In cartilaginous fishes, a ghrelin-like peptide was newly identified in an elasmobranch, the red stingray (Figs. 1 and 2). This peptide, at 16 amino acid residues length, is the smallest ghrelin identified so far, and glycosylation, which is not seen in shark ghrelin-like peptide or other ghrelins, is found in the molecule (Fig. 2). This modification is likely to be involved in maintaining the biological activity of the peptide through physicochemical features such as the polar carbohydrate moieties that stabilize ghrelin-like peptide in the circulation [38]. *Elasmobranchii*, including *Carcharhiniformes* such as hammerhead and blacktip reef sharks, express primitive characteristics of vertebrates. *Rajiformes* such as the stingray are considered to have evolved later. Thus, the shark ghrelin-like peptide structure may represent the primitive form of ghrelin molecule.

A schematic phylogenetic tree of ghrelins was made based on the above-described features of the vertebrate ghrelins (Fig. 9). The ghrelin of *Agnatha* has not been identified yet. *Gnathostomata* is roughly divided into *Chondrichthiomorphi* and *Teleostomi*. *Teleostomi* is divided roughly into *Actinopterygii* and *Sarcopterygii*, and bony fish belongs to *Actinopterygii* (*Teleostei*). On the other hand, tetrapods including mammals, birds, reptiles and amphibians evolved from *Sarcopterygii*. The structures of ghrelin are likely to reflect these evolutionary processes. The N-terminal 7 amino acids have been well conserved in teleost, amphibians, reptiles and mammals; the ghrelin type shown in *Rana*, which has Thr-3, was derived within amphibians. And bony fish ghrelin has become shorter in length during the evolutionary process, and it has decreased the number of charged amino acid residues by the added C-terminal amide structure. In another major branch, *Chondrichthyes*, ghrelin retains its ancestral form as seen in the stingray, and it has acquired physicochemical features of peptide chain shortening and intramolecular glycosylation. Consideration of molecular evolution of ghrelin based on current information is still quite speculative.

7. Comparison of ghrelin receptor sequence

Since GHS-R is comprised of two forms, a biologically active GHS-R1a and a functionally-undetermined GHS-R1b (see [43]), in our discussion here we focus on the GHS-R that is involved in functional ghrelin receptor activity, namely GHS-R1a. Fig. 4 shows alignments of deduced GHS-R1a proteins, which are identified in eleven fish species, one frog and two birds, and mammals. Incomplete receptor protein sequences were omitted.

Based on structure, GHS-R1a proteins are roughly divided into two groups: GHS-R1a and GHS-R1a-like receptors (GHSR1a-LR). In general, the former are identified in mammals, birds, amphibians and some of the bony fishes including *Cypriniformes* and *Siluriformes*. The latter receptor group has been found only in some bony fishes including *Salmoniformes*, *Perciformes*, *Gastrosteiformes*, *Beloniformes* and *Tetraodontiformes*. GHSR1a-LR is commonly characterized by a longer 2nd extracellular loop that connects transmembrane segments 4 and 5, compared with that of GHS-R1a (seen in the third row of Fig. 4). Studies by the author's group demonstrated no response by rainbow trout (*Salmoniformes*) and Mozambique tilapia (*Perciformes*) receptors expressed in transformed cells even when homologous ghrelin was treated [44,45]. Seabream and southern pufferfish GHS-R1a receptors are activated at high doses of ghrelin or GHSs [10,77]. No data are available on the receptor activity of GHSR1a-LR for stickleback, medaka and Wami tilapia. The author's group has proposed that GHS-R1a receptors that are not demonstrably functional in ghrelin signaling and receptors with the long second extracellular loop should be called GHSR1a-LR. Our proposed distinction of receptor types is supported by our observations on GHS-R1a of goldfish (*Cypriniformes*) has a short second extracellular loop as seen in GHS-R1a of tetrapods, and is activated by homologous goldfish ghrelin [42]. The protein sequence of channel catfish GHS-R1a is a little different from other GHS-R1a, and functional activities of channel catfish, zebrafish and Jian carp receptors have not been confirmed yet. The protein sequence of the clawed frog (*Xenopus laevis*) receptor has been deposited in the database, but the receptor signaling activity is unknown.

Channel catfish (*Siluriformes*), and goldfish, zebrafish and Jian carp (*Cypriniformes*), possess two types of functionally-active GHS-Ra, namely GHS-R1a and 2a, and the sequence identity between the two is approximately 70%. In zebrafish, the GHS-R1a and 2a genes are located on different chromosomes (chromosomes 4 and 24, respectively). To date, the presence of multiple GHS-Ras has not been reported in tetrapods. These paralogs are considered to derive from the teleost-specific whole genome duplication (TSGD) that occurred in the ray-finned fish lineage (see [42]). In goldfish, furthermore, variants are present for each GHS-R1a or 2a, namely 1a-1, 1a-2, and 2a-1, 2a-2, which share 96% identity among variants. These variants are encoded by four distinct genes [42], and may have been derived by tandem duplication of each gene. In rainbow trout (*Salmoniformes*), on the other hand, similar variants are present, namely DQTA/LN-type and ERAT/IS-type, that share 98% identity at the amino acid level. Interestingly, the two receptor proteins derive from three distinct genes [44]; therefore, the evolutionary derivation may be the same as for goldfish receptor variants. The evolutionary process for GHS-Ra receptor is not yet clear. Two-types of GHS-Ra, as seen in goldfish, have not been found in the above-mentioned *Salmoniformes*, *Perciformes*, *Gastrosteiformes*, *Beloniformes* and *Tetraodontiformes*, which belong to *Acanthopterygii* or *Protacanthopterygii* (Superorder) and are considered to be so-called advanced fishes. On the other hand, the two-types of GHS-Ra are common in the second-largest superorder of fish, namely *Ostariophysi*. Our understanding of the evolutionary history of GHS-Ra will depend on future exploration of GHS-Ra in other fish stocks, and in land animals including amphibians and reptiles.

8. Perspective

Research on the identification of ghrelins in non-mammalian vertebrates, especially in fish and birds, rapidly advanced in recent years. Remarkably, ghrelin can be also identified in organisms that are phylogenetically distant from the vertebrates. Aydin et al. [3] reported the detection of ghrelin immunoreactivity in the appetite-stimulating plants, *Syzygium aromaticum* (SA) and *Salvadora persica* (SP), in amounts equal to or greater than the ghrelin content in human salivary gland tissue. The ghrelin-like substance seems to be localized in the trachea and parenchyma cells in flower buds of SA, but no immunopositive reaction was observed in the trachea of SP branches. Furthermore, Peric-Mataruga et al. [81] reported ghrelin effects on the feeding behavior of fourth instar caterpillars of the pest insect, *Lymantria dispar* L., and found a positive influence on daily food intake, frass elimination, body mass and locomotor activity, and a decrease in stadium duration. Ghrelin presence is not evident in *Drosophila*, in which genomic sequences has been deciphered. Accumulating evidence indicates that ghrelin is a multifaceted hormone more than we imagined, and that its species-specific actions are remarkable in non-mammalian vertebrates, especially in the fishes. These species-specific activities might reflect the diversity of adaptive responses to various environments. The phylogenetic study of ghrelin reflects not only the diversity of ghrelin actions, but also the importance of ghrelin in living animals, and perhaps even in plants. We anticipate that further advancements in ghrelin research on non-mammalian vertebrates, including reptiles, will improve our understanding of this important biological molecule.

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Ghrelin ameliorates bleomycin-induced acute lung injury by protecting alveolar epithelial cells and suppressing lung inflammation

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ABSTRACT

Acute lung injury is a critical illness syndrome consisting of acute respiratory failure with bilateral pulmonary infiltrates that is refractory to current therapies. Acute lung injury is characterized by injury of the alveolar capillary barrier, neutrophil accumulation, and induction of pro-inflammatory cytokines followed by devastating lung fibrosis. Ghrelin, an acylated peptide produced in the stomach, increases food intake and growth hormone secretion, suppresses inflammation, and promotes cell survival. We investigated the pharmacological potential of ghrelin in the treatment of acute lung injury by using a bleomycin-induced acute lung injury model in mice. Ghrelin or saline was given to mice daily starting 1 day after bleomycin administration. Ghrelin-treated mice showed a definitively higher survival rate than saline-treated ones. They also had smaller reductions in body weight and food intake. The amelioration of neutrophil alveolar infiltration, pulmonary vascular permeability, induction of pro-inflammatory cytokines, and subsequent lung fibrosis were notable in ghrelin-treated mice. Additionally, ghrelin administration reduced the injury-induced apoptosis of alveolar epithelial cells. Our results indicate that ghrelin administration exerts a protective effect against acute lung injury by protecting the alveolar epithelial cells and regulating lung inflammation, and highlight ghrelin as a promising therapeutic agent for the management of this intractable disease.

1. Introduction

Acute lung injury and its severe clinical manifestation, acute respiratory distress syndrome, are critical syndromes consisting of acute respiratory failure associated with extensive pulmonary infiltrates (Ware and Matthay, 2000). Acute lung injury and acute respiratory distress syndrome frequently lead to persistent respiratory failure with prolonged dependence on mechanical ventilation, and increasing susceptibility to multiorgan dysfunction with a high mortality rate over 40% (Rubenfeld et al., 2005). The pathological characterization of acute lung injury includes injuries of alveolar epithelial cells and endothelial cells, alveolar neutrophilic infiltration, and increases in pro-inflammatory cytokines, which cause destruction of the alveolar capillary barrier and subsequent devastating lung fibrosis (Matute-Bello et al., 2008; Ware and Matthay, 2000). Fibrosing alveolitis is well correlated with mortality (Martin et al., 1995). Despite extensive investigations aiming at early diagnostic and pathogenetic factors of acute lung injury, clinical management of acute lung injury is mainly supportive, and no specific therapy has been proven effective, including anti-inflammatory agents or a neutrophil elastase inhibitor (Wheeler and Bernard, 2007; Zeiher et al., 2004).

Disruption of epithelial integrity has a considerable effect on the pathogenesis of acute lung injury. A marked increase in extravascular lung water in acute lung injury is caused mainly by an increase in epithelial permeability (Matthay and Wiener-Kronish, 1990). Furthermore, denudation of alveolar membranes induced by exfoliation of alveolar epithelial cells from alveolar septa leads to the activation of fibroblasts and subsequent increases in extracellular matrix deposition (Ware and Matthay, 2000). In fact, the degree of alveolar epithelial injury is an important predictor of the outcome of acute lung injury (Matthay and Wiener-Kronish, 1990). Protection of the alveolar epithelial cell integrity may therefore have a favorable effect on the control of acute lung injury progression, but specific strategies to exert a protective effect on alveolar epithelial cells have not been contrived.

Ghrelin is a 28-amino-acid peptide initially isolated from human and rat stomach as an endogenous ligand for the growth hormone secretagogue receptor (Kojima et al., 1999). Ghrelin is predominantly produced by a distinct type of endocrine cells of the gastric oxyntic glands and acts on the pituitary to stimulate growth hormone release and on the hypothalamus to enhance food intake (Kojima et al., 1999; Nakazato et al., 2001). The wide distribution of the growth hormone secretagogue receptor in various organs suggests a potentially broad array of actions for ghrelin. Li et al. (2004) have reported that ghrelin mitigates pro-inflammatory cytokine production and mononuclear

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cell binding in human endothelial cells (Li et al., 2004). Ghrelin has an inhibitory effect on apoptotic cell death in several types of cells, including neurons (Lee et al., 2010), cardiomyocytes, and endothelial cells (Baldanzi et al., 2002). The anti-inflammatory effect of ghrelin was also shown in a rat model of sepsis-induced acute lung injury (Wu et al., 2007); however, the impact of ghrelin on the integrity of the alveolar capillary barrier and its therapeutic effect on lung fibrosis remain unknown. In the present study, we investigated the efficacy of ghrelin on the amelioration of acute lung injury using a bleomycin-induced acute lung injury model in mice (Matute-Bello et al., 2008), and tested the hypothesis that ghrelin administration mitigates alveolar epithelial cells injury and subsequent lung fibrosis.

2. Materials and methods

2.1. Animals

Male 8-week-old C57BL/6N mice (20–22 g; Charles River Japan Inc., Yokohama, Japan) were housed in a temperature-controlled room (23 ± 1 °C) on a 12-h light (08:00–20:00 h)/12-h dark cycle and fed a standard laboratory chow with *ad libitum* access to food, unless otherwise stated in the study protocol. All experimental procedures were performed in accordance with the Japanese Physiological Society's guidelines for animal care and were approved by the Ethics Committee on Animal Experimentation of the University of Miyazaki.

2.2. Administration of bleomycin and ghrelin

Mice were anesthetized by intraperitoneal injection of pentobarbital sodium, and then 6–8 U/kg of bleomycin (Sigma-Aldrich Japan Inc., Tokyo, Japan) dissolved in 50 μl sterile phosphate-buffered saline (PBS) or 50 μl PBS was intratracheally administered. One day after this treatment, intraperitoneal administration of human ghrelin (10 nmol/mouse; Asubio Pharma Co., Tokyo, Japan) in 200 μl saline or saline alone at 07:00 and 19:00 was started and continued during the 7-day or 14-day study interval. We here designated bleomycin-injected, ghrelin-treated mice as the bleomycin/ghrelin group; bleomycin-injected, saline-treated mice as the bleomycin/saline group; PBS-injected, ghrelin-treated mice as the PBS/ghrelin group; and PBS-injected, saline-treated mice as the PBS/saline group. For the survival study under condition of *ad libitum* feeding, we used the bleomycin/ghrelin group (n = 18) and bleomycin/saline group (n = 20), both of which were administered 8 U/kg of bleomycin, and monitored for 14 days to record survival. To evaluate the dose-dependent effect of ghrelin on the survival of bleomycin-injected mice under condition of *ad libitum* feeding, we used the three different types of bleomycin/ghrelin groups that were administered 10 (n = 19), 3 (n = 20), or 1 (n = 17) nmol/mouse of ghrelin as mentioned above, respectively, and bleomycin/saline group (n = 20). All groups were previously administered 8 U/kg of bleomycin, and monitored for 14 days to record survival. For the survival study under the pair-fed condition, we used the bleomycin/ghrelin pair-fed group (n = 18) and bleomycin/saline group (n = 18) both of which were administered 8 U/kg of bleomycin. Bleomycin/saline group was allowed *ad libitum* access to food, and bleomycin/ghrelin pair-fed group was fed the same quantity of diet as consumed by animals of bleomycin/saline group. Both groups were monitored for 14 days to record survival. For immunohistochemical analyses and TUNEL assay, we used the bleomycin/ghrelin group and bleomycin/saline group (n = 5 per each group), both of which were administered 6 U/kg of bleomycin and examined on day 7. For Sircol collagen assay (n = 5 per group) and histological assay (n = 3 per group), we used additional couple of the bleomycin/ghrelin and bleomycin/saline groups administered 6 U/kg of bleomycin and examined on day 14. For the measurements of body weight and food intake (n = 5 per group), the histological assay (n = 3 per group), and the bronchoalveolar lavage (n = 5 per group), we used four additional groups administered 6 U/kg of bleomycin or PBS and examined on day 7. Body weights were measured daily from one day before bleomycin or PBS administration to day 7. The 7-day food intake amounts were also measured.

2.3. Bronchoalveolar lavage

Mice were anesthetized 7 days after bleomycin injection, and the trachea was cannulated with a 20-gauge catheter. Bronchoalveolar lavage was repeated four times with 0.8 ml saline by the use of a trachea tube. Bronchoalveolar lavage fluid was centrifuged at 1000 rpm for 5 min, and the supernatant was stored at −70 °C until used. The cell pellet was used to determine the number and types of cells by light microscopy.

2.4. Measurement of cytokine and protein concentrations in bronchoalveolar lavage fluid

The amount of total protein in the bronchoalveolar lavage fluid supernatant was measured by the Bradford assay (Frank et al., 2008). An ELISA was run using the bronchoalveolar lavage fluid supernatant to measure the concentrations of interleukin-6 (IL-6), interleukin-1β (IL-1β), CXCL2/macrophage inhibitory protein-2 (MIP-2), and insulin-like growth factor-1 (IGF-1) with commercially available ELISA kits specifically designed for each protein (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

2.5. Measurement of lung collagen

Total lung collagen levels were determined by measuring soluble collagen in lungs using a Sircol collagen assay kit (Biocolor, Ltd., Carrickfergus, UK) according to the manufacturer's instructions.

2.6. Histological and immunohistochemical analyses and TUNEL assay

The lungs were fixed in 10% buffered formalin solution and embedded in paraffin. Lung sections (4 μm thickness) were mounted on slides for hematoxylin–eosin (HE) staining, Masson's trichrome staining (Diagnostic BioSystems, Pleasanton, CA), and immunostaining with an antibody recognizing surfactant protein-C (SP-C; Santa Cruz Biotechnology Inc., Santa Cruz, CA). To evaluate the apoptosis of alveolar epithelial cells, a TUNEL assay of SP-C-immunostained lung tissue sections was performed using an *in situ* cell death detection kit, TMR red (Roche Diagnostics, Basel, Switzerland). The sections were counterstained with DAPI (WAKO, Saitama, Japan). Ten high-power fields per one slide were randomly selected and the numbers of TUNEL-positive cells and SP-C-positive cells were counted.

2.7. Statistical analysis

All results were subjected to statistical analysis using one-way Mann–Whitney *U* test and expressed as the means ± standard deviation. A *t*-test was used for comparison between groups, and values of $P<0.05$ were considered statistically significant. Kaplan–Meier survival analysis was performed using Prism 5 (GraphPad Software, La Jolla, CA) and statistical significance was determined by log-rank test.

3. Results

3.1. Ghrelin administration attenuated mortality, weight loss, and suppression of food intake in bleomycin-injected mice

A survival study was first performed to explore the effect of ghrelin administration on a mouse model of acute lung injury induced by 8 U/kg of bleomycin. The survival rate of the bleomycin/saline group

was 85% (17/20) on day 7, and 35% (7/20) on day 14 (Fig. 1A). In contrast, ghrelin administration maintained a survival rate of 100% (18/18) on day 7 and 94% (17/18) on day 14. Kaplan–Meier analysis showed that the overall survival rate of bleomycin/ghrelin mice was significantly higher than that of the bleomycin/saline group (log-rank, $P=0.0002$, Fig. 1A). As shown in Fig. 1B, ghrelin administration improved the survival of bleomycin-injected mice in a dose-dependent manner. In addition to bleomycin/ghrelin mice receiving ghrelin at a dose of 10 nmol/mouse, the survival rate of bleomycin/ghrelin mice receiving ghrelin at a dose of 3 nmol/mouse was also significantly higher than that of bleomycin/saline mice. We also analyzed the effects of ghrelin on body weight change and food intake in four condition groups. Administration of 6 U/kg of bleomycin significantly decreased body weight and food intake compared to the PBS groups (Fig. 1, C and D). The body weight gain and food intake in the PBS/ghrelin group were significantly higher than those of the PBS/saline group. Ghrelin administration significantly attenuated the weight loss and reduction of food intake in the bleomycin groups. To validate whether the improvement of survival rate in the ghrelin-treated group was dependent on its orexigenic effect, we carried out survival study for the bleomycin/saline group and bleomycin/ghrelin group under pair-fed condition. Kaplan–Meier analysis showed that the survival rate of bleomycin/ghrelin pair-fed mice (16/18, 89%) was significantly higher than that of bleomycin/saline mice (11/18, 56%) 14 days after 8 U/kg of bleomycin instillation (log-rank, $P=0.018$, Fig. 1E). There was no significant difference in the survival rate between bleomycin/ghrelin *ad libitum* fed group and bleomycin/ghrelin pair-fed group.

3.2. Ghrelin administration reduced neutrophil alveolar infiltration, vascular permeability, and levels of pro-inflammatory cytokines in bleomycin-injected mice

The lungs of bleomycin/saline mice exhibited markedly extensive neutrophil accumulation in the alveolar space and intra-alveolar

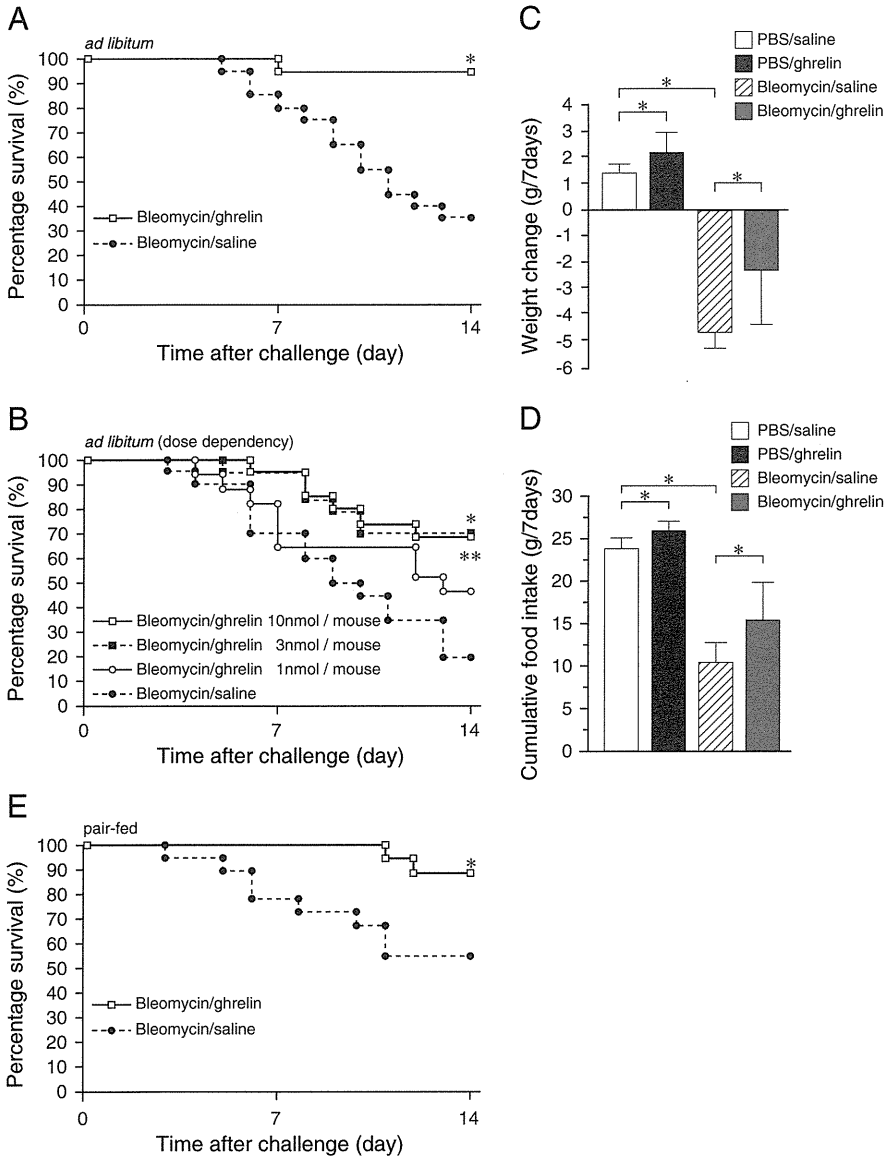

Fig. 1. Effects of ghrelin administration on survival rate, weight change, and food intake in acute lung injury model mice. (A) Kaplan–Meier survival curves for the bleomycin/saline group (n=20) and bleomycin/ghrelin group (n=18) for 14 days after challenge with 8 U/kg of bleomycin under condition of *ad libitum* feeding. *$P=0.0002$. (B) Kaplan–Meier survival curves for the bleomycin/saline group (n=20), and bleomycin/ghrelin groups administered a dose of 1 nmol/mouse (n=17), 3 nmol/mouse (n=20), and 10 nmol/mouse (n=19) of ghrelin for 14 days after challenge with 8 U/kg of bleomycin under condition of *ad libitum* feeding. *$P=0.0024$, bleomycin/saline *versus* bleomycin/ghrelin (3 nmol/mouse); **$P=0.003$, bleomycin/saline *versus* bleomycin/ghrelin (10 nmol/mouse). Alterations in body weight (C) and cumulative food intake (D) (n=5 per group) over 7 days after instillation of 6 U/kg of bleomycin or PBS. Data are expressed as the means ± SEM. *$P<0.05$. (E) Kaplan–Meier survival curves for the bleomycin/saline group (n=18) and bleomycin/ghrelin group (n=18) for 14 days after challenge with 8 U/kg of bleomycin under pair-fed condition.

edema (Fig. 2A, upper-right panel) compared to PBS/saline lungs (Fig. 2A, upper-left panel). Ghrelin administration did not affect the histological findings of the lungs in the PBS groups (Fig. 2A, left panels). Ghrelin administration to the bleomycin group reduced neutrophil accumulation and alveolar flooding (Fig. 2A, right panels). Bleomycin treatment significantly increased the numbers of total cells and neutrophils in bronchoalveolar lavage fluid in the saline groups (Fig. 2B). Ghrelin administration significantly diminished these parameters in the bleomycin groups. The total protein concentrations in bronchoalveolar lavage fluid were less than 0.05 mg/ml in the PBS groups (Fig. 2C). Bleomycin treatment significantly increased them in the saline group. Ghrelin administration significantly reduced these values in the bleomycin groups. The concentrations of IL-6, CXCL2/MIP-2, IL-1β and IGF-1 in the bronchoalveolar lavage fluid of the bleomycin/saline group were significantly higher than those of the PBS/ghrelin group (Fig. 2D). Ghrelin administration significantly reduced these cytokines in the bleomycin groups.

3.3. Ghrelin administration reduced lung fibrosis in bleomycin-injected mice

The lung sections of bleomycin/saline mice obtained on day 14 showed extensive lung fibrosis associated with destruction of the normal lung architecture (Fig. 3A, upper left panel); however, those of the bleomycin/ghrelin mice demonstrated an amelioration of lung fibrosis and comparatively preserved lung architecture (Fig. 3A, upper right panel). Masson's trichrome staining showed dense collagen deposition in bleomycin/saline mice, whereas this deposition was markedly reduced in bleomycin/ghrelin mice (Fig. 3A, lower panels). Sircol assay revealed a significant reduction of collagen contents in the bleomycin/ghrelin lungs ($P<0.05$; Fig. 3B).

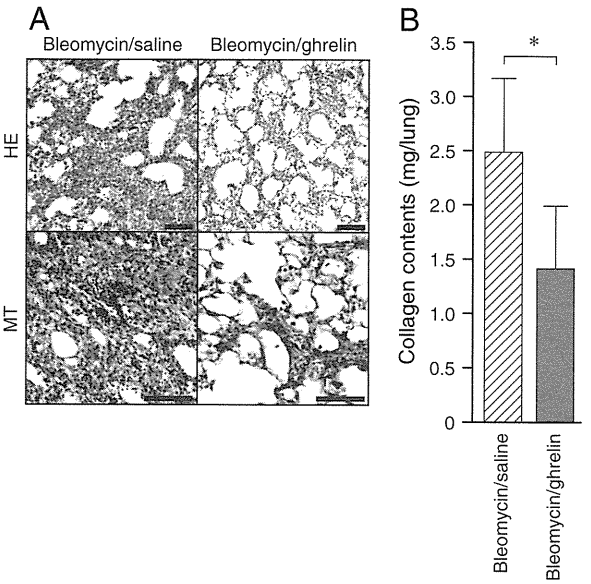

Fig. 3. Effects of ghrelin administration on pulmonary fibrosis in acute lung injury model mice. (A) Representative HE staining and Masson's trichrome (MT) staining of lung sections of bleomycin/saline (left panels) and bleomycin/ghrelin (right panels) mice on day 14 after instillation of 6 U/kg bleomycin. Scale bars: 100 μm. (B) Collagen contents in the lungs of bleomycin/saline and bleomycin/ghrelin mice (n = 5 per group). *$P<0.05$.

3.4. Ghrelin administration reduced apoptosis of alveolar epithelial cells in bleomycin-injected mice

We studied the immunohistochemistry of SP-C, a marker of type II alveolar epithelial cells, and TUNEL staining to detect apoptosis of

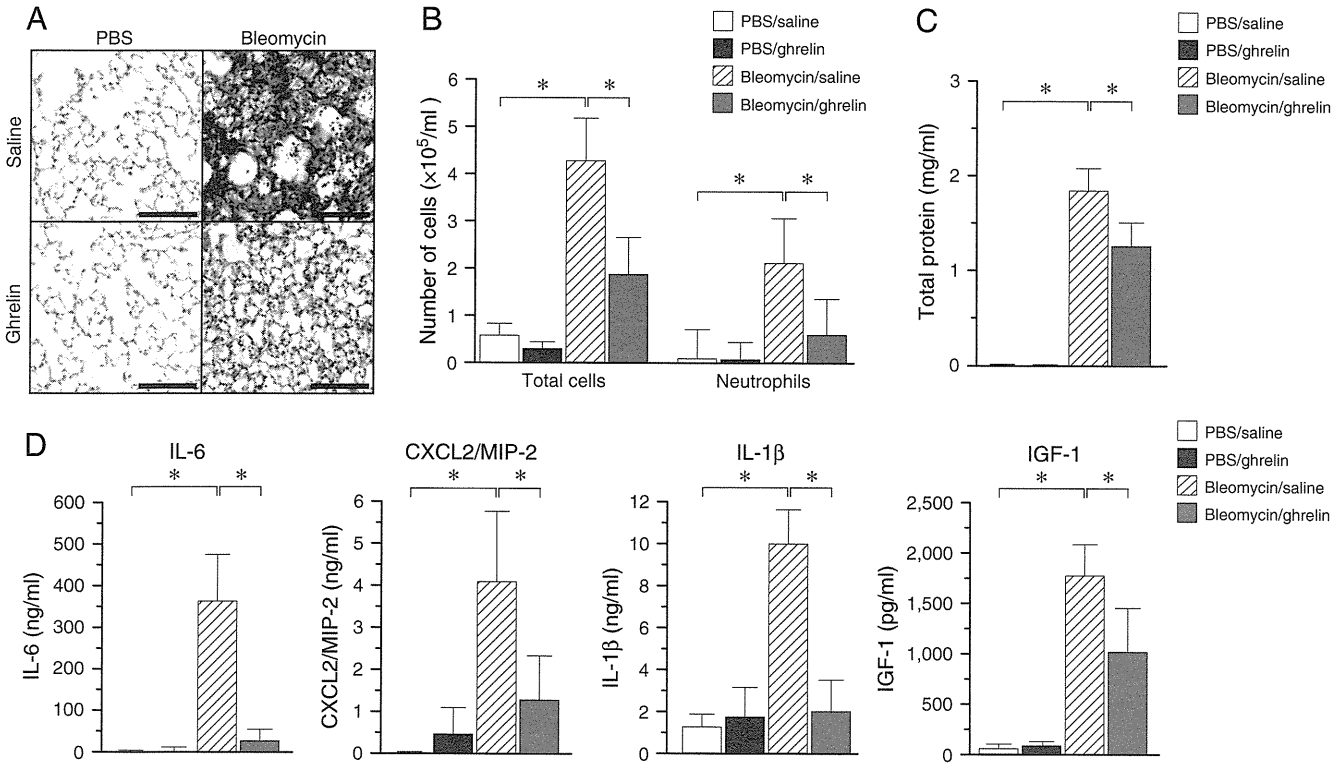

Fig. 2. Effects of ghrelin administration on neutrophil alveolar infiltration, vascular permeability, and levels of pro-inflammatory cytokines in acute lung injury model mice. (A) Representative HE staining of lung sections of saline-treated (upper panels) and ghrelin-treated (lower panels) mice after instillation of PBS (left panels) or 6 U/kg bleomycin (right panels). Scale bars: 100 μm. (B) Cell counts in the bronchoalveolar lavage fluid of saline- and ghrelin-treated mice (n = 5 per group) 7 days after bleomycin administration. *$P<0.05$. (C) Total protein concentrations in the bronchoalveolar lavage fluid. *$P<0.05$. (D) Concentrations of IL-6, CXCL2/MIP-2, IL-1β, and IGF-1 in the bronchoalveolar lavage fluid (n = 5 per group). *$P<0.05$.

alveolar epithelial cells. TUNEL and SP-C double-positive cells were frequently detected in the lung sections of bleomycin/saline mice, whereas these cells were significantly reduced in bleomycin/ghrelin mice ($P<0.05$; Fig. 4).

4. Discussion

In this study, we demonstrated for the first time the protective effects of ghrelin against bleomycin-induced acute lung injury. Ghrelin inhibited the induction of early-response cytokines and chemokines, suppressed recruitment of neutrophils into the alveolar spaces, protected alveolar epithelial cells from lung injury, ameliorated lung fibrosis, and consequently improved the survival rates in the mouse model of acute lung injury. From a therapeutic perspective, it is important to take into account that the delayed treatment with ghrelin after bleomycin administration could prevent the progression of acute lung injury. Ghrelin has been tested in patients with a variety of disorders, including cancer-related anorexia (Neary et al., 2004), chronic heart failure (Nagaya et al., 2004), and chronic obstructive pulmonary disease (Nagaya et al., 2005). These studies demonstrated that ghrelin improved these pathologies by exerting its pleiotropic effects, including induction of positive energy balance (Nagaya et al., 2004, 2005; Neary et al., 2004), stimulation of food intake (Nagaya et al., 2004, 2005; Neary et al., 2004), inhibition of sympathetic nerve activity (Nagaya et al., 2005), and protection of the cardiovascular system (Nagaya et al., 2004). These studies also showed that ghrelin administration had no side effects. Our results suggest that ghrelin could also provide a hopeful therapeutic strategy for acute lung injury/acute respiratory distress syndrome patients.

Apoptotic injury to the alveolar epithelial cells is an important component of the pathophysiology of acute lung injury and a critical element of outcome. The failures of anti-inflammatory drugs probably reflect the importance of epithelial apoptosis in the pathomechanisms of acute lung injury, which is not targeted by these anti-inflammatory agents. We here showed that ghrelin inhibited cell apoptosis of alveolar epithelial cells in the acute lung injury model. Ghrelin's protective effect on alveolar epithelial cells may prevent subsequent lung fibrosis by minimizing the denudation of the alveolar membrane. Reduction of neutrophil-mediated injury through a decrease in neutrophil alveolar accumulation may contribute to the protection of alveolar epithelial cells in ghrelin-treated mice. In addition, ghrelin has been reported to inhibit cell apoptosis in various types of cells (Baldanzi et al., 2002; Kui et al., 2009; Wang et al., 2010). Previous studies showed that ghrelin exhibited antiapoptotic effect mediated through the mitochondrial-dependent apoptotic pathway, ERK1/2 pathway, and PI3-kinase/Akt pathway, independent of its acylation, growth hormone release, or growth hormone secretagogue receptor expression on the target cells (Baldanzi et al., 2002; Kui et al., 2009; Lee et al., 2010). The direct protective mechanism of ghrelin against injury of alveolar epithelial cells will need to be verified in future studies.

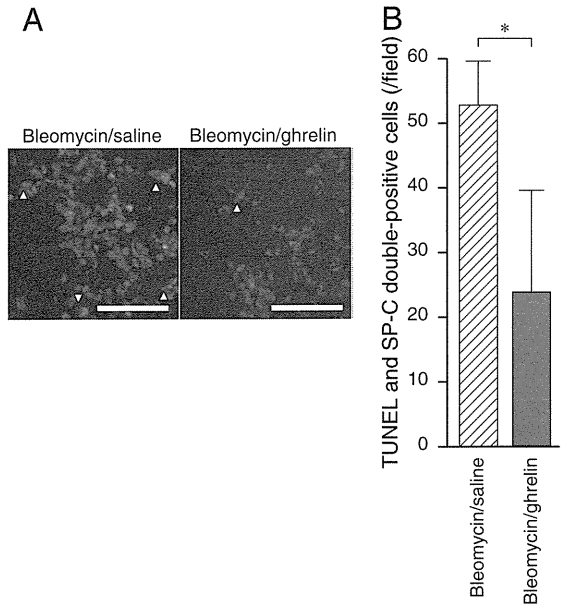

Fig. 4. Effect of ghrelin administration on cell apoptosis of alveolar epithelial cells in acute lung injury model mice. (A) Representative profiles of immunohistostaining of SP-C (green) and TUNEL staining (red) of lung sections of bleomycin/saline and bleomycin/ghrelin mice on day 7 after 6 U/kg bleomycin instillation. SP-C and TUNEL double-positive cells (arrowheads) in bleomycin/ghrelin lungs are fewer than those of bleomycin/saline lungs. DAPI was used in counterstaining of the nucleus (blue). Scale bars: 50 μm. (B) Quantification of SP-C and TUNEL double-positive cells. *$P<0.05$.

A decrease in pro-inflammatory cytokines levels is another important mechanism by which ghrelin exerted its protective effect on bleomycin-induced acute lung injury. Early-response cytokines, such as IL-1β, participate not only in neutrophil recruitment into alveolar spaces but also in acceleration of the induction of other pro-inflammatory cytokines, including IL-6 and CXCL2/MIP-2. Previous studies indicated that ghrelin has a direct effect on the production of pro-inflammatory cytokines by monocytes, T cells (Dixit et al., 2004), endothelial cells (Li et al., 2004), and alveolar epithelial cells (Hou et al., 2009) through the activation of growth hormone secretagogue receptor (Dixit et al., 2004; Li et al., 2004) and NF-κB (Li et al., 2004). The inhibitory effects of ghrelin on endothelial cells against mononuclear cell binding (Li et al., 2004) might also be involved in the amelioration of lung inflammation.

This is the first report demonstrating the antifibrotic effect of ghrelin in the injured lungs. With regard to the mechanisms of mitigation of lung fibrosis in ghrelin-treated mice, we demonstrated a significant reduction of IL-1β and IGF-1 in the bronchoalveolar lavage fluid of bleomycin/ghrelin mice. IGF-1 was reported to upregulate the production of collagen in cultured fibroblasts (Goldstein et al., 1989). In addition, a previous study showed that overexpression of IL-1β induced pulmonary fibrosis (Kolb et al., 2001). Ghrelin may therefore have an antifibrotic effect through the reduction of IL-1β and IGF-1, in addition to its effect on the maintenance of an intact alveolar epithelial cells layer which suppresses fibroblast proliferation and matrix deposition.

We also showed that ghrelin was effective in ameliorating body weight loss and food intake reduction in acute lung injury mice. Our observation that ghrelin retained its ability to stimulate food intake under the condition of acute lung injury is striking, because acute lung injury induces a series of profound and deleterious effects, including hypermetabolism and loss of lean body weight mass (Norbury et al., 2007). Although there was no difference in the survival rate between bleomycin/ghrelin *ad libitum* fed group and bleomycin/ghrelin pair-fed group, we assume that ghrelin's effects including stimulation of food intake and induction of positive energy balance are still important in acute lung injury. The fact that body mass index is independently associated with hospital mortality in mechanically ventilated patients with acute lung injury (O'Brien et al., 2006) indicates the significance of the orexigenic and anabolic effects of ghrelin on this pathology. Since IL-1β and IL-6 were reported to act on the central nervous system to control food intake and energy homeostasis (Dantzer, 2001), a reduction of IL-1β and IL-6 levels in ghrelin-treated mice may also be the cause of a comparatively maintained body weight and feeding after lung injury.

5. Conclusion

Our results demonstrated the efficacy of ghrelin administration in a rodent model of acute lung injury. Ghrelin administration protected

alveolar epithelial cells, reduced lung inflammation, ameliorated lung fibrosis, and ultimately, saved mice from bleomycin-induced acute lung injury. The pleiotropic effects of ghrelin against acute lung injury shown in this study suggest a novel attractive therapeutic strategy for the treatment of acute lung injury in humans.

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Identification of the endogenous cysteine-rich peptide trissin, a ligand for an orphan G protein-coupled receptor in *Drosophila*

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ABSTRACT

There are many orphan G protein-coupled receptors (GPCRs), for which ligands have not yet been identified, in both vertebrates and invertebrates, such as *Drosophila melanogaster*. Identification of their cognate ligands is critical for understanding the function and regulation of such GPCRs. Indeed, the discovery of bioactive peptides that bind GPCRs has enhanced our understanding of mechanisms underlying many physiological processes. Here, we identified an endogenous ligand of the *Drosophila* orphan GPCR, CG34381. The purified ligand is a peptide comprised of 28 amino acids with three intrachain disulfide bonds. The preprotein is coded for by gene CG14871. We designated the cysteine-rich peptide "trissin" (it means for triple S–S bonds) and characterized the structure of intrachain disulfide bonds formation in a synthetic trissin peptide. Because the expression of trissin and its receptor is reported to predominantly localize to the brain and thoracicoabdominal ganglion, trissin is expected to behave as a neuropeptide. The discovery of trissin provides an important lead to aid our understanding of cysteine-rich peptides and their functional interaction with GPCRs.

1. Introduction

G protein-coupled receptors (GPCRs) constitute a large protein superfamily, which shares a 7-transmembrane motif as a common structure. Human genome sequencing has identified several hundred orphan GPCRs, for which ligands have not yet been identified [1]. Meanwhile, the recent sequencing of the *Drosophila melanogaster* genome [2] has enabled the identification of at least 160 fly GPCRs [3]. *Drosophila* is an excellent animal model for genetic analysis, as it is a small organism with a relatively short lifecycle and can be bred easily under laboratory conditions. Combined with the possibility of manipulating the *Drosophila* genome, this offers a powerful tool for studying developmental and behavioral processes. GPCRs play crucial roles in cell-to-cell communication involved in a variety of physiological phenomena, and are the most common target of pharmaceutical drugs. Therefore, identification of endogenous ligands for orphan GPCRs will lead to clarification of novel physiological regulatory mechanisms and potentially facilitate development of new GPCR-targeted therapeutics. Recently, many bioactive molecules have been discovered or identified as endogenous ligands of orphan GPCRs by using reverse pharmacology [4]. These include nociceptin, prolactin-releasing peptide, orexin, apelin, ghrelin, metastin, and neuromedin S [5–12]. Presently, the discovery of novel endogenous ligands for orphan GPCRs in mammals is challenging. In part, this may be due to the very restricted timing of expression or distribution pattern of GPCR ligands. Structural or sequence comparison of newly discovered peptides in *Drosophila* with those in mammals may lead to the discovery of new peptide signaling modules. Here, we report the identification of trissin, a ligand for the orphan GPCR CG34381

Abbreviations: GPCRs, G protein-coupled receptors; CHO, Chinese hamster ovary; CM, carboxymethyl; ACN, acetonitrile; TFA, trifluoroacetic acid; PTH, phenyl thiohydantoin.

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in *D. melanogaster*. Trissin has 28 amino acids, three disulfide bonds, and no significant structural similarities to known endogenous peptides. Cysteine-rich peptides are known to have antimicrobial or toxicant activities, although frequently their mechanism of action is poorly understood. Our data provide an important lead to aid our understanding of cysteine-rich peptides and their functional interaction with GPCRs in both insect and mammals.

2. Materials and methods

2.1. Construction of an assay system using CG34381-expressing cells

The full-length cDNA of *Drosophila* CG34381 (GenBank Accession No. NM_135118; residues 515–2656) was obtained by RT-PCR, with *Drosophila* cDNA as the template. The sense and antisense primers were 5′-gcagcgacgtcctttattgg-3′ and 5′-aagtgggtccatccttcgat-3′, respectively. The amplified cDNA was ligated into a pcDNA3.1 vector (Invitrogen, Tokyo, Japan). The expression vector CG34381-pcDNA3.2 was transfected into Chinese hamster ovary (CHO) cells. Thereafter, stably expressing cells were selected using 1 mg/ml G418 (Nacalai Tesque, Kyoto, Japan). The selected cell line CHO-CG34381-line 9–26 showed the highest expression of CG34381 mRNA. The cells were cultured in a humidified environment of 95% air:5% CO_2. Changes in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) were measured using the FlexStation 3 fluorometric imaging plate reader (Molecular Devices, CA, USA). CHO-CG34381 cells (3×10^4 cells) were plated into 96-well black-wall microplates (Corning, NY, USA) 20 h before each assay. The cells were incubated with 100 μl of the Calcium 4 assay kit (Molecular Devices) for 1 h, and then 50 μl of each sample was added to the CHO-CG34381 cells. The maximum $[Ca^{2+}]_i$ change was then determined as a response.

2.2. Purification of Drosophila trissin

A total of 400 g of *D. melanogaster* flies (Canton S.) was collected on dry ice. A basic peptide fraction was prepared as described previously [13] and then fractionated on a Sephadex G-50 gel filtration column (2.9 × 142 cm; GE Healthcare, Tokyo, Japan). A portion of each fraction, equivalent to 1.16 g of flies, was subjected to the Calcium 4 assay using CHO-CG34381 cells. The active fraction was separated by carboxymethyl (CM)-ion exchange high-performance liquid chromatography (HPLC) on a TSK CM-2SW column (4.6 × 250 mm; Tosoh, Tokyo, Japan) with an ammonium acetate ($HCOONH_4$) (pH 6.5) gradient of 10 mM to 1 M in the presence of 10% acetonitrile (ACN) at a flow rate of 1 ml/min for 180 min. The active fraction was further purified by fractionation on the same column at pH 4.8. The active fractions were separated by reverse-phase (RP)-HPLC with a μBondasphere C18 column (3.9 × 150 mm; Waters, MA, USA) with a 10–60% ACN/0.1% trifluoroacetic acid (TFA) linear gradient at a flow rate of 1 ml/min for 80 min. The active fractions were further purified by RP-HPLC using a diphenyl column (2.1 × 150 mm; 219TP5125, Vydac, Hesperia, CA, USA) for 80 min under a linear gradient of 10–60% ACN/0.1% TFA at a flow rate of 0.2 ml/min. Fractions corresponding to absorption peaks were collected, and an aliquot of each fraction (2 g tissue equivalent) was assayed by the FLEX system. Approximately 5 pmol of the final purified peptides were analyzed with a protein sequencer (Model 494; Applied Biosystems, CA, USA). Approximately 0.5 pmol of the active fraction was used for molecular weight determination by matrix-assisted laser desorption–ionization time of flight (MALDI-TOF) mass spectrometry with a Voyager-DE PRO instrument (Applied Biosystems).

2.3. Cloning of Drosophila prepro-trissin cDNA

A tblastn search of the *Drosophila* genome resources was performed using the sequence of the purified peptides, and a *D. melanogaster* mRNA sequence (CG14871; NM_142214) derived from an annotated genomic sequence was obtained. We searched for the open reading frame both upstream and downstream of the genome sequence of CG14871 by using specific primers (5′-gttcacatgcccactggagtc-3′; 5′-ctggggatatccttaggggtagtag-3′). The candidate PCR product was subcloned into the pCR-II TOPO vector and sequenced. The nucleotide sequence of the isolated cDNA fragment was determined by automated sequencing (DNA sequencer: model 3100, Applied Biosystems) according to the protocol for the BigDye terminator cycle sequencing kit (Applied Biosystems).

2.4. Peptide synthesis and structural analysis

Trissin was synthesized by Peptide Institute, Inc (Osaka, Japan). Three disulfide bonds (S–S bonds) were spontaneously formed by oxidation. Synthetic S–S bonded peptides was digested by thermolysin or endoproteinase Asp-N and analyzed with a protein sequencer or mass spectrometry.

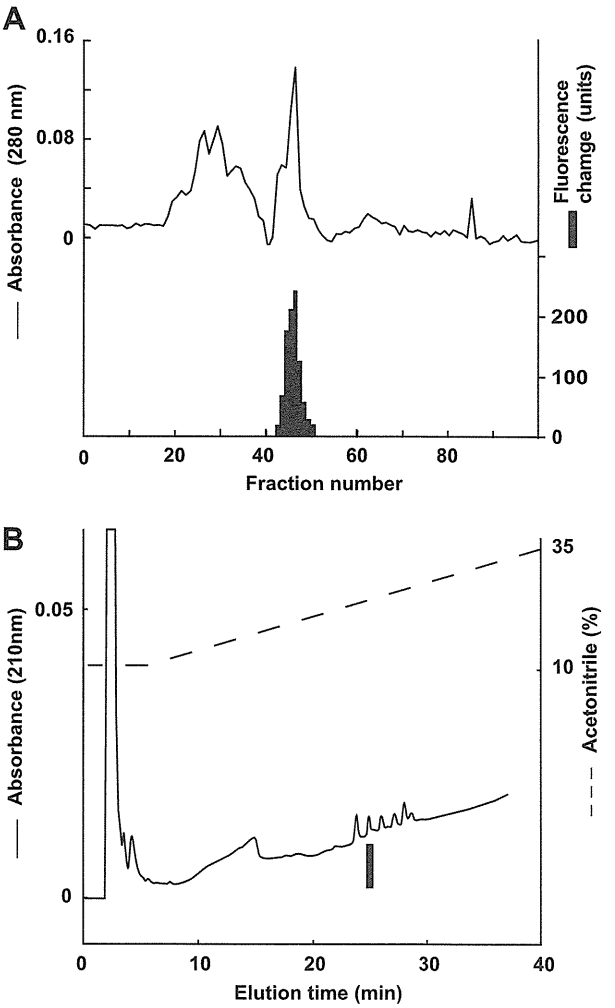

Fig. 1. Purification of trissin from fly extracts. *Black bars* indicate fluorescence signal changes of $[Ca^{2+}]_i$ in CHO-CG34381 cells. (A) G-50 gel filtration of the basic peptide fraction of fly extracts. Active fraction was subjected to two steps of CM-ion exchange HPLC and 2 steps of RP-HPLC. (B) Final purification of the active fraction by RP-HPLC.

3. Results and discussion

The first $[Ca^{2+}]_i$ assays were performed using the gel filtration samples as part of the effort to isolate the endogenous ligand of CG34381 (Fig. 1A). Nine sequential fractions exhibited activity (numbers 43–51). Fractions 46–47, which contained particularly high activity, were further separated by CM-ion exchange HPLC at pH 6.5 and pH 4.8. The active fraction was purified as a single peak in the final RP-HPLC (Fig. 1B). The final yield of the purified peptide was approximately 5.5 pmol. A partial N-terminal amino acid sequence of the purified peptide was determined to be IKXDTXGKEXASAXGTKHFRTXXFNY, using a protein sequencer (where X is a position that was not identified). For elucidating the complete amino acid sequence of the peptide, a *Drosophila* cDNA encoding the purified peptide was isolated by RT-PCR. The cDNA encoded a 108-residue protein (CG14871) that contained

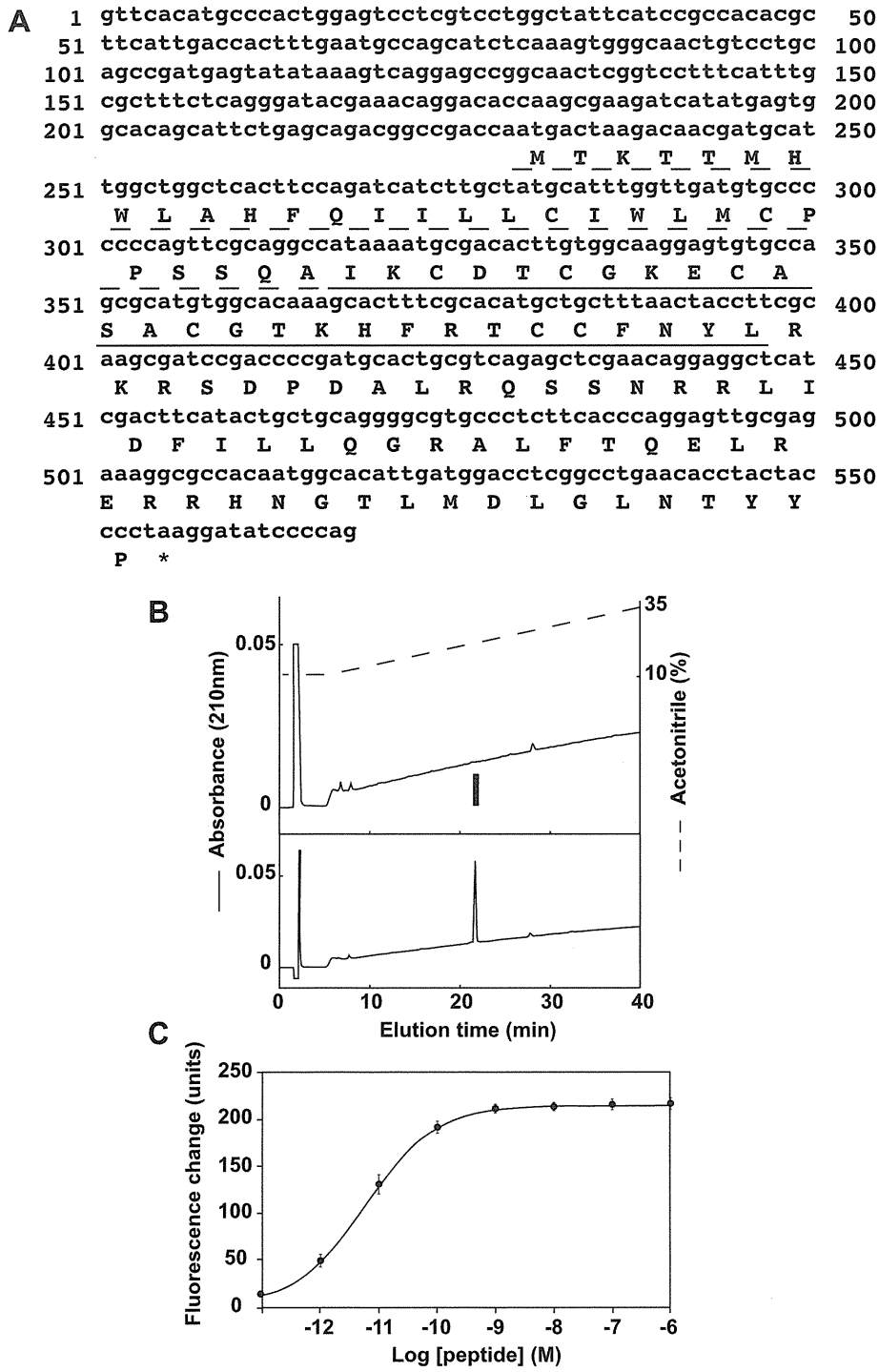

Fig. 2. (A) Nucleotide sequence and deduced amino acid sequence of trissin. The trissin cDNA encodes a 108-residue protein. The dotted line denotes the predicted signal peptide, which preceded the sequence of trissin itself (continuous underline). (B) Chromatographic comparison by RP-HPLC of natural trissin (upper panel) and synthetic trissin (lower panel). *Black bars* indicate the fluorescence signal changes of $[Ca^{2+}]_i$ in CHO-CG34381 cells (upper panel). Each peptide was applied to a symmetry C18 column with a linear gradient elution for 80 min. (C) Dose–response relationship of changes in $[Ca^{2+}]_i$ for synthetic trissin in CHO-CG34381 cells. Each symbol on the line graph represents the mean ± S.E.M. of data from six replicates for each dose.

features characteristic of an N-terminal signal peptide immediately preceding the purified peptide sequence. It also indicated that every X residue was cysteine, while the rest of the sequence was identical to that determined by peptide sequencing (Fig. 2A). Sequencing resulted in a very low yield of phenyl thiohydantoin (PTH) at the steps involving X, suggesting that all these cysteines might form disulfide bonds (S–S bonds). This preproprotein contained a potential processing site at the C-terminal end of the purified peptide sequence. We therefore deduced the primary structure of the newly purified 28-residue peptide to be IKCDTCGKECASACGTKHFRTCCFNYL. Mass spectrometric analysis revealed that the observed monoisotopic *m/z* value of the purified peptide (2996.0182) was very close to the theoretically predicted value for this peptide (2996.2446), which has three intrachain disulfide bonds. Since the exact positions of S–S bonds in purified peptide were unknown, we generated a synthetic peptide and subjected it to oxidizing conditions. This lead to the formation of three S–S bonds that were confirmed by amino acid analysis and mass spectrometry. The synthetic peptide had an identical retention time to the natural peptide according to RP-HPLC (Fig. 2B). We also examined the interaction of the synthetic peptide with the GPCR CG34381. This peptide induced dose-dependent, robust increases in $[Ca^{2+}]_i$ in CHO-CG34381, with half-maximal response concentrations (EC_{50}) of 5.73×10^{-12} M (Fig. 2C). The natural peptide and synthetic peptide showed comparable potency and efficacy for this receptor. Neither peptide induced a response in CHO cells transfected with vector alone (data not shown). These results suggest that the structure of the synthetic peptide is nearly identical to that of the natural peptide. To identify the structure of three intrachain disulfide bonds, we first digested the synthetic peptide with thermolysin. Three digested segments (TH1, TH2, and TH3) were obtained and each segment was analyzed by mass spectrometry (Fig. 3A). In addition, TH1 was analyzed with a protein sequencer. Because of sequences information, there are three segments which were bridged by three S–S bonds in TH1. We then digested the TH1 segment with endoproteinase Asp-N. Two digested segments (TH1/D1 and TH1/D2) were obtained and each segment was analyzed by mass spectrometry (Fig. 3B). These data suggest that TH1/D1 has an S–S bond (Cys3–Cys14) and TH1/D2 has two potential S–S bonds, although the mode of disulfide pairings in the latter peptide remained unclear. Theoretically, two types of S–S modes could be considered for TH1/D2, i.e. A-type and B-type as illustrated in Fig. 3B. Sequence analysis confirmed that TH1/D2 has the A type S–S bonds (Cys6–Cys23 and Cys10–Cys22), since $PTH(Cys)_2$ was observed at the fifth and seventh cycles. Therefore, we propose that the structure of this peptide is IKCDTCGKECAS ACGTKHFRTCCFNYL (Cys3–Cys14, Cys6–Cys23, Cys10–Cys22) and designate it 'trissin' (Fig. 3C). We have not found this disulfide linkage type, C^{I}–C^{IV}, C^{II}–C^{VI}, C^{III}–C^{V}, in other cysteine-rich peptides. Trissin shares high sequence similarity with peptides from various insects (Fig. 4). Kaplan et al. reported raalin as a predicted toxin-like peptide from *in silico* studies of *Apis mellifera* [14]. The amino acid sequence of raalin is DQCGRKCANICGTQQFPACCFN, and it therefore shares some similarity with trissin. However, the deduced sequence of raalin has only five cysteines, and characteristic N-terminal signal peptide or C-terminal processing sites were not identified. Therefore, we suggest that raalin may be derived from a longer polypeptide homologous to the *Drosophila* trissin preproprotein. Further studies are required to determine whether the structure of honeybee raalin is similar to that of trissin.

Cysteine-rich peptides have diverse physiological functions, and further studies are now required to place trissin within this polypeptide class. The cysteine-rich defensin family exhibits antimicrobial activity in vertebrates, invertebrates, and plants [15,16], whereas scorpion toxins and conus snail venoms have toxicant activities [17,18]. In addition, LUREs attract competent pollen tubes

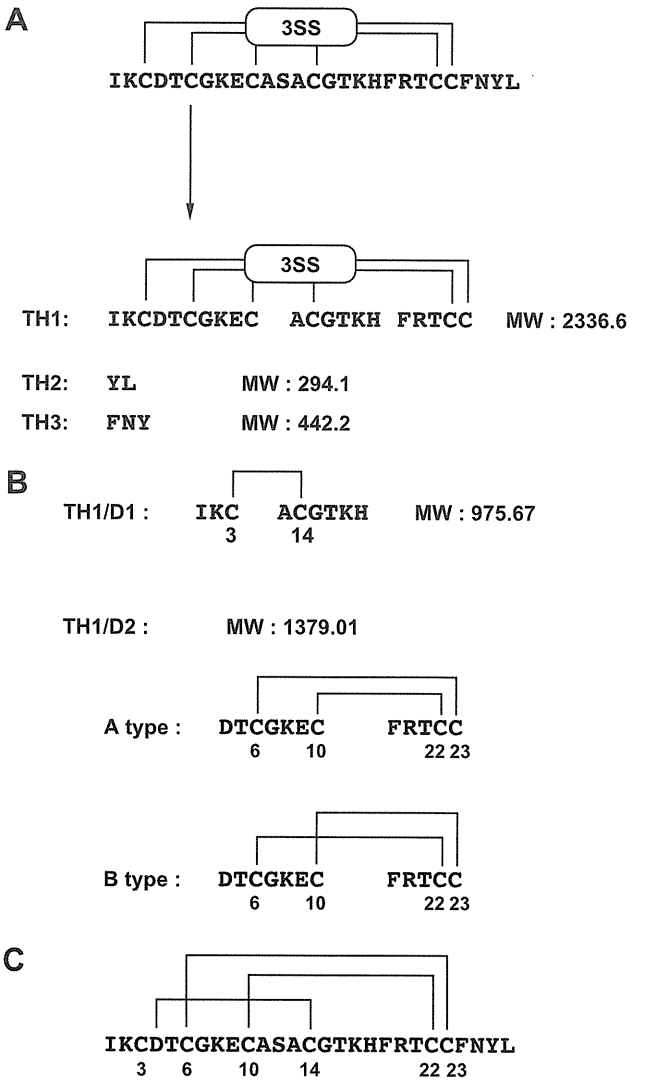

Fig. 3. Structure determination of three intrachain disulfide bonds in trissin. (A) Structures of the peptides obtained by digesting the synthetic trissin with thermolysin. (B) Structures of TH1/D1 and TH1/D2 obtained by digesting TH1 with endoproteinase Asp-N. Two possible disulfide structures, i.e. A-type and B-type could be considered for TH1/D2. (C) Structure of trissin.

in plants [19]. The activities of defensins and LUREs are not mediated by GPCRs. Rather, defensins act as effectors of immunity via a direct interaction with the microbial membrane that leads to disruption or lesion formation, or their translocation into the cytoplasm, where they can reach intramolecular targets [20]. On the other hand, most receptors recognized by scorpion toxins and conus snail venoms are ion channels [20,21]. Prokineticin-1 and -2, which are mammalian homologs of the black mamba peptide VPRA/MIT1 and frog skin peptide Bv8, contain 10 cysteine residues. These polypeptides are the cognate ligands for two closely related GPCRs that couple either to *Gi* or *Gq* [22–24]. The distribution of defensins is consistent with their defensive role, as they are mainly found in immunocompetent cells or surface epithelia [20]. Prokineticins are expressed not only in peripheral tissues, but also in the central nervous system [25]. Intracerebroventricular injection of prokineticin-2 controls the circadian rhythm from the SCN [26]. Like prokineticins, we show here that trissin is a cognate GPCR ligand. However, the physiological role of trissin remains to be determined. Both trissin (CG14871) and the trissin receptor (CG34381) are expressed predominantly in the brain and thoracicoabdominal

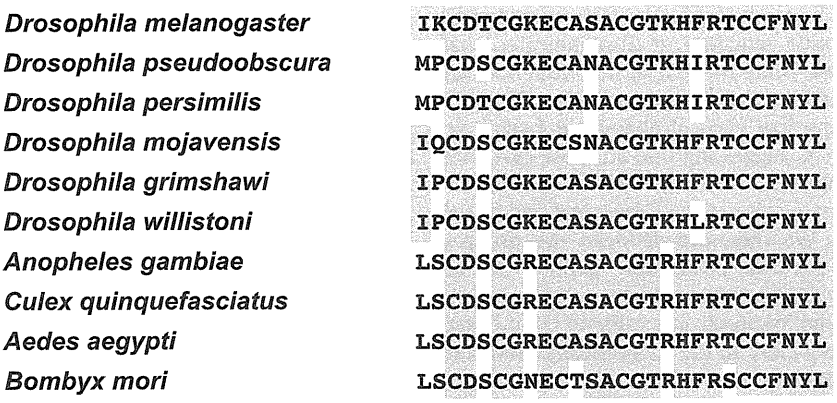

Fig. 4. Sequence comparison of trissin between insects. *Drosophila melanogaster*, *D. pseudoobscura*, *D. persimilis*, *D. mojavensis*, *D. grimshawi*, *D. willistoni*, *Anopheles gambiae*, *Culexquinque fasciatus*, *Aedes aegypti*, and *Bombyx mori* sequences are aligned. Residues identical between peptides are shaded.

ganglion (by FlyAtlas; http://www.flyatlas.org/; University of Glasgow). These distributions raise the possibility that trissin functions as a neuropeptide. Thus, future functional genetic studies of the role of trissin and its receptor at these sites in *Drosophila* and in mammalian systems will provide greater insight into its biological role, and into GPCR modulation in general.

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Identification of the novel bioactive peptides dRYamide-1 and dRYamide-2, ligands for a neuropeptide Y-like receptor in *Drosophila*

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ABSTRACT

A number of bioactive peptides are involved in regulating a wide range of animal behaviors, including food consumption. Vertebrate neuropeptide Y (NPY) is a potent stimulator of appetitive behavior. Recently, *Drosophila* neuropeptide F (dNPF) and short NPF (sNPF), the *Drosophila* homologs of the vertebrate NPY, were identified to characterize the functions of NPFs in the feeding behaviors of this insect. Dm-NPFR1 and NPFR76F are the receptors for dNPF and sNPF, respectively; both receptors are G protein-coupled receptors (GPCRs). Another GPCR (CG5811; NepYR) was indentified in *Drosophila* as a neuropeptide Y-like receptor. Here, we identified 2 ligands of CG5811, dRYamide-1 and dRYamide-2. Both peptides are derived from the same precursor (CG40733) and have no significant structural similarities to known bioactive peptides. The C-terminal sequence RYamide of dRYamides is identical to that of NPY family peptides; on the other hand, dNPF and sNPF have C-terminal RFamide. When administered to blowflies, dRYamide-1 suppressed feeding motivation. We propose that dRYamides are related to the NPY family in vertebrates, similar to dNPF and sNPF.

1. Introduction

The neuropeptide Y (NPY) family of peptides is widely conserved among vertebrates and has been implicated in feeding behavior, circadian rhythm, anxiety, and other physiological processes [1–6].

The vertebrate NPY family consists of NPY, peptide YY (PYY), and pancreatic polypeptide (PP), and these are grouped together based on structural similarities and evolutionary relationships. The C-terminal RYamide is conserved among peptides of the NPY family. In invertebrates, NPY homologs are designated as neuropeptide F (NPF), because the C-terminal residue in all cases appears to be an amidated phenylalanine (F) residue, in contrast to the typical amidated tyrosine (Y) residue in vertebrate NPY family peptides [7]. NPFs have been isolated and identified in several invertebrates [7,8]. In 1999, the first NPF, a 36-residue neuropeptide, was isolated from *Drosophila melanogaster*, and the gene encoding this neuropeptide was identified [9]. In addition to long NPF peptide, another group of peptides was discovered in insects; these peptides ranged in size from 6 to 11 amino acid residues and showed a similar carboxyterminus. These are referred to as short neuropeptide F (sNPF) [7,8]. The *sNPF* (CG13968) and *dNPF* (CG10342) genes have been characterized in *D. melanogaster* genome. The gene *dNPF* is expressed in the brain and midgut of larvae and adult flies and is known to regulate larval feeding behavior [9,10]. Also, the function of sNPF was found to control food intake and regulate body size [11]. Overexpression of sNPF promotes food intake and knockdown of sNPF results in the opposite phenotype. Recently, it was shown that *Drosophila* sNPF regulates extracellular signal-related kinase (ERK)-mediated insulin-like peptide (DILP2, 3, and 5) expression to modulate growth and metabolism [12]. These functions of dNPF and sNPF as feeding modulators are similar to the role of vertebrate NPY.

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The vertebrate NPY receptors can be divided into five subgroups: Y1–Y6. In *Drosophila*, *Dm-NPFR* (CG1147), the receptor for dNPF, was cloned and found to be homologous to the vertebrate NPY receptor family of seven-transmembrane G protein-coupled receptors (GPCRs) [13]. *NPFR76F* (CG7395), the receptor for sNPF, has also been cloned [14,15]. Both receptors show structural similarities to vertebrate Y2 receptors and are expressed in the brain and gut. NPFR76F is not activated by other RFamide peptides, indicating that the fourth arginine from C-terminal is crucial for receptor activation [15]. Another GPCR (CG5811; NepYR) was identified in *Drosophila* that could be activated by mammalian NPY and PYY and that shows some sequence similarities to NPY and NPF receptor [16]. CG5811 did not specifically bind radiolabeled dNPF, in contrast to Dm-NPFR. The natural ligand has not yet been found for CG5811.

We were interested in the role of this orphan receptor CG5811, because the novel bioactive peptide for this receptor may provide new insights into feeding mechanisms. In this study, we identified dRYamide-1 and dRYamide-2, the ligands for CG5811 in *D. melanogaster*. Injection of dRYamide resulted in the suppression of feeding motivation in the blowfly. Our data suggest that dRYamides are novel bioactive peptides for CG5811, and that these bioactive peptides are candidate factors for NPY family and the factor of feeding control in *Drosophila*.

2. Materials and methods

2.1. Construction of an assay system using CG5811-expressing cells

The full-length cDNA of *Drosophila* CG5811 (GenBank accession number NM_079801; residues 84–1583) was obtained by RT-PCR, with *Drosophila* cDNA as the template. The sense and antisense primers were 5′-ttctaccattgacgcgcttg-3′and 5′-cggtgcttcttatgtttgcttc-3′, respectively. The amplified cDNA was ligated into a pcDNA3.1 vector (Invitrogen, Tokyo, Japan). The expression vector, CG5811-pcDNA3.1, was transfected into Chinese hamster ovary (CHO) cells. Thereafter, stably expressing cells were selected using 1 mg/ml G418 (Nacalai Tesque, Kyoto, Japan). The selected cell line, CHO-CG5811-line 7-4, showed the highest expression of CG5811 mRNA. The cells were cultured in a humidified environment of 95% air: 5% CO_2. Changes in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) were measured using the FlexStation 3 fluorometric imaging plate reader (Molecular Devices, CA, USA). CHO-CG5811 cells (3×10^4 cells) were plated into 96-well black-wall microplates (Corning, NY, USA) 20 h before each assay. The cells were incubated with 100 μl of Calcium 4 assay kit (Molecular Devices) for 1 h, and then 50 μl of each sample was added to the CHO-CG5811cells. The maximum $[Ca^{2+}]_i$ change was then determined as a response.

2.2. Purification of Drosophila dRYamide-1 and dRYamide-2

A total of 350 g of *D. melanogaster* flies (Canton S.) was collected on dry ice. A basic peptide fraction (SP-III) was prepared as described previously [17] and then fractionated on a Sephadex G-50 gel filtration column (2.9 × 142 cm; GE Healthcare, Tokyo, Japan). A portion of each fraction, equivalent to 1.16 g of flies, was subjected to the assay using CHO-CG5811 cells. The active fraction was separated by carboxymethyl (CM) ion-exchange high-performance liquid chromatography (HPLC) on a TSK CM-2SW column (4.6 × 250 mm; Tosoh, Tokyo, Japan) with an ammonium acetate ($HCOONH_4$) (pH 6.5) gradient of 10 mM–1 M in the presence of 10% acetonitrile (ACN) at a flow rate of 1 ml/min for 180 min. The active fraction was further purified by fractionation on the same column at pH 4.8. The active fractions were separated by reverse-phase (RP)-HPLC with a μBondasphere C18 column (3.9 × 150 mm, Waters, MA, USA) with a 10–60% ACN/0.1% trifluoroacetic acid (TFA) linear gradient at a flow rate of 1 ml/min for 80 min. The active fractions were further purified by RP-HPLC using a diphenyl column (2.1 × 150 mm; 219TP5125, Vydac, Hesperia, CA, USA) for 80 min under a linear gradient of 10–60% ACN/0.1% TFA at a flow rate of 0.2 ml/min. Fractions corresponding to absorption peaks were collected, and an aliquot of each fraction (2 g tissue equivalent) was assayed by the FLEX system. Approximately 10 pmol and 20 pmol of the final purified peptides were analyzed with a protein sequencer (model 494; Applied Biosystems, CA, USA).

2.3. Cloning of Drosophila prepro-dRYamide cDNA

A tblastn search of the *Drosophila* genome resources was performed using the sequence of the purified peptides, and a *D. melanogaster* mRNA sequence (CG40733; NM_001110912) derived from an annotated genomic sequence was obtained. The sequence was not a full-length open reading frame cDNA encoding prepro-dRYamide-1 and dRYamide-2. Thus, we searched for the open reading frame both upstream and downstream of the genome sequence of CG40733 by using specific primers (5′-cttcgtccccttgttattattgtct-3′; 5′-cgttctagacgacgggatagatatt-3′). The candidate PCR product was subcloned into the pCR-II TOPO vector and sequenced. The nucleotide sequence of the isolated cDNA fragment was determined by automated sequencing (DNA sequencer: model 3100, Applied Biosystems) according to the protocol for the BigDye terminator cycle sequencing kit (Applied Biosystems).

2.4. Peptides

dRYamide-1, dRYamide-2, dRYamide-1 C-terminal free, and dRYamide-2 C-terminal free were synthesized by Medical and Biological Laboratories (Nagano, Japan). Neuropeptide Y (NPY) (human, rat), Peptide YY (PYY) (human 3–36), and RFamide-related peptide-1 (RFRP-1) were purchased from the Peptide Institute, Inc. (Osaka, Japan). Pancreatic Polypeptide (PP) (human), neuropeptide FF (NPFF) (human), neuropeptide AF (NPAF) (human), and neuropeptide VF (NPVF) (rat, mouse) were purchased from Phoenix Pharmaceuticals, Inc. (CA, USA).

2.5. Proboscis extension reflex test for appetite measurements

The proboscis extension reflex (PER) test and the feeding test were performed in the blowfly *Phormia regina* as described previously [18]. The blowflies had been raised in the laboratory and fed on 0.1 M sucrose at 22 °C under the 16 h/8 h LD cycle. Behavioral analyses were performed on 5–7 days-old adult flies, which had been starved for 24 h and provided with only water. dRYamide-1 was dissolved in the blowfly linger solution to be 10 pmol per μl. The 20 flies were secured by their wings with washing pins, and the first PER test was performed with 12 steps of sucrose concentrations that had been prepared by 2-fold serial dilutions with distilled water, beginning from a sucrose concentration of 1 M. We investigate the PER in three different groups of 20 flies each; no injection, fly linger injection, and, fly linger plus peptide injection. The PER tests were performed 30 min after 1 μl of blowfly linger solution with or without peptide was injected into the shoulder in each flies. We repeated four sets of PER test each of which 20 flies were used in a same batch.

2.6. Statistical analysis

Results are presented as mean ± S.E.M. for each group. To compare the PER thresholds between four groups, we used a

nonparametric Steel–Dwass test. The criterion for statistical significance was $P < 0.05$ for all the tests. We used the statistical software program GraphPad PRISM (GraphPad software, CA, USA).

3. Results

3.1. Structural determination of dRYamide-1 and dRYamide-2

The first $[Ca^{2+}]_i$ assays were performed using the gel filtration samples as part of the effort to isolate the endogenous ligands of CG5811 (Fig. 1A). The active fractions were observed in 20 sequential fractions (numbers 41–61). When the fractions (51–54) with particularly high activity were separated by CM-ion-exchange HPLC at pH 4.8, two separate agonist activities were revealed (Fig. 1B, P1 and P2). Each activity was purified as a single peak in the final RP-HPLC (Fig. 1C and D). The amino acid sequences of the purified peptides were determined as NEHFFLGSRY (P1) and PVFFVASRY (P2) by a protein sequencer. For elucidating the complete amino acid sequence of these peptides, a *Drosophila* cDNA encoding the purified peptides was isolated by RT-PCR. The *D. melanogaster* cDNA encoded a 109-residue protein (CG40733) (Fig. 2A). The P1 and P2 C-terminal sequences contained Gly, which presumably serves as an amide donor for C-terminal amidation. We generated the synthetic peptides PVFFVASRY, PVFFVASRY-NH_2, NEHFFLGSRY, and NEHFFLGSRY-NH_2. The retention times of the P1 and P2-active fractions were identical to the those of the synthetic NEHFFLGSRY-NH_2 and PVFFVASRY-NH_2 peptides on RP-HPLC, respectively (Fig. 2B). Thus, these data suggest that both natural peptides have C-terminal amidation. PVFFVASRY-NH_2 and NEHFFLGSRY-NH_2 were named dRYamide-1 and dRYamide-2, respectively. Each peptide shares high sequence similarity with peptides from various mosquitoes (Fig. 2C).

3.2. Pharmacological characterization

The interaction of dRYamide-1 and dRYamide-2 with CG5811 was examined using synthetic peptides. dRYamide-1 and dRYamide-2 induced dose-dependent, robust increases in $[Ca^{2+}]_i$ in CHO-CG5811, with half-maximal response concentrations (EC_{50}) of 1.31×10^{-11} and 3.97×10^{-11}, respectively (Fig. 3A). Neither dRYamide-1 nor dRYamide-2 induced a response in CHO cells transfected with vector alone (data not shown). Non-C-terminal amidated synthetic peptides activated CG5811 only slightly (EC_{50}; 1.78×10^{-6} and 1.59×10^{-8}, respectively) (Fig. 3A). Fig. 3B shows the amino acid sequences of mammalian NPY and RFamide family peptides and their interaction with CG5811. Dose–response curves are presented in Fig. 3C (NPY family peptides) and Fig. 3D (RFamide family peptides). The effect of almost all NPY and RFamide family peptides on CG5811 was weaker than that of the dRYamides (EC_{50}; submicromolar vs. subnanomolar range). However, NPFF was substantially more active in the nanomolar range.

3.3. Proboscis extension reflex (PER) test for measuring feeding sensitivity

As shown in Fig. 4, a significant increase in the mean PER threshold defined as the sucrose concentration, at which 50% of flies show PER, was observed, after the injection of 10 pmol dRYamide-1; 230 mM (no injection) to 320 mM (30 min after linger solution injection) ($p > 0.05$ vs. no injection) or 2300 mM

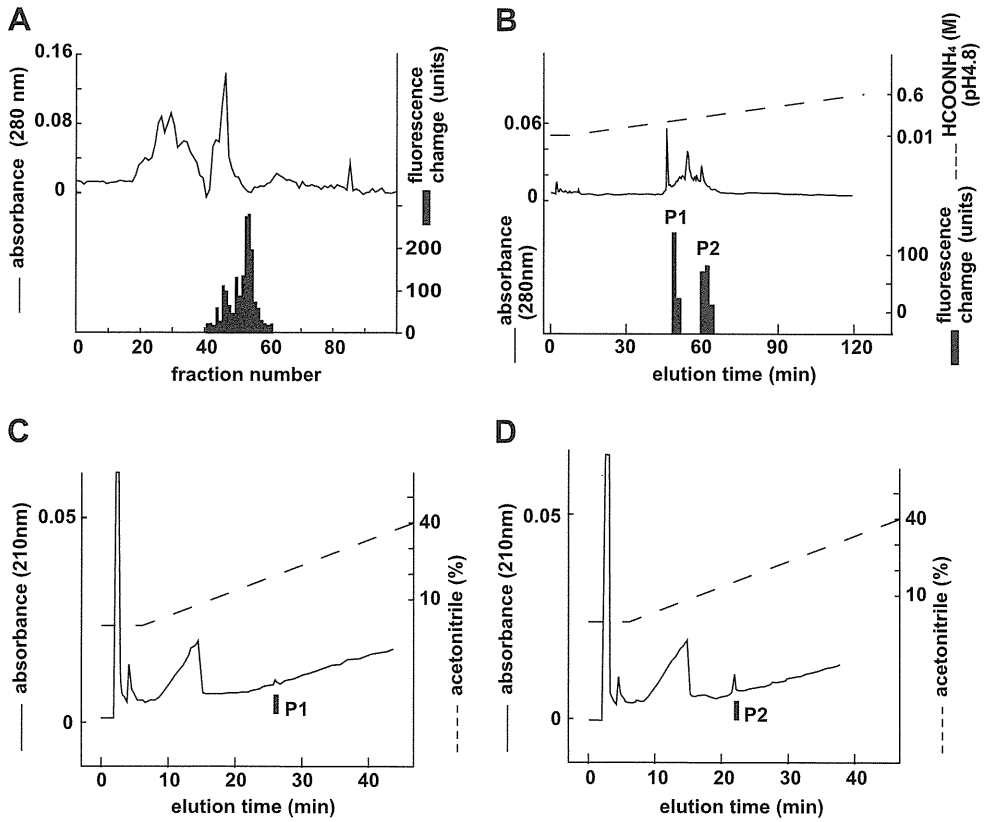

Fig. 1. Purification of dRYamide-1 and dRYamide-2 from fly extracts. *Black bars* indicate fluorescence signal changes of $[Ca^{2+}]_i$ in CHO-CG5811 cells. (A) G-50 gel filtration of the SP-III fraction of fly extracts. (B) CM ion-exchange HPLC (pH 4.8) of fr51–54 on the gel filtration. Each active fraction (P1 and P2) was subjected to 3 steps of RP-HPLC. (C and D) Final purification of the active fraction P1 (C) and P2 (D) obtained from CM-HPLC by RP-HPLC.

A

```
   1  cttcgtccccttgttattattgtctgaatttttaagtatatataataata   50
  51  atgaatgaatgtgtaaacaaattattacatttaaagtttttgttctattt  100
       M  N  E  C  V  N  K  L  L  H  L  K  F  L  F  Y  F
 101  cattctaggaattcaaaaacgcccagttttttttgttgctagtcgctatg  150
         I  L  G  I  Q  K  R(1) P  V  F  F  V  A  S  R  Y
 151  gacgaagtactacatacgatgaaagtttaaaatccagacgcattttcatc  200
       G*  R  S  T  T  Y  D  E  S  L  K  S  R  R  I  F  I
 201  gtacctcgaaacgagcatttctttcttggctctcggtacggtaaacgaag  250
         V  P  R(2) N  E  H  F  F  L  G  S  R  Y  G*  K  R  S
 251  cggcaaatatttatgtctttcgagggaaataaataagctgatagtcagaa  300
         G  K  Y  L  C  L  S  R  E  I  N  K  L  I  V  R
 301  aaagattacgaaacaatgataaagaacgaactccaactttaagctttata  350
       K  R  L  R  N  N  D  K  E  R  T  P  T  L  S  F  I
 351  accaaacactttctgatgcgaaatacttaactagtatattaaaagaaatt  400
         T  K  H  F  L  M  R  N  T stop
 401  tcaatgtagagaccgccagttattgattgaccaatattctgagtttgtaa  450
 451  tggcattcgactctgacatgaatgccaattactaatgcttttgttaaat  500
 501  ataacatataat----------tggctgcagttt 1250
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1301  atctatcccgtcgtctagaacg                             1350
```
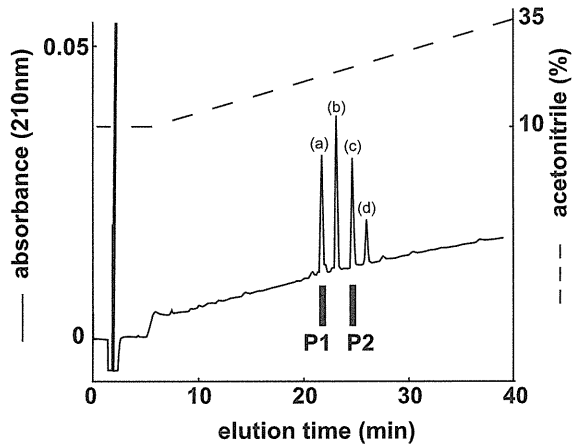

B



C

	dRYamide-1	dRYamide-2
Drosophila melanogaster	PVFFVASRY-NH_2	NEHFFLGSRY-NH_2
Drosophila pseudoobscura	PAFFVGSRY-NH_2	NDRFFLGSRY-NH_2
Drosophila ananassae	TAFFVGSRY-NH_2	NDRFFLGSRY-NH_2
Anopheles gambiae	P-FFVGSRY-NH_2	NDRFFLGSRY-NH_2
Culex quinquefasciatus	P-FFVGSRY-NH_2	NDRFFLGSRY-NH_2
Anopheles aegypti	P-FFVGSRY-NH_2	NDRFFLGSRY-NH_2

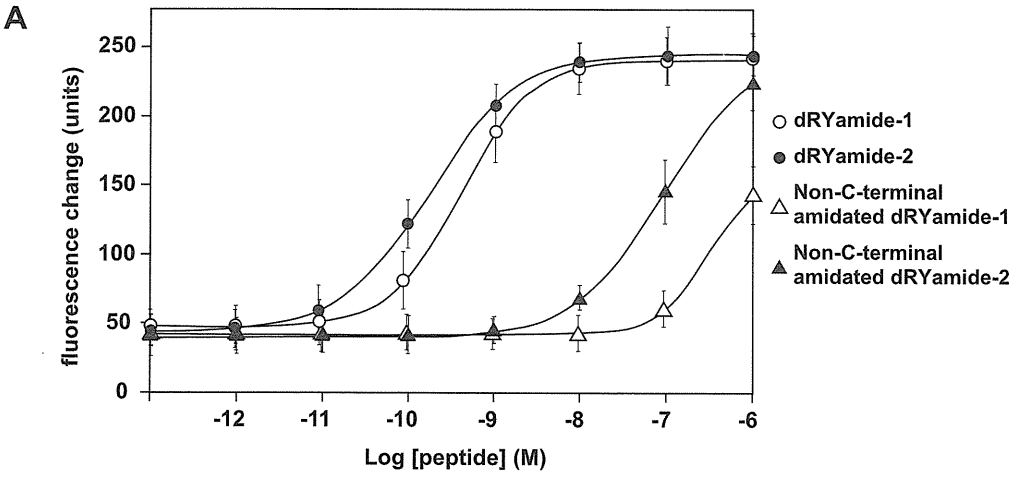
Fig. 2. (A) Nucleotide sequence and deduced amino acid sequence of dRYamide-1 and dRYamide-2 cDNA. The dRYamide-1 and dRYamide-2 cDNA encodes a 109-residue protein. The asterisks show a glycine residue, which serves as an amide donor for C-terminal amidation. The dRYamide-1 and dRYamide-2 sequences are underlined (1) and (2), respectively. (B) Chromatographic comparison by RP-HPLC of natural dRYamides and synthetic dRYamides. *Black bars* indicate the fluorescence signal changes of $[Ca^{2+}]_i$ in CHO-CG5811 cells. Each peptide was applied to a Symmetry C18 column with a linear gradient elution for 80 min. P1 and P2 represent active fractions containing natural dRYamides. (a–d) synthetic peptides, (a) dRYamide-2, (b) non-C-terminal amidated dRYamide-2, (c) dRYamide-1, (d) non-C-terminal amidated dRYamide-1. (C) Sequence comparison of dRYamide-1 and dRYamide-2. *Drosophila melanogaster*, *Drosophila pseudoobscura*, *Drosophila ananassae*, *Anopheles gambiae*, *Culex quinquefasciatus*, and *Aedes aegypti* sequences are aligned. Residues identical between peptides are shaded.

(30 min after dRYamide-1 injection) ($p < 0.05$ vs. linger solution injection).

4. Discussion

In this study, we purified 2 novel peptides, dRYamide-1 and dRYamide-2, as endogenous ligands for the orphan GPCR CG5811 (NepYR). These peptides do not correspond to any known peptide.

The dRYamide preproprotein is 109 amino acids residues long and contains both dRYamide-1 and dRYamide-2. Pharmacological characterization using CHO cells expressing receptors indicated that dRYamide-1 and dRYamide-2 share a highly similar potency for activating recombinant CG5811. However, non-C-terminal amidated synthetic peptides only slightly activated CG5811. These results show that both dRYamide-1 and dRYamide-2 are cognate ligands for CG5811. CG5811 has been shown to be expressed predominantly in the hindgut (by FlyAtlas; http://www.flyatlas.org/; University of Glasgow). The gene expression levels of *dRYamides* (CG40733) have not been published in FlyAtlas yet. We generated specific antisera against dRYamide-1 and dRYamide-2. In an immunohistochemical analysis using dRYamide-2-specific antiserum,



B

	amino acid sequence	EC_{50}(M)
dRYamide-1	PVFFVASRY-NH_2	3.98 x 10^{-10}
dRYamide-2	NEHFFLGSRY-NH_2	1.72 x 10^{-10}
NPY family		
NPY	YPSKPDNPGEDAPAEDMARYYSALRHYINLITRQRY-NH_2	3.19 x 10^{-7}
PYY3-36	YPAKPEAPGEDASPEELSRYYASLRHYLNLVTRQRY-NH_2	2.51 x 10^{-8}
PP	APLEPMYPGDYATPEQMAQYETQLRRYINTLTRPRY-NH_2	1.51 x 10^{-7}
RF-amide peptide family		
NPFF	SPAFLFQPQRF-NH_2	1.74 x 10^{-9}
NPAF	AGEGLNSQFWSLAAPQRF-NH_2	1.10 x 10^{-7}
RFRP-1	SLNFEELKDWGPKNVIKMSTPAVNKMPHSFANLPLRF-NH_2	1.21 x 10^{-7}
NPVF	FPSLPQRF-NH_2	5.65 x 10^{-8}

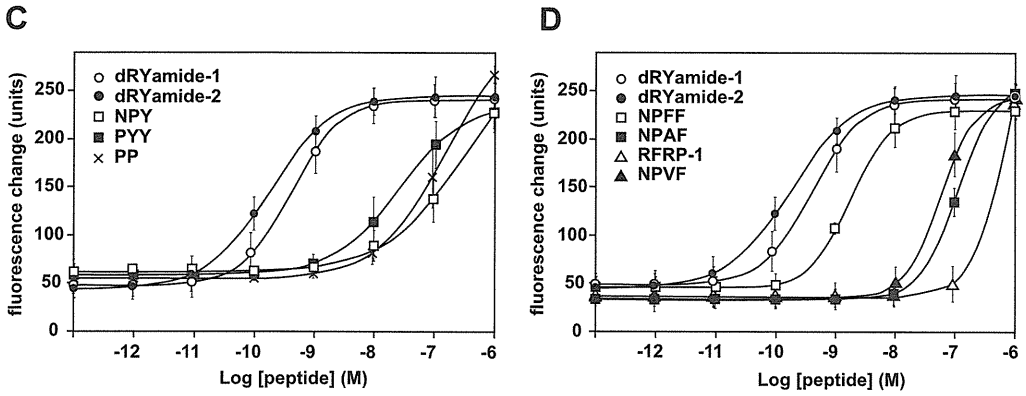


Fig. 3. Pharmacological characterization of synthetic peptides using CG5811 stably expressed in CHO cells. (A) Dose–response relationships of changes in $[Ca^{2+}]_i$ for dRYamide-1 (open circle), dRYamide-2 (filled circle), non-C-terminal amidated dRYamide-1 (open triangle), and non-C-terminal amidated dRYamide-2 (filled triangle) in CHO-CG5811 cells. (B) Sequences of synthetic peptides and half-maximal response concentrations (EC_{50}) (M) for changes in $[Ca^{2+}]_i$ by using CHO-CG5811. Residues identical between peptides are shaded. (C and D) Dose–response relationships of changes in $[Ca^{2+}]_i$ for various peptides, dRYamide-1 (open circle), and dRYamide-2 (filled circle) in CHO-CG5811 cells. (C) NPY (open square), PYY (filled square), and PP (cross) (D) NPFF (open square), NPAF (filled square), RFRP-1 (open triangle), and NPVF (filled triangle). Each symbol on the line graph represents the mean ± S.E.M. of data from six times for each experiment.

dRYamide-2 was distributed in the larval central nervous system (CNS), midgut, and hindgut [Sano et al., in preparation]. Therefore, dRYamides are suspected to be brain–gut peptides in insects.

It is generally accepted that NPY family peptides regulate feeding behavior in mammals [1–3]. Therefore, we evaluated their effects using the PER test in the blowfly *P. regina*. In flies and certain other insects, the PER test has long been used to investigate behavioral sensitivity to phagostimulative tastes [18]. Flies extend their proboscis when the contact chemosensilla on their labella detect sweetness of sugar above a certain threshold concentration. Thus, we estimated appetite or feeding motivation of the fly by the PER test to sucrose, in which the threshold concentration of sucrose was evaluated as an indicator of feeding sensitivity. The present study showed that the injection of dRYamide-1 increased the threshold for feeding on a sucrose solution. These data suggest that dRYamides attenuated the feeding motivation of the flies. Indeed, administration of dRYamides significantly reduced sucrose intake. Instead, the administration of dRYamides did not affect the