomach of an elasmobranch, the red stingray. Only a few peptide hormones have been identified in the stingray, notably calcitonin [28], relaxin-like molecule [29], pituitary adenylate cyclase-activating polypeptide (PACAP) [30] and proopiomelanocortin [31]. The result of the present study would become one of valuable information on peptide that exists in stingray.

GRLN is characterized by the modification of Ser-3 with fatty acids such as *n*-octanoic or *n*-decanoic acid in both mammals and non-mammals [3,8]. This acylation is

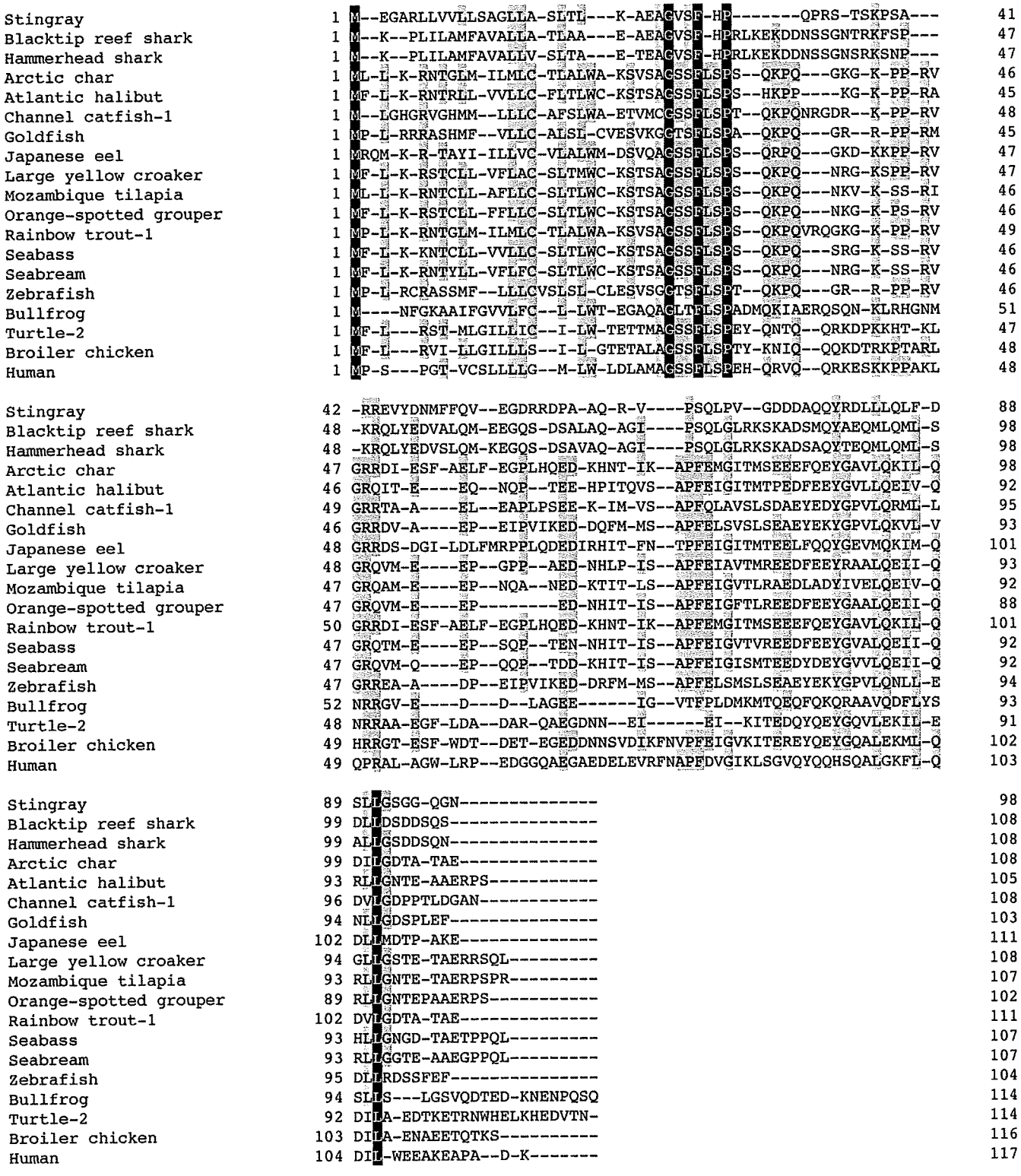

Figure 3

Multiple comparisons of amino acid sequence of stingray GRLN-LP. Sequence alignment was performed using GENETYX-Mac ver 15.0.1. Amino acids that are identical to all species are densely shadowed, and amino acids conserved in more than two species are thinly shadowed.

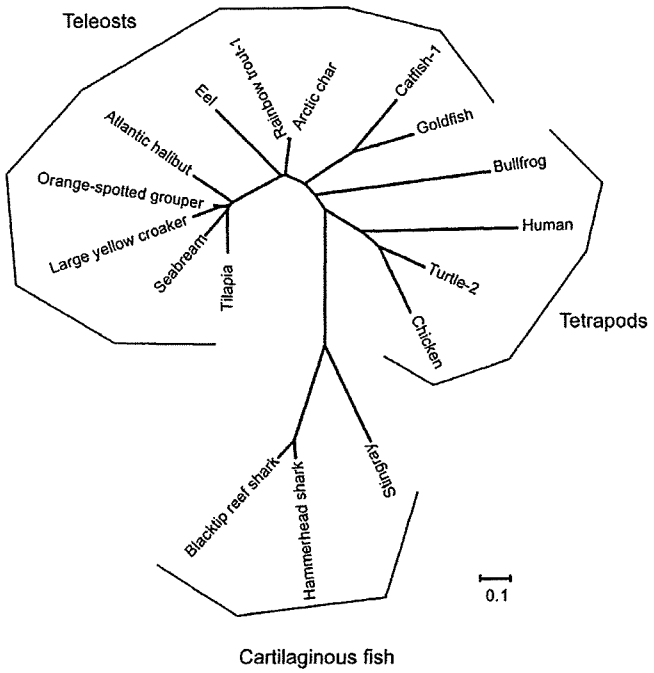

Figure 4
Phylogenetic analysis of stingray GRLN-LP. Phylogenetic tree was generated using the neighbour-joining method by MEGA4 http://www.megasoftware.net/(Tamura et al., 2007). Amino acid sequences are available from the DDBJ/EMBL/GenBank databases as shown in Table 2.

essential for GRLN to bind to GHS-R1a and elicit GRLN activity [2]. Isolated stingray peptides activated a rat GHS-R1a-expressing cell line, suggesting that all peptides have GRLN-like structures, and the amino acid at position 3 may be acylated. In fact, peptide sequence and mass spectrometric analyses revealed that amino acid sequences of isolated stingray peptides are similar to shark GRLN-LP [25], and the isolated peptides were octanoylated. These data strongly suggest that the identified peptides are stingray GRLN or GRLN-LP.

We have identified GRLN-LP in another elasmobranch shark [25]. The peptide was named GRLN-LP because it had the following characteristics: (1) rat GHS-R1a activation properties, (2) presence of acyl-modifications at Ser-3, (3) predominant mRNA expression in the stomach and (4) similar gene organization compared to the GRLN gene. The newly identified stingray peptide satisfies three of these four structural features with an exception (4). Furthermore, the six N-terminal amino acids (GVSFHP) are identical between shark and stingray. In a phylogenetic analysis of the precursor protein, the stingray peptide and shark GRLN-LPs belong to the same clade. Although it is necessary to confirm that the stingray peptide possesses the characteristic GRLN activities such as stimulation of GH release and hyperphagia using a stingray or rodent model, we designate this peptide stingray GRLN-LP. Strictly, all structures, including glycosylation as mentioned below, are present in stingray GRLN-LP. It is interesting to note that mechanisms governing the acylation of Ser-3 are conserved from elasmobranches to mammals. Recently an acyltransferase, namely ghrelin-*O*-acyltransferase (GOAT), was identified [32,33], although the detailed mechanisms of acylation are still unknown [34,35].

Stingray GRLN-LP possessed *O*-glycosylation at Ser-10 and Thr-11, in addition to the acyl-modification at Ser-3. This glycosylation has not been seen in other GRLN/GRLN-LP so far. To date, little is known about the presence of *O*-glycosylated peptide hormones other than glycoproteins or peptides such as gonadotropins,

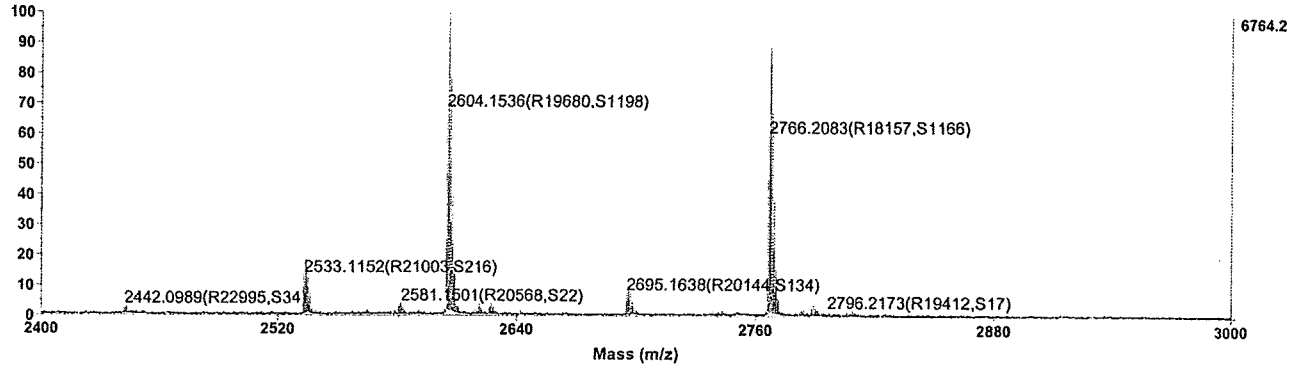

Figure 5
MALDI-TOF mass spectrum of an isolated stingray GRLN-LP. Peak 5 peptide, which is indicated in Table 1, was analyzed.

Table 3: Expected mass spectra detected from GRLN-LP modified by glycan chains

No.	Peptide	Predicted glycan chains	Sum of glycan chains	Expected mass
1	GRLN-LP			1832.8*
2		HexNAc1	203.07	2035.87
3		HexNAc1-Hex	365.12	2197.92
4		HexNAc1-Hex2	527.17	2359.97
5		HexNAc2	406.14	2238.94
6		HexNAc2-Hex	568.19	2400.99
7		HexNAc2-Hex2	730.24	2563.04
8		HexNAc3	609.21	2442.01
9		HexNAc3-Hex	771.26	2604.06
10		HexNAc3-Hex2	933.31	2766.11

Note: * Detected mass is GRLN-LP that was modified by octanoic acid (*m/z* 1808.8 $[M+H]^+$) containing unidentifed adduct (*m/z* 24).

prohormone, and vespulakinins (a wasp venom) [36-39]. These glycosylation positions of stingray GRLN-LP were found by using the NetOGlyc program http://www.cbs.dtu.dk, suggesting a glycosylation motif may be present. Mass spectrometric analyses of purified peptides revealed that stingray GRLN-LP possesses an elongated glycan structure based on a type-2 core (Figs. 6F and 7). For the formation of this glycan, β-1,3-galactose transferase and β-1,6-*N*-acetylglucosamine-transferase would be involved in the elongation. Glycosylation, as well as acylation by GOAT [32,33], is a post-translational modification. Further studies are required to determine if these transferases and GOAT co-localize in GRLN-LP-producing cells in the stingray stomach.

The functions of carbohydrates that are attached to proteins range from effects on protein folding to the formation of antigen-recognition sites. The functional significance of the glycosylation of stingray GRLN-LP is still unknown. This modification is likely involved in maintaining the biological activity of GRLN-LP because the native stingray GRLN-LP deglycosylating by *O*-glycanase resulted in a decrease in intracellular Ca^{2+} concentrations in rat GHS-R1a-expressing cells (Fig. 8B). However, the glycosylation was not necessary to exhibit GRLN-like activity because unglycosylated synthetic stingray GRLN-LP could activate a rat GHS-R1a-expressing cell line (Fig. 8A). Furthermore, the C-terminal portion of GRLN, except the seven N-terminal amino acids, is important to maintain GRLN activity in the circulation. Consistent with these findings, truncated GRLN molecules with portions of the C-terminus deleted, do not stimulate GH release in rats *in vivo* [40]. The C-terminal portion of GRLN is rich in basic amino acid residues such as arginine (Arg), lysine (Lys) and histidine, *e.g.*, eight residues in human GRLN8-28 and five in eel GRLN8-21. On the other hand, stingray GRLN-LP has only two basic amino acids, Arg-9 and Lys-13. It is speculated that the mucin-type glycan chains may regulate the physicochemical features of this peptide and act as polar molecules that stabilize GRLN-LP in the circulation of the stingray. Further studies are required to understand the functional relevance of glycosylation of stingray GRLN-LP, such as determining the feeding-stimulatory or GH-releasing activity of glycosylated or deglyc-

Table 4: Predicted compositions of glycan chains in isolated stingray GRLN-LP

Peak	Mass $[M+H]^+$	Possible compositions of glycan chains	Remarks
1	2580.15	Thr-11+(HexNAc)3(Hex)	Single peak
2	3351.68	Ser-10+(HexNAc)3(Hex) Thr-11+(HexNAc)3(Hex)	m/z 2580.14 was detected
	3513.74	Ser-10+(HexNAc)3(Hex) Thr-11+(HexNAc)3(Hex)2	m/z 2580.14 was detected
3	3375.42	Ser-10+(HexNAc)3(Hex) Thr-11+(HexNAc)3(Hex)	m/z 24 adduct of peak 2
	3537.46	Ser-10+(HexNAc)3(Hex) Thr-11+(HexNAc)3(Hex)2	m/z 24 adduct of peak 2
4	2580.26	Thr-11+(HexNAc)3(Hex)	
	2742.33	Thr-11+(HexNAc)3(Hex)2	
5, 6	2604.15	Thr-11+(HexNAc)3(Hex)	m/z 24 adduct of peak 1
	2766.20	Thr-11+(HexNAc)3(Hex)2	m/z 24 adduct of peak 1
7	2594.15	Thr-11+(HexNAc)3(Hex)	m/z 14 adduct of peak 4
	2756.21	Thr-11+(HexNAc)3(Hex)2	m/z 14 adduct of peak 4

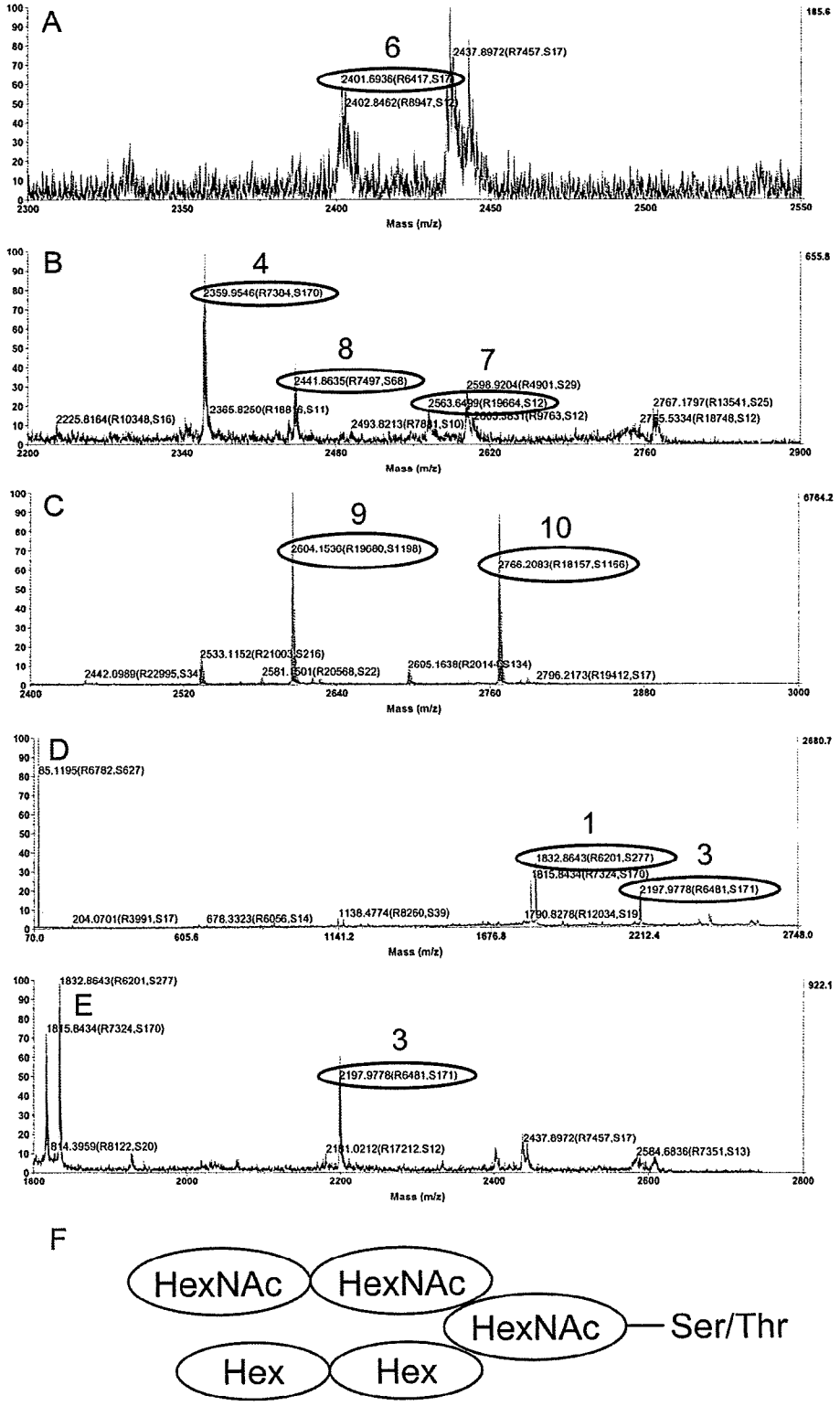

Figure 6
MALDI-TOF MS/MS spectra of an isolated stingray GRLN-LP. Peak 5 peptide (parent mass of m/z 2766.2), which is indicated in Table 1, was analyzed by MALDI-TOF MS/MS spectrometry. The glycan chains that are predicted from the fragment masses (A-E) are shown in Table 3. (F) Predicted structure of the glycan chains with a type-2 core.

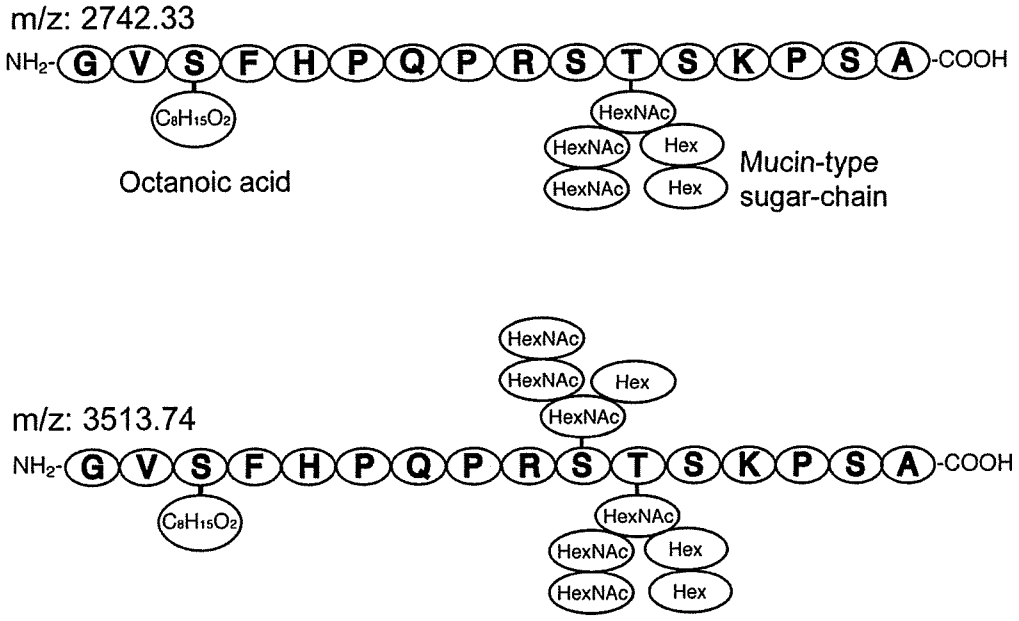

Figure 7
Predicted primary structures of stingray GRLN-LP. Stingray GRLN-LP is modified by *n*-octanoic acid at Ser-3, and mucin-type sugar chains at Thr-11 or both Ser-10 and Thr-11. These structures were predicted based on protein sequencing and MALDI-TOF MS/MS of peak 4 (mass-2, m/z 2742.33) and peak 2 (mass-2, m/z 3513.74) that are indicated in Table 1. The predicted compositions of the glycan chains of other isolated stingray GRLN-LPs are shown in Table 4.

osylated GRLN-LP after injection into the stingray circulation *in vivo*.

GRLN-LP mRNA was predominantly expressed in the stomach. This expression pattern is consistent with that of GRLN in other vertebrates. GLRN has multiple physiological functions such as regulation of GH release, appetite regulation, cardiovascular function, gastrointestinal motility, pancreatic function and reproductive function from fish to mammals as endocrine/paracrine factors [3-6,8]. GRLN-LP could play some roles in homeostatic regulation in the stingray. However, physiological functions of GRLN-LP in the stingray have been still undetermined at present. It is interesting to note that Caminos et al. [41] reported that GRLN has a role in cartilage metabolism in humans, mice and rats. This could be a dominant effect in cartilaginous fish such as stingray. Further research is necessary to clarify physiological roles of GRLN-LP in the stingray.

Elasmobranchii is a species that have primitive characteristics of vertebrates. It is interesting to note that the GRLN-LP of the red stingray has more complicated post-translational modifications. We have determined GRLN-LP in two elasmobranch sharks, hammerhead shark and blacktip reef shark [25], and their GRLN-LPs do not have the glycosyl modification. Which is more primitive form of GRLN-LP? Chondrichthyes include two subclasses, the Elasmobranchii and the Holocephali [42]. Stingrays and sharks belong to the formers, and it is divided roughly as superorders, Batoidea and Selachimorpha, respectively. Hammerhead and blacktip reef shark, and stingray belong to the order Carcharhiniformes and Rajiformes, respectively. It has been considered that Rajiformes evolved later than Carcharhiniformes [43,44]. The modification of *O*-glycosylation seems to be specific to the red stingray for two reasons: (a) both the Ser-10 and Thr-11 are not evolutionarily conserved; and (b) the more ancient GRLN-LP in shark has no *O*-glycosylation. Based on this division, shark GRLN-LP might exhibit a more primitive structure, and the stingray GRLN-LP structure may be an advanced form.

Lastly, it has been reported that a novel appetite-inhibiting hormone, obestatin, is present in the C-terminal peptide of the GRLN precursor in mammals [45]. In this study, the potential processing signals that would release obestatin were not found in the stingray GRLN-LP precursor as well as in sharks [25], suggesting that obestatin-like peptide does not exist in the stingray from the structural view point.

Conclusions

We characterized both the structure and tissue expression of GRLN-LP in a primitive vertebrate (cartilaginous fish), the red stingray. The structure of stingray GRLN-LP is very

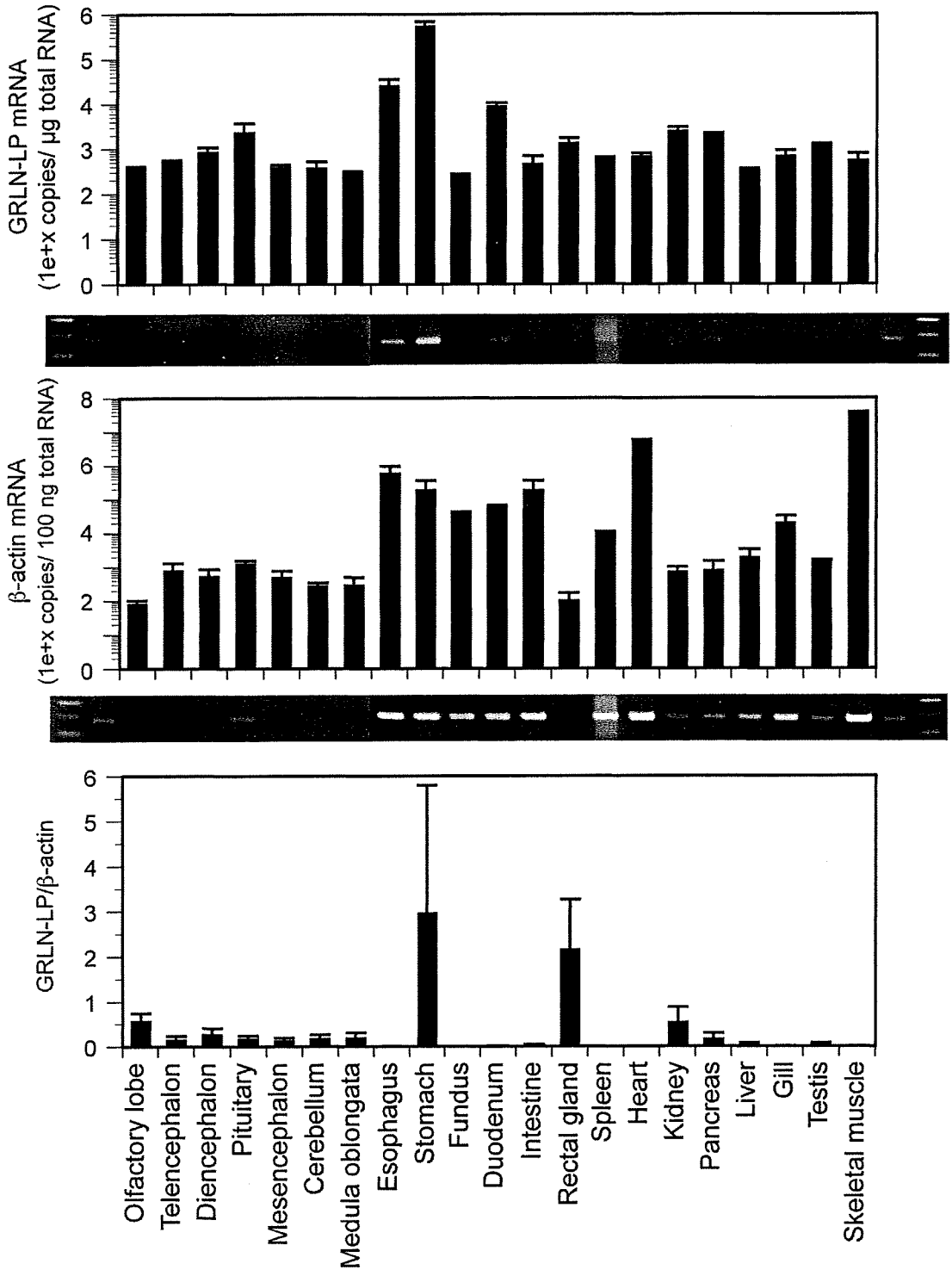

Figure 8

Tissue expression pattern of GRLN-LP mRNA in the red stingray. Quantitative real-time PCR results of GRLN-LP (top) and for β-actin (middle) are shown. Ratio of GRLN-LP to the β-actin gene is shown in the bottom. The values represent the means (± SD) of two different stingrays. Representative gel views obtained after 30 amplification cycles are displayed under each graph. The first and last two lanes contain the 100-bp ladder and the positive control in which PCR product of 1000-copies plasmid DNA standard finished by 35 amplification cycles.

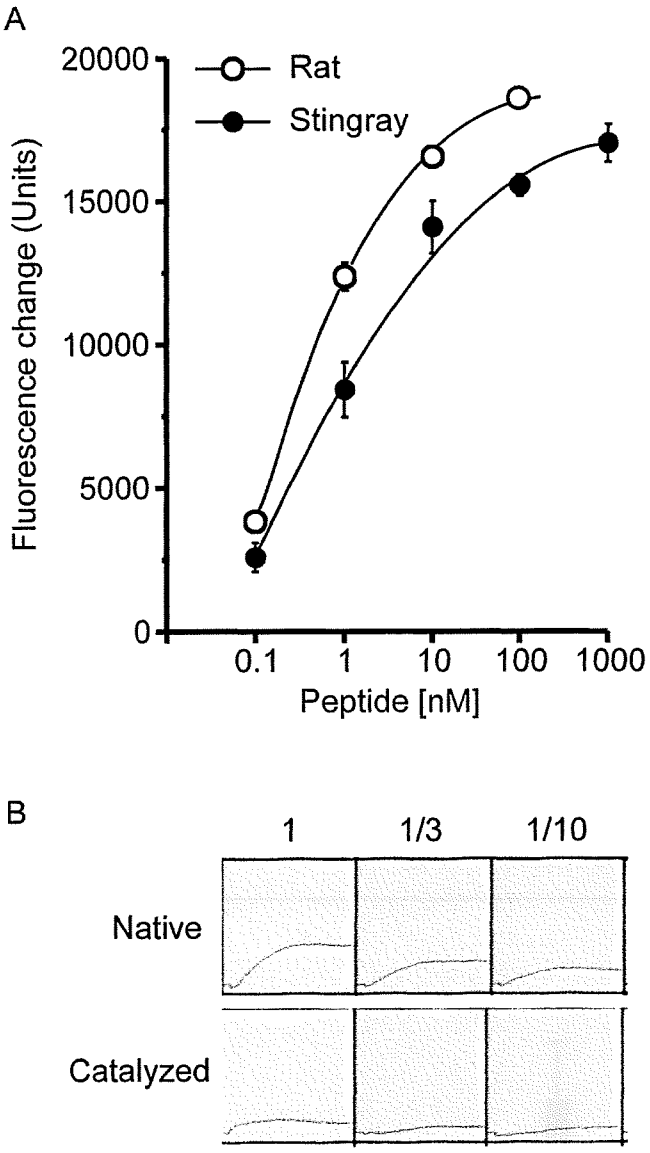

Figure 9
GRLN-like activity of stingray GRLN-LP. (A) Dose-response effects of rat GRLN (open circle) and unglycosylated stingray GRLN-LP (closed circle) on intracellular Ca^{2+} concentrations in rat GHS-R1a-expressing cell line. Values represent the means (± SE) of samples tested in triplicate. (B) Reduction of GRLN-like activity after the native stingray GRLN-LP was catalyzed. The changes in intracellular Ca^{2+} levels are shown for both the native (upper) and catalyzed (lower) peptides. The increases in intracellular Ca^{2+}concentrations decreased after the native stingray GRLN-LP was catalyzed by *O*-glycanase. A dose response was obtained by diluting the original sample 1/3 and 1/10.

unique and is modified by both a mucin-type sugar chain and an *n*-octanoyl modification, indicating that stingray GRLN-LP reflects an ancestral form of the GRLN molecule. However, we are unable to conclude definitively that this peptide is GRLN because the characteristic biological properties of GRLN peptides, such as GH-releasing activity or appetite-stimulating activity, have not been confirmed for this peptide. Since the unglycosylated synthetic stingray GRLN-LP activated rat GHS-R1a-expressing cells in an *in vitro* assay system, further studies are necessary to elucidate the physiological functions of GRLN-LP in the stingray as well as determining the functional importance of the glycosylation of this peptide.

Authors' contributions

HK performed almost all of the experiments, such as peptide purification, cDNA cloning, real-time PCR, phylogenetic analysis, functional analysis of the peptide, and drafted the manuscript. SK participated in the structural analyses of the glycan chains. KM, KI and MU extracted the stingray tissues and isolated the tissue RNA. MM and KK participated in the design and coordination of this study and helped draft the manuscript. All authors read and approved the final manuscript.

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Difference Between Japanese and Caucasian Populations in the Allelic Frequency of Growth Hormone Receptor Polymorphisms

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ABSTRACT

***Background*: Polymorphisms in the growth hormone receptor (*GHR*) gene were reported in Caucasian populations. However, the frequency of those in other ethnic backgrounds remains unclear.**

***Aim*: We investigated the presence of polymorphisms in the *GHR* gene in a Japanese population and compared the frequencies with those reported in Caucasian populations.**

***Population*: We selected 30 children with idiopathic short stature and 30 adult Japanese of normal height.**

***Methods*: The sequences of exons 6 and 10 in the *GHR* gene were determined by direct sequencing by polymerase chain reaction (PCR). The genomic deletion of exon 3 (GHR-d3) was investigated by multiplex PCR.**

***Results*: The frequency of the GGG genotype at codon 168 was significantly higher than that reported in Caucasian populations. The frequency of GHR-d3 in Japanese was significantly lower than that in Caucasian populations.**

***Conclusions*: The frequencies of the G168G polymorphism and GHR-d3 in Japanese are different from those in Caucasians.**

KEY WORDS

growth hormone receptor, idiopathic short stature, single nucleotide polymorphism, ethnic difference

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INTRODUCTION

The major role of growth hormones (GH) during childhood is to promote longitudinal bone growth[1]. In adults, GH affects body composition, bone mineralization[2], and lipid[3] and glucose metabolism[4]. The actions of GH are mediated by its binding to a specific, high affinity cell surface receptor, i.e., GHR. Genetic abnormalities in the *GHR* gene cause the GH insensitivity syndrome that results in severe short stature, osteopenia, obesity, insulin resistance, and hypercholesterolemia at a later stage in life if the function of the GHR is severely disrupted[5]. Therefore, it is possible that single nucleotide polymorphisms (SNPs) in the *GHR* gene that cause mild dysfunction may result in mild short stature, altered body composition, osteopenia, or metabolic syndromes such as diabetes mellitus or hyperlipidemia in normal populations. SNPs in the *GHR* gene have been identified in children with idiopathic short stature (ISS). Goddard *et al.*[6] reported that some heterozygous mutations and/or polymorphisms in the *GHR* gene might cause ISS and be responsible for short stature in Caucasian populations. Sanchez *et al.*[7] and Sjoberg *et al.*[8] investigated the *GHR* gene in 17 and 26 Caucasian children with ISS, respectively, and identified only one heterozygous mutation as well as two high frequency SNPs, i.e., G168G (adenine to guanine at position 3 of codon 168) and L526I (cytosine to adenine at position 1 of codon 526). Bonioli *et al.*[9] reported that 22 of 37 children with ISS and 23 of 50 normal children possess the GGG genotype at codon 168 of the *GHR* gene in an Italian population. On the other hand, Pilotta *et al.*[10] reported that 44 of 54 children with GH deficiency (GHD) possess the GGG genotype at codon 168.

Deletion of exon 3 of the *GHR* gene is associated with increased sensitivity to GH[11]. There are several reports from Europe in terms of the frequencies of GHR-d3 in the normal population[12-14], and ISS[11], GHD[10], and small for gestational age (SGA) groups[13,14]. In Japan, we previously reported several mutations[15,16] and/or polymorphisms[17-19] in the *GHR* gene. We hypothesized that the frequency of polymorphisms in the *GHR* gene might differ between Caucasian and Japanese populations and might partly explain ethnic differences in stature or the incidence of metabolic disorders such as diabetes mellitus, hyperlipidemia, or osteoporosis.

In this study, we examined part of the *GHR* gene in 30 children with ISS and 30 adult Japanese of normal height, and compared the frequencies of the identified SNPs with previously published results from Caucasian populations.

POPULATION AND METHODS

Population

For gene analysis, 30 children with ISS (16 boys and 14 girls) were selected from the outpatients of the Dr. Nose's Clinic and Kobe University Hospital. Candidates for the ISS group were selected based on the following criteria[20]: (1) short stature (height 2.0 SD or more below the mean for age and gender of the Japanese population[21]), (2) normal serum GH level (GH >10 μg/l) after a provocative test with insulin or clonidine, and (3) low or low normal serum insulin-like growth factor I (IGF-I) levels for age and gender of the Japanese population. Patients with known causes of short stature, such as identifiable endocrine, genetic, and skeletal dysplasias, were excluded from the study. Thirty adults (18 males and 12 females) of normal stature were selected at random from the in-patients and outpatients of our hospital. They were treated at the hospital for various disorders, excluding severe osteoporosis, diabetes mellitus, and hyperlipidemia. Informed consent was obtained from the participants, and the investigation was approved by Kobe University's ethics committee.

Genetic analysis

Genomic DNA was isolated from peripheral blood leukocytes using the Wizard genomic DNA extraction kit (Promega Corp., Madison, WI) according to the manufacturer's instructions. Exons 6 and 10, which included high frequency SNPs, were amplified by polymerase chain reaction (PCR), as described previously[18]. The amplification products were purified using the QIAquick Gel Extraction Kit (Qiagen, Tokyo, Japan) and were analyzed by direct sequencing with the BigDye Terminator Ready Reaction Kit (Perkin Elmer, PE Applied Biosystems, Foster, CA) using a DNA sequencer (model 310, Perkin Elmer). The multiplex PCR assay was performed for genotyping of the GHR-exon 3 locus[12].

Statistical analysis

The allelic frequency in each group was estimated, and statistical analysis was performed using the chi-squared test (GraphPad Software Inc., San Diego, CA). A p value of <0.05 was considered significant.

RESULTS

In this study, we focused on two frequent SNPs, in exon 6 (G168G) and exon 10 (L526I), as well as GHR-d3 of the *GHR* gene. Based on a previous report[22], we presumed that the wild-type sequence at codon 168 is GGA, while GG(G/A) and GGG are polymorphisms. In this case, the coded amino acid is unchanged (G168G). The frequencies of polymorphisms at codon 168 in the study population are listed in Table 1, and were compared with previously published results from Caucasian populations[6-10]. The frequency of GGG at codon 168 was 100% and 97% in the adult control group and children with ISS, respectively; in contrast, in the Cuban, Caucasian, Chilean, and Italian populations reported[6-10], the GGA genotype was more frequent (Table 1). The difference between the Japanese and Caucasian populations in terms of the allelic frequency of codon 168 was statistically significant.

In codon 526, we presumed that the wild-type sequence is CTC, while (A/C)TC and ATC are

TABLE 1

Allelic frequency of polymorphisms at codon 168

Reference (Country)	Group	No. analyzed	GGG at codon 168 (%)	p value
Goddard *et al.*[6] (USA)	Control	102	20	<0.0001[b]
	ISS	100	30	<0.0001[c]
Sanchez *et al.*[7] (Cuba)	Control	21	35	<0.0001[b]
	ISS	17	30a	<0.0001[c]
Sjoberg *et al.*[8] (Chile)	Control	ND	ND	ND
	ISS	26	35	<0.0001[c]
Bonioli *et al.*[9] (Italy)	Control	50	39	<0.0001[b]
	ISS	37	46	<0.0001[c]
Pilotta *et al.*[10] (Italy)	GHD	54	82[a]	<0.05
Present study (Japan)	Control	30	100	–
	ISS	30	97	–

ISS = idiopathic short stature; GHD = growth hormone deficiency; ND = not determined.
[a] This percentage includes heterozygous and homozygous GGG.
[b] Versus the control group in our study; [c] versus the ISS group in our study.

TABLE 2

GHR genotype frequencies reported in patient and control groups

Reference (Country)	Group	No. analyzed	GHR genotype (%)		
			d3/d3	*d3/fl*	*fl/fl*
Pantel *et al.*[12] (France)	Control	150	9	33	58[b]
Dos Santos *et al.*[11] (France)	ISS	96	8	40	52[c]
Binder *et al.*[13] (Germany)	Control	62	15	40	45[b]
	SGA	60	14	38	48[b]
Audi *et al.*[14] (Spain)	Control	289	15	58	27[b]
Pilotta *et al.*[10] (Italy)	GHD	54	48*	52	b
Present study (Japan)	Control	50	2	20	78
	ISS	20	5	15	80

ISS = idiopathic short stature; GHD = growth hormone deficiency; SGA = small for gestational age.
* Homozygotes plus heterozygotes.
[b] $p < 0.05$ versus the control group in our study; [c] $p < 0.05$ versus the ISS group in our study.

polymorphisms (L526I)[22]. The frequency of ATC at codon 526 was 40% and 48% in the adult control group and children with ISS, respectively. There were no statistical differences between the Japanese and Caucasian populations in terms of the frequency of the L526I SNP. In addition, no significant difference was observed in height associated with the L526I polymorphism, in agreement with previous studies in Caucasian populations[6,7,9].

Next, we examined the frequencies of full-length GHR (GHR-fl) and deletion of exon 3 of the *GHR* gene (GHR-d3). As shown in Table 2, the frequencies of homozygous GHR-fl, heterozygous GHR-fl/GHR-d3, and homozygous GHR-d3 in Japanese children with ISS were 80%, 15% and 5%, respectively, whereas those in Japanese adults with normal height were 78%, 20% and 2%, respectively. The difference between the Japanese and Caucasian populations in terms of the allelic frequency of homozygous GHR-fl was statistically significant[10-14].

DISCUSSION

In this study, we identified the different frequencies of SNPs and GHR-d3 in the *GHR* gene, and compared them with those in Caucasian populations reported by other investigators[6-10]. Several studies have suggested the presence of homozygous or heterozygous polymorphisms in the *GHR* gene in ISS, and these might account for ISS in approximately 5% of the selected patients[6]. However, all available data on polymorphisms in the *GHR* gene were from Caucasian populations. We believe that this is the first report on the variations of SNPs and GHR-d3 in the *GHR* gene that is based on ethnic background.

Goddard *et al.*[6] reported that some heterozygous mutations and/or polymorphisms in the *GHR* gene affect its receptor function and result in partial GH insensitivity. They also suggested that G168G and L526I were two frequent SNPs in the *GHR* gene.

In the present study, one of the SNPs that we focused on was a so-called silent mutation because the amino acid remained unchanged (G168G). However, more than 98% of the alleles examined in the present study showed a GGG variant at codon 168. Although the number of individuals examined in this study was relatively small, the allelic frequency is statistically different from that of Caucasian populations[6-10]. These results led us to conclude that the frequency of the GGG variant at codon 168 was extremely high in the Japanese population, irrespective of body height. Although this alteration is unlikely to impair the function of the GHR protein *per se*, other unidentified functional variants may be present in linkage disequilibrium. These variants are unlikely to be coding sequences in the *GHR* gene, because we examined all coding sequences in several Japanese children of short stature and failed to detect additional SNPs except for G168G and L526I, as described previously[15-19]. However, it is possible that the G168G variant may be linked with a mutation in the regulatory elements and may affect the expression levels of GHR in tissues. Alternatively, the G168G variant may be linked with the alteration of another unidentified gene that is located adjacent to the *GHR* gene. It is noteworthy that the G168G variant was also identified in a patient with classical Laron syndrome (LS) with a heterozygous R43X mutation in the *GHR* gene[23]. Since a heterozygous mutation alone does not express the typical phenotype of LS, the G168G variant may contribute to the reduced function of the GHR.

There is evidence that demonstrates differences between Caucasians and Japanese with respect to allelic frequency in other genes. For instance, a dinucleotide repeat polymorphism in intron 1 of the epidermal growth factor receptor (EGFR) gene was reported to regulate EGFR expression and be associated with EGFR-mediated cancer cell growth. Liu *et al.*[24] reported that there were significant inter-ethnic differences in the allelic frequencies of the intron 1 polymorphism of the EGFR gene between Asian and Caucasian or African-American populations, that may contribute to ethnic differences in response to anti-cancer drugs and EGFR inhibitors.

Besides longitudinal body growth, GH is known to play a role in the regulation of glucose, lipid and bone metabolism as well as local growth. The P561T variant of GHR was reported to be associated with mandibular height[25], although this variant was not found to have any correlation with body

height[17,25]. Takada *et al.*[26] and Ihara *et al.*[27] reported that the L526I variant of *GHR* modified the plasma HDL-cholesterol phenotype in familial hypercholesterolemia and serum cholesterol level in children with GHD during GH treatment, respectively, although this variant was one of the most frequent SNPs and did not affect body height[6].

Recently, GHR genotype frequencies with or without exon 3 were reported in the Caucasian population[10-14]. As shown in Table 2, our study demonstrated that the frequency of the GHR-d3 genotype in the Japanese population was significantly lower than those in Caucasian populations. The low frequency of GHR-fl may be associated with decreased sensitivity to GH in Japanese populations. However, further studies are required to elucidate the physiological significance of the difference in frequencies of polymorphisms.

In conclusion, we determined the difference between Caucasian and Japanese populations in terms of allelic frequency of the *GHR* gene. The frequency of the GGG genotype at codon 168 was significantly higher than that reported in Caucasian populations. The frequency of GHR-d3 in Japanese was significantly lower than that in Caucasian populations.

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Ghrelin suppresses noradrenaline release in the brown adipose tissue of rats

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Abstract

To clarify the role of ghrelin in the regulatory mechanism of energy metabolism, we analyzed the effects of centrally and peripherally administered ghrelin on noradrenaline release in the brown adipose tissue (BAT) of rats using a microdialysis system. I.c.v. administration of ghrelin at a dose of 500 pmol suppressed noradrenaline release in BAT, and microinjection of ghrelin (50 pmol) into the paraventricular nucleus (PVN) or arcuate nucleus (ARC) of the hypothalamus also suppressed noradrenaline release in BAT. In addition, i.v. administered ghrelin (30 nmol) suppressed noradrenaline release in BAT, and this suppression was blocked by a vagotomy. Neither i.c.v. nor i.v. administration of des-acyl ghrelin, which does not bind to GH secretagogue receptor type 1a (GHS-R1a), affected noradrenaline release in BAT. These results indicate that ghrelin increases energy storage by suppressing the activity of the sympathetic nerve innervating BAT. It seems that the PVN and ARC, which express GHS-R1a, are the sites of action of ghrelin in the brain and that the action of peripheral ghrelin on the sympathetic nerve activity innervating BAT is mediated by the vagal nerve, which also expresses GHS-R1a.

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Introduction

Ghrelin was isolated from the rat stomach extracts (Kojima *et al.* 1999) as an endogenous ligand for GH secretagogue receptor (GHS-R; Howard *et al.* 1996, Smith *et al.* 1997). Alternative mRNA processing of the rat GHS-R gene transcript generates two products, GHS-R1a and GHS-R1b (McKee *et al.* 1997). The former isoform functions by binding ghrelin/GHS while the latter is non-functional (Howard *et al.* 1996). Ghrelin/GHS stimulates not only GH secretion but also food intake through GHS-R1a when administered centrally or peripherally (Okada *et al.* 1996, Nakazato *et al.* 2001, Date *et al.* 2002, Tamura *et al.* 2002). Ghrelin increases Fos expression in the ventromedial nucleus, dorsomedial nucleus, suprachiasmatic nucleus, arcuate nucleus (ARC), and paraventricular nucleus (PVN) of the hypothalamus when administered i.c.v. or peripherally, and GHS-R1a is expressed in these nuclei (Lawrence *et al.* 2002, Rüter *et al.* 2003, Kobelt *et al.* 2005, Mano-Otagiri *et al.* 2006, Zigman *et al.* 2006). The GHS-R1a expressed in GHRH neurons is mainly involved in ghrelin/GHS-induced GH secretion (Tannenbaum *et al.* 2003). We have shown that GHS-R1a expressed in GHRH neurons upregulates GHRH gene expression (Mano-Otagiri *et al.* 2006). Ghrelin administered i.c.v. or peripherally also increases the respiratory quotient (RQ), indicating its inhibitory action on fat expenditure (Tschöp *et al.* 2000). Furthermore, the microinjection of ghrelin into the PVN or ARC increases food intake and RQ (Currie *et al.* 2005). Therefore, the central effect of ghrelin on energy homeostasis may be mediated through the activation of GHS-R1a in the PVN and ARC.

Brown adipocytes are important in the regulation of energy metabolism by increasing energy expenditure through thermogenesis (Lowell & Spiegelman 2000), and the activity of brown adipocytes is stimulated by the sympathetic nerves (Lowell & Spiegelman 2000). I.c.v. administration of ghrelin suppresses the electrophysiological activity of the sympathetic nerves innervating brown adipose tissue (BAT) in rats anaesthetized with urethane and chloralose (Yasuda *et al.* 2003). These findings suggest that ghrelin induces a positive energy balance not only by increasing food intake but also by decreasing energy expenditure.

We developed a microdialysis system in which the release of noradrenaline, an important stimulator of brown adipocytes, is monitored in BAT in freely moving rats. In the present study, we used this system to test the effect of i.c.v. administration of ghrelin or microinjection of ghrelin into the PVN or ARC on noradrenaline release in BAT. Our goal was to determine if i.c.v. administration of ghrelin inhibits noradrenaline release in BAT and if those hypothalamic nuclei are involved in the inhibitory action of ghrelin on the sympathetic nerves innervating BAT. Furthermore, the effect

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of i.v. administration of ghrelin on noradrenaline release in BAT was analyzed; the effect was further examined in bilateral vagotomized rats to clarify the pathway of peripheral ghrelin signals to BAT, since GHS-R1a is expressed in vagal nodose ganglion (Sakata *et al.* 2003, Burdyga *et al.* 2006).

Materials and Methods

Animals

Male Sprague–Dawley rats (SLC, Shizuoka, Japan), 7–8 weeks old, were used in this study. Rats were housed individually in each cage (20×25×18 cm) and maintained at 24 °C on a 12 h light:12 h darkness (light on at 0800 h, off at 2000 h). They were allowed access to laboratory chow and distilled water *ad libitum*. All experimental procedures were conducted in accordance with the guidelines for the use and care of laboratory animals from the ethics committee of Nippon Medical School.

Surgical procedure

To administer samples i.c.v., a polyethylene cannula was implanted into the right lateral ventricle and fixed to the skull with three screws and acrylic dental cement under sodium pentobarbital anesthesia (50 mg/kg of body weight, i.p.), as previously described, 5 days before the experiment (Mano-Otagiri *et al.* 2006).

For microinjection of samples into the PVN or ARC, a 24-gauge stainless guide cannula was implanted and fixed to the skull with three screws and acrylic dental cement. Stereotaxic coordinates, as determined from the atlas of Paxinos & Watson (1996) were just above the PVN (rostral, −1·8 mm; lateral, 0·3 mm; and ventral, −6·2 mm, relative to the bregma and the brain surface) or ARC (rostral, −3·8 mm; lateral, 0·3 mm; and ventral, −8·1) under sodium pentobarbital anesthesia (50 mg/kg of body weight, i.p). A dummy cannula was inserted into the entire length of each guide cannula in order to prevent obstruction. After surgery, all rats were individually housed and allowed to recover for at least 6 days.

For i.v. injection of sample, an i.v. catheter was inserted into the right external jugular vein of rats under sodium pentobarbital anesthesia (50 mg/kg of body weight, i.p.; Thrivikraman *et al.* 2002) 4 days before the experiments.

To examine the effect of a vagotomy, a truncal vagotomy (or a sham operation as a control) was performed 7 days before the i.v. catheter insertion. After a midline incision of the abdominal wall, the lower part of the esophagus was exposed and the anterior and posterior branches of the vagal nerve were incised under anesthesia with sodium pentobarbital (50 mg/kg of body weight, i.p.), as previously described (Smith *et al.* 1981). During the sham operations, the vagal trunks were similarly exposed without any cutting of the vagal nerve.

Microdialysis of noradrenaline in BAT

On the day of the experiment, the skin in the interscapular area was shaved and cleaned with 70% ethanol, and a small incision was made along the midline, exposing a depot of white fat and the underlying interscapular brown fat. A microdialysis probe (OP-100-05; Eicom Corp., Kyoto, Japan) with a 5-mm dialyzable membrane was inserted into either lobe of the brown fat lobes under light anesthesia with ether (Gabaldón *et al.* 2003). The end of the probe was exteriorized through the midscapular skin incision, and the skin incision was then sutured close. After the rat had recovered from anesthesia, the probe was connected with tubing for microdialysis, and microdialysis was performed under free-moving conditions without chow and water between 1100 and 1800 h. The probe was continuously perfused with Ringer's solution (147 mM NaCl, 4 mM KCl, and 2·3 mM $CaCl_2$, pH 7·0) at a flow rate of 2 μl/min, and the dialysate was collected every 20 min. Noradrenaline concentrations in dialysates (40 μl/20 min) were determined by a combination of HPLC and electrochemical detection by an Eicompak CA-5ODS column (2·1 mm i.d.×150 mm; Eicom) and a WE-3G graphite electrode (Eicom) set at +450 mV against an Ag/AgCl reference electrode. The electrode's current sensitivity was 0·1 nA. The minimum detection limit of noradrenaline in the system was 0·1 pg. The mobile phase in the HPLC column was 0·1 M sodium phosphate buffer (pH 6·0) containing 1·85 mM sodium octanesulfonic acid, 0·17 mM EDTA, and 5·0% (v/v) methanol. After a 3 h stabilization period, the baseline noradrenaline levels were defined as the average release in three consecutive fractions immediately preceding the injection and were considered as 100%. Rats then received an injection of rat ghrelin (Peptide Institute, Inc., Osaka, Japan).

Ghrelin at a dose of 50 or 500 pmol dissolved in 2 μl of saline (or 2 μl of saline as a control) was administered i.c.v. through the guide cannula. Ghrelin at a dose of 50 pmol dissolved in 0·5 μl of saline (or 0·5 μl of saline as a control) was microinjected into the PVN or ARC. Ghrelin at doses of 6 or 30 nmol dissolved in 0·5 ml saline (or 0·5 ml saline as a control) was i.v. administered. Each administered dose of ghrelin was determined by its stimulatory effect on food intake as determined in previous reports, 10 pmol to 1 nmol for i.c.v. administration, 15–60 pmol for microinjection into the brain, and 1·5–10 nmol for i.v. administration (Nakazato *et al.* 2001, Date *et al.* 2002, Currie *et al.* 2005). We also administered rat des-acyl ghrelin (Peptide Institute, Inc.), which does not bind to GHS-R1a, at a dose of 500 pmol for i.c.v. experiment or 30 nmol for i.v. experiment to certify that the action of ghrelin is mediated by GHS-R1a.

Histology

To verify the placement of the cannula, rats were deeply anesthetized with sodium pentobarbital at the end of the experiment, and dye (0·5 μl Brilliant Blue 6B; Tokyo Kasei,