In vitro selection of a peptide antagonist of growth hormone secretagogue receptor using cDNA display

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G protein-coupled receptors (GPCRs) are major drug targets, and their ligands are currently being explored and developed by many pharmaceutical companies and independent researchers. Class A (rhodopsin-like) GPCRs compose a predominant GPCR family; therefore, class A GPCR ligands are in demand. Growth hormone secretagogue receptor (GHS-R) is a class A GPCR that stimulates food intake by binding to its peptide ligand, ghrelin. Therefore, antagonists of GHS-R are expected to exert antiobesity function. In this article, we describe the use of cDNA display to screen for successfully and identify an antagonistic peptide of GHS-R. The antagonistic peptide inhibited the ghrelin-induced increase in intracellular Ca^{2+} in vitro (IC_{50} = approximately 10 μM) and repressed the contraction of isolated animal stomach in response to ghrelin. Furthermore, peripheral administration of the peptide inhibited the food intake of mice. This work provides new insight into the development of antiobesity drugs and describes a method for the discovery of unique peptide ligands for class A GPCRs.

aptamer | in vitro display | peptide drug | ligand screening | cell-based selection

Ghrelin, a 28-amino acid peptide hormone with a unique *N*-octanoyl modification at Ser[3], is an endogenous ligand of growth hormone secretagogue receptor (GHS-R), which is a member of the class A (rhodopsin-like) G protein-coupled receptor (GPCR) family (1). Ghrelin is mainly secreted in the stomach and, through binding to GHS-R, stimulates not only the release of growth hormone but also weight gain by increasing food intake and decreasing energy consumption (2). It has been reported that peripheral administration of ghrelin increases food intake and weight gain in animals (3–5) and humans (6). Additionally, with the exception of ghrelin, no hormones that stimulate food intake via peripheral administration are known. Therefore, GHS-R antagonists are expected to perform antiobesity functions by suppressing food intake and weight gain. In fact, small-molecule GHS-R antagonists and [D-Lys-3]-GHRP-6, which is one of the few known peptide antagonists of GHS-R, decrease food intake and weight gain via peripheral administration (7–10).

Because a peptide can be chemically synthesized and is unlikely to act as an antigen, peptide drugs are attractive candidates to replace antibodies in drug therapies targeting specific molecules. The exploration of how novel peptides bind to a cell surface receptor from a randomized peptide library has been achieved using phage display (11). Drugs developed from peptides that bind to thrombopoietin (12, 13) and erythropoietin receptors (14) were discovered by phage display and have been used in therapies (15). Alternatively, in vitro display techniques, such as mRNA display (16, 17) and ribosome display (18, 19), provide advantages over phage display in available library size and selection rapidity. In fact, novel peptide (20) or proteins (21–26) binding to a cell surface receptor were also selected by in vitro display techniques, some of which (20, 22, 24) are physiologically active. However, bioactive peptides that bind to class A GPCRs, which constitute a major GPCR family (27), have not been selected via in vitro display techniques.

To identify a ligand for class A GPCRs, the complete structure of the receptor in question is required. The extracellular domain of a receptor, although useful in ligand selection, is not sufficient to identify a ligand for class A GPCRs because the ligand-binding site of class A GPCRs encompasses the transmembrane region, which is composed of multiple α-helices (28). Living cells, which express the target receptors on their surfaces, are appropriate for binding target because receptors expressed on living cells, unlike receptors in membrane fractions, assume their native structure and display only their extracellular side. In fact, living cells were used to screen DNA or RNA aptamers (29–35), as well as both peptides and antibodies, using phage display libraries (36–41), which bind to membrane proteins expressed on the cell surface. Nevertheless, selection with live cells has not been performed with in vitro display techniques because mRNA display and ribosome display (in which a peptide is displayed on mRNA) are labile and easily degraded by ribonucleases in serum-supplemented cell culture medium.

Recently, we have developed an in vitro display system, termed cDNA display, which is an improved system of mRNA display; some peptides were successfully screened via this method (42–46). Because a peptide is displayed on a cDNA in cDNA display (instead of being displayed on mRNA, as with mRNA display), peptide-cDNA fusion libraries resist degradation by ribonucleases and can be used under severe selection conditions, such as cell culture medium.

In this study, we used cDNA display to screen a unique GHS-R-binding peptide with antagonistic activity against GHS-R. In doing so, we used live cells expressing GHS-R on their surfaces as binding targets. To the best of our knowledge, this report describes a previously uncharacterized selection of novel bioactive peptide ligands for class A GPCRs via an in vitro display technique.

Results

The Selection Procedure. The selection scheme used in this study is illustrated in Fig. 1. Initially, a randomized DNA library was prepared and converted to mRNA and mRNA/cDNA-peptide fusion library. Nonspecifically bound peptides were removed via exposure to the cells that were not expressing GHS-R. The remaining peptides were exposed to cells expressing GHS-R. Peptides bound to the cells were collected and amplified to prepare the DNA library used in the next round of selection. GHS-R-

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The authors declare no conflict of interest.

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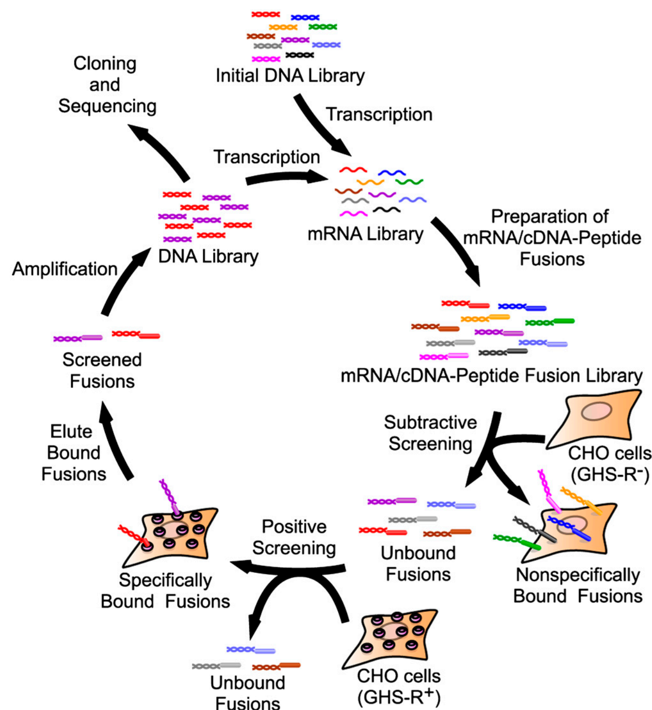

Fig. 1. Schematic representation of the selection of GHS-R-binding peptides. The initial DNA library was transcribed into a mRNA library and subsequently converted to a mRNA/cDNA-peptide fusion library. The peptide fusion library was incubated with normal cells, which did not express GHS-R. Bound fusions were discarded and the unbound fusions were incubated with GHS-R-expressing cells. The bound fusions were collected and their DNA moieties were amplified via PCR. The amplified DNA moieties were used for a DNA library in the next round of selection. After performing this selection several times, peptides binding to GHS-R were enriched, and its sequences were analyzed via cloning and sequencing of the bound DNA.

binding peptides were selected by repeatedly performing this selection process. Details of the selection conditions are shown in Table S1 and described in *Materials and Methods*.

Pilot Selection. Prior to the actual selection with a library of randomized peptides, we performed a pilot experiment to confirm the feasibility of our selection procedure. Although octanoyl modification at Ser^3 in ghrelin is essential for its activity (1), it has been reported that replacing octanoyl Ser^3 with Trp preserves its activity with minimal inhibition (47). Therefore, we constructed the mRNA/cDNA-peptide fusions encoding a Trp^3-mutant ghrelin (Trp^3-ghrelin) and des-octanoyl ghrelin (desacyl-ghrelin) as model binding and unbinding peptides, respectively (Fig. 2). A mixture of mRNA/cDNA-peptide fusions comprising Trp^3-ghrelin and desacyl-ghrelin at a ratio of 1:10 was incubated with normal CHO cells or CHO-GHSR62 cells (1), of which only the latter expressed GHS-R. Peptide fusions bound to the cells were collected, and their DNA moieties were amplified via PCR and analyzed via polyacrylamide gel electrophoresis. As a result, it was possible to enrich molecules encoding Trp^3-ghrelin from a mixture containing an order of magnitude of excess desacyl-ghrelin molecules in a single round of selection for CHO-GHSR62 cells relative to normal CHO cells (Fig. 3). This result indicates that cDNA-display selection is practical for use with live cultured cells as binding targets.

Actual Selection. The N-terminal region of ghrelin is important for its binding activity (47) and for the determination of ligand characteristics (i.e., agonist or antagonist) (48, 49). In addition, it is thought that the C-terminal region of ghrelin provides stability against degradation in blood (50). Therefore, we used a randomized ghrelin mutant library for the initial selection in which eight N-terminal amino acids were randomized and 20 C-terminal amino acids were conserved without modification (Fig. 2). After five rounds of selection (the details of which are summarized in Table S1), DNA molecules in the library were cloned, and 14 clones were sequenced. As a result, the library converged to three variants for which the sequence G5-1 was most common (11/14 clones), whereas the others were single substitutes of G5-1 (Table 1). Converged sequences had no homology to ghrelin or other known peptide ligands of GHS-R. The eight N-terminal amino acids of G5-1 displayed no homology to the known proteins of humans and common experimental animals, including mice and rats, as assessed by a BLAST search.

Intracellular Calcium Mobilization Assay. Activities of the selected peptides were investigated via an intracellular calcium mobilization assay. Stimulated GHS-R activates the G protein subunit Gα 11, leads to the formation of inositol triphosphate, and induces Ca^{2+} release. Fig. 4 depicts the results of the intracellular calcium

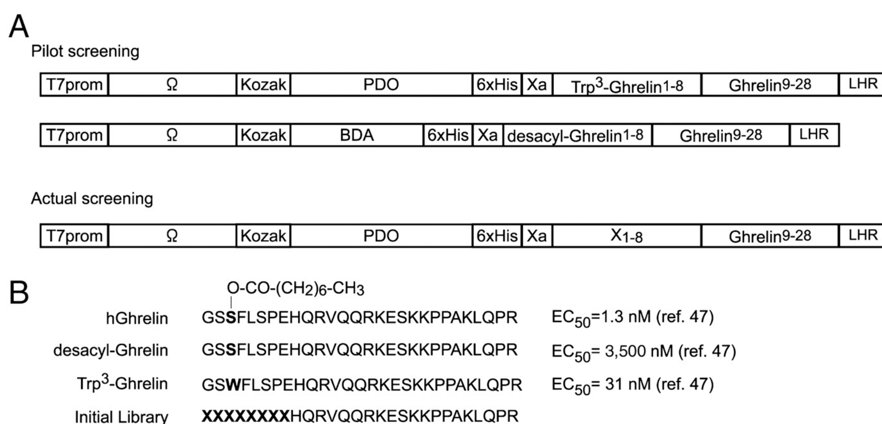

Fig. 2. DNA constructs and amino acid sequences used in this study. (*A*) DNA constructs used in the pilot screen and actual screen. The amino acid sequences of Trp^3-ghrelin$^{1–8}$, desacyl-ghrelin$^{1–8}$, and $X_{1–8}$ are described in (*B*). Additional symbols and abbreviations are defined as follows: T7prom, T7 promoter; Ω, translation enhancer of tobacco mosaic virus; Kozak, Kozak sequence for translation initiation; PDO and BDA, POU-specific DNA-binding domain of Oct-1 (PDO) or B domain of protein A (BDA) as a scaffold protein; 6xHis, hexahistidine tag; Xa, the recognition site for factor Xa protease; Trp^3-ghrelin$^{1–8}$, desacyl-ghrelin$^{1–8}$; $X_{1–8}$, eight N-terminal amino acids of the ghrelin mutant or eight randomized amino acids; ghrelin$^{9–28}$, 20 C-terminal amino acids of ghrelin; and LHR, the hybridization region for the puromycin linker. (*B*) Amino acid sequences of the regions of ghrelin used in the selection process. hGhrelin is native human ghrelin listed as a reference, desacyl-ghrelin and Trp^3-ghrelin were used in the pilot Selection, and “initial library” was used in the actual Selection. “X” represents a randomized amino acid.

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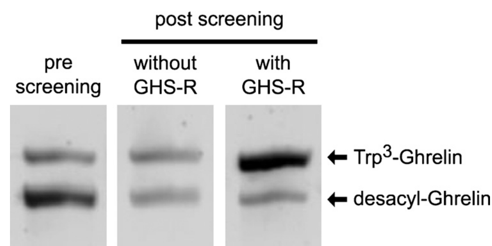

Fig. 3. Result of the pilot selection. Single-round selection was performed with CHO-GHSR62 cells and normal CHO cells as binding targets for the library, which was a mixture of two DNA constructs encoding Trp^3-ghrelin and desacyl-ghrelin at a ratio of 1 : 10. After binding procedure, DNA moieties of the bound molecules were amplified by PCR and subjected to analysis. Trp^3-ghrelin gene was significantly enriched during selection for CHO-GHSR62 cells relative to normal CHO cells.

mobilization assay, in which costimulation with 5 nM ghrelin and various concentrations of the selected peptides were tested on CHO-GHSR62 cells. As illustrated by Fig. 4, G5-1 suppressed the intracellular calcium increase caused by ghrelin binding to GHS-R in a dose-dependent manner. The suppression was equivalent to that of [D-Lys-3]-GHRP-6, a known peptidic antagonist, with an IC_{50} of approximately 10 μM. However, G5-2, which contained a single amino acid substitute of G5-1, and G5-1-N8, which contains only eight N-terminal G5-1 amino acids, exhibited no suppression.

Binding Assay. The binding affinity of G5-1 to GHS-R was examined by a competitive binding assay. Cell membranes of CHO-GHSR62 cells were incubated with [^{125}I]-ghrelin (51) and increasing concentrations of the test ligands (ghrelin, [D-Lys-3]-GHRP-6, and G5-1). As shown in Fig. S1, these ligands suppressed the binding of [^{125}I]-ghrelin to GHS-R. The IC_{50} and K_i values of G5-1 were 2.50 ± 0.56 and 2.40 ± 0.60 μM, respectively. The affinity of G5-1 for GHS-R was comparable to that of [D-Lys-3]-GHRP-6. This comparable effect was equivalent to the result of physiological inhibition observed in the intracellular calcium mobilization assay.

Organ Bath Assay. *Suncus* stomachs were used for in vitro contraction experiments. Motilin is a peptide hormone that stimulates gastric contraction similarly to ghrelin, and it is not expressed in rodents such as mice and rats (52). *Suncus murinus* is a small mammal that expresses both motilin and ghrelin; thus, it can be used to study motilin-ghrelin family peptides (53). *Suncus* stomachs pretreated with low concentrations of motilin (100 pM) underwent contraction in response to ghrelin in a dose-dependent manner (Fig. S2). Co-pretreatment with 100 pM motilin and either 1 μM [D-Lys-3]-GHRP-6 or G5-1 nearly eliminated the contraction of *Suncus* stomachs induced with ghrelin (Fig. 5). It is noteworthy that motilin began to evoke gastric contraction of *S. murinus* at 1 nM but was unable to do so at 100 pM in vitro (54). Stomach contractions occurred in response to 100 pM ghrelin after pretreatment with motilin alone, but only small contractions occurred when stomachs were pretreated with [D-Lys-3]-GHRP-6 or G5-1 in conjunction with motilin, even at high doses of ghrelin. This result indicates that, during pretreatment, [D-Lys-3]-GHRP-6 and G5-1 bound to GHS-R expressed on the stomach and blocked binding of ghrelin.

Table 1. Selected and analyzed peptides

	Sequence	No. of clones
Library	XXXXXXXX HQRVQQRKESKKPPAKLQPR	—
G5-1	FQFLPFMF HQRVQQRKESKKPPAKLQPR	11
G5-2	FQFLPFMS HQRVQQRKESKKPPAKLQPR	2
G5-3	FQFLPVMF HQRVQQRKESKKPPAKLQPR	1
G5-1-N8	FQFLPFMF-NH_2	—

Three peptides (G5-1, G5-2, and G5-3) were selected and three peptides (G5-1, G5-2, and G5-1-N8) were chemically synthesized and analyzed.

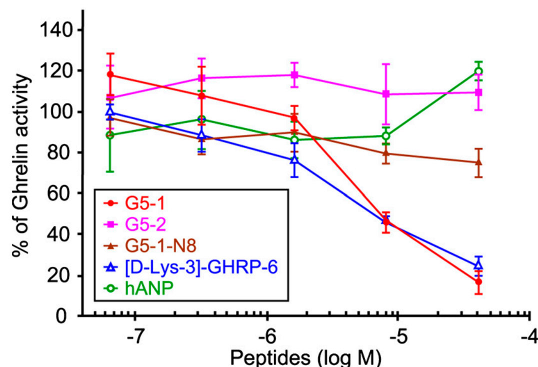

Fig. 4. Antagonistic activity of selected peptides. CHO-GHSR62 cells were stimulated with 5 nM ghrelin and the appropriate concentration of each selected peptide, and the resulting Ca^{2+} release was measured. The ghrelin effect was suppressed by G5-1 and [D-Lys-3]-GHRP-6, a control peptide, in a dose-dependent manner. G5-2 and G5-1-N8 showed no inhibition. Human atrial natriuretic peptide (hANP) was used as a negative control. The IC_{50} of G5-1 was approximately 10 μM. All values are presented as means ± SEM ($n = 4$).

Feeding Tests. It is known that the level of ghrelin in serum increases during fasting and decreases upon refeeding in rodents (3). Next, G5-1 was administered peripherally to determine how it affects ghrelin-induced food intake in fasting mice.

[D-Lys-3]-GHRP-6 (1.5 mg/kg; 1.6 μmol/kg) and G5-1 (2.0 mg/kg; 0.57 μmol/kg) were intravenously administrated to mice that had fasted for 16 h. [D-Lys-3]-GHRP-6 significantly suppressed food intake by 1 and 2 h postadministration. Similarly,

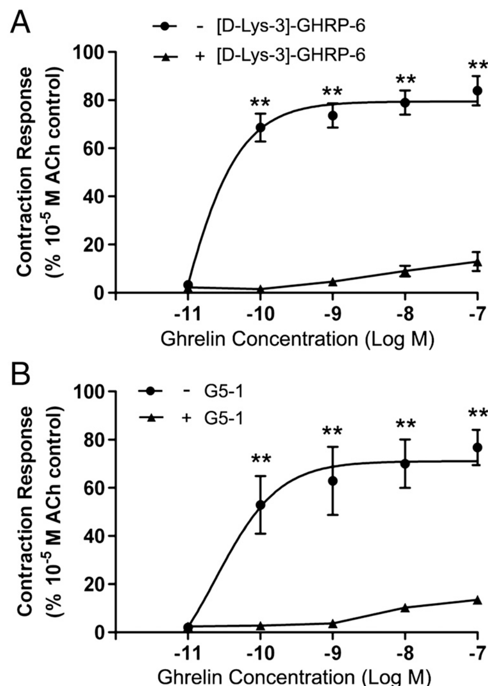

Fig. 5. Effect of [D-Lys3]-GHRP-6 and G5-1 on ghrelin-induced gastric contractions in *S. murinus*. The *Suncus* stomach was pretreated with 100 pM motilin and either 1 μM [D-Lys3]-GHRP-6 (*A*) or G5-1 (*B*). Subsequently, stomachs were treated with each concentration of ghrelin and the resulting contractile response was measured. The contractile response is expressed as a percentage of the contraction induced by 10 μM of ACh. Ghrelin-induced *Suncus* gastric contraction was nearly abolished by pretreatment with G5-1 or [D-Lys-3]-GHRP-6. All values are presented as means ± SEM ($n = 4$). **$P < 0.01$.

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G5-1 significantly suppressed food intake at 1 h postadministration relative to the vehicle control group (Fig. 6 *A* and *B*). Fig. 6*C* shows integrated data of cumulative food intake at 1 h postadministration of G5-1 (0.5–2.0 mg/kg; 0.14–0.57 μmol/kg), [D-Lys-3]-GHRP-6 (1.5 mg/kg; 1.6 μmol/kg), and *Suncus* motilin (4.0 mg/kg; 1.5 μmol/kg). G5-1 suppressed food intake in a dose-dependent manner, whereas motilin, a negative control, had no effect. These results indicate that peripheral administration of G5-1, as well as [D-Lys-3]-GHRP-6, can inhibit food intake in mice.

Discussion

We used cDNA display to screen a peptide library and identified G5-1, a novel peptide that exhibits antagonistic activity against the class A GPCR, GHS-R. The amino acid sequence of G5-1 differs from that of ghrelin and other known GHS-R peptide ligands. Nearly all known GHS-R ligands, including ghrelin, have one or two basic residues and many hydrophobic residues that interact with E124 and the hydrophobic pocket of GHS-R, respectively (55, 56). Similarly, G5-1 is composed of a N-terminal amino group and many hydrophobic residues in its N-terminal region. Therefore, G5-1 appears to bind to GHS-R via the same mechanism as other ligands. Ghrelin binds to GHS-R at its N-terminus, and its C-terminus is thought to regulate its stability in blood. In fact, five N-terminal amino acids of ghrelin are essential to its activity, and truncation of the C-terminus did not decrease its activity in vitro (47). Nevertheless, G5-1-N8, a mutant that contains only eight N-terminal G5-1 amino acids, exhibited no activity in a calcium mobilization assay in vitro. This finding suggests that the C-terminal sequence of ghrelin may play a role in G5-1 binding to GHS-R. Additionally, G5-2, a mutant containing a single amino acid substitute of G5-1, exhibited no activity. This result suggests that there are strict requirements for amino acid interactions between G5-1 and GHS-R.

A number of small-molecule antagonists of GHS-R have been discovered with IC_{50} values in the nM range (7–9). However, peptide GHS-R antagonists are restricted to only two peptides with weak IC_{50} values of approximately 10 μM, [D-Lys-3]-GHRP-6 and G5-1, the latter of which was characterized in this study. Although G5-1 is a weak antagonist, it is the first peptide GHS-R antagonist composed of only L-amino acids. Therefore, G5-1 could be the basis for a short-acting antiobesity drug with minimal side effects, and it could also be prepared as a fusion with other proteins (such as the Fc portion of IgG and albumin) to extend the half-life of the peptide (57, 58), as observed with the thrombopoietin-binding peptide selected by phage display (13, 59, 60). Additionally, as G5-1 is a relatively short peptide, it could be easily stabilized against peptidase by chemical modifications—such as PEGylation, glycosylation, acetylation, amidation, and D-amino acid substitution (61, 62)—for its use as a therapeutic drug. Alternatively, drug-delivery systems (DDS) could be efficiently used to prevent the undesired degradation of peptide-based drugs. In DDS of peptide-based drugs, liposomes, microparticles, and nanoparticles are used for controlled or sustained release of the drug (63), and mucoadhesive polymers are used for transmucosal drug delivery (i.e., nasal, ocular) (64).

The use of in vitro display techniques to screen for novel peptide GPCR ligands has been limited to several reports because of the difficulty of preparing GPCRs as binding targets. Because ligand-binding sites of class B GPCRs are in the extracellular domain, purified extracellular domains immobilized on beads can be used as a binding target. Ja et al. (20) screened the peptide ligand of Mth, a class B GPCR, via mRNA display with the extracellular domain of Mth. However, because ligand-binding sites of Class A GPCRs include transmembrane regions composed of multiple transmembrane α-helices, the use of complete receptors is required to recreate molecular structures accurately. Although detergent-solubilized receptors from the membrane fraction are used to select ligands, they generally display both intra-and extracellular surfaces without distinction. In fact, Milovnik et al. (26) selected the proteins that bound to the neurotensin receptor 1, a class A GPCR, via ribosome display with detergent-solubilized receptors; the selected proteins bound to both extra- and intracellular sides of the receptor. Although the use of whole cells to express the target receptor as a binding target facilitates the preparation of receptors in their native conformation and active states, this method has not previously been used in in vitro display techniques. In this paper, we have successfully used live cells in conjunction with cDNA display to screen and identify novel peptide ligands for class A GPCRs.

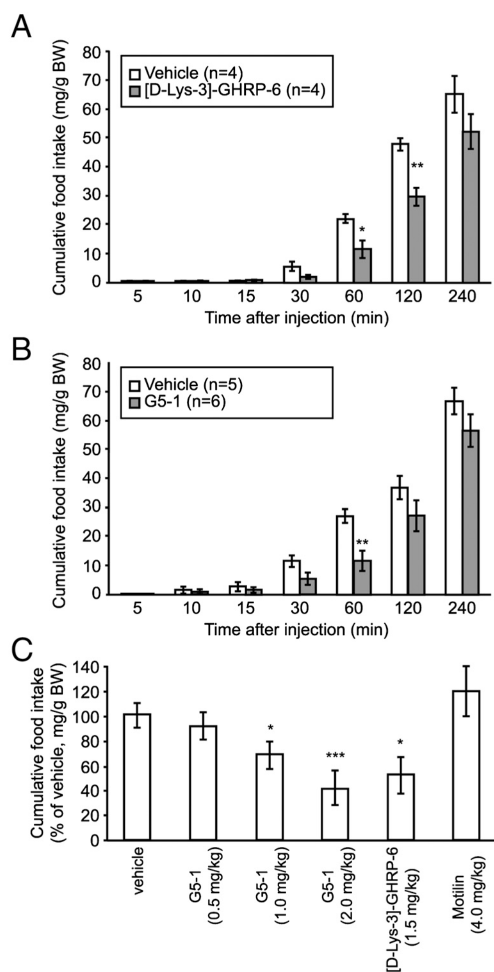

Fig. 6. Suppression of food intake by intravenous administration of G5-1. (*A*) Effect of intravenously administered [D-Lys-3]-GHRP-6 (1.5 mg/kg; 1.6 μmol/kg) on cumulative food intake in fasting mice. (*B*) Effect of intravenously administered G5-1 (2.0 mg/kg; 0.57 μmol/kg) on cumulative food intake in fasting mice. (*C*) Effect of intravenously administered G5-1 (0.5–2.0 mg/kg; 0.14–0.57 μmol/kg), [D-Lys-3]-GHRP-6 (1.5 mg/kg; 1.6 μmol/kg), and *Suncus* motilin (4.0 mg/kg; 1.5 μmol/kg) on cumulative food intake at 1 h postadministration in fasting mice. Motilin was used as a negative control. Statistical comparisons were made against the control group using Student's *t* test (*A*, *B*) and one-way ANOVA (Dennett's posttest) (*C*). All values are presented as means ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Materials and Methods

Preparation of mRNA/cDNA-Peptide Fusions. The procedures for preparation of DNA constructs and mRNA/cDNA-peptide fusions are illustrated in Figs. S3 and S4. Puromycin-linker and mRNA/cDNA-peptide fusions were prepared as previously described (43, 65). Detailed procedures are provided in *SI Materials and Methods*.

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Pilot Selection. The mixture of the mRNA/cDNA-peptide fusions containing the fusion of Trp^3-ghrelin and an excess of the desacyl-ghrelin fusion was incubated with 5.0×10^4 CHO-GHSR62 cells for 1 h in 50 μL of selection buffer (10 mM Hepes-NaOH, pH 7.4: 135 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 0.8 mM $MgCl_2$, 10 mM Glucose, 0.2% BSA, and 0.6 mM $NaHCO_3$). The cells were washed three times with ice-cold selection buffer on ice and incubated with 50 μL of 0.1 M glycine-HCl (pH 3.5) for 10 min at room temperature. The supernatant was collected and followed by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation. The precipitate was dissolved in water and amplified via PCR. The amplified product was analyzed via 8 M urea-denatured PAGE. Detailed procedures are provided in *SI Materials and Methods*.

Actual Selection. The mRNA/cDNA-peptide fusion library was dissolved in selection buffer and incubated with CHO cells on ice. The supernatant was collected and incubated on ice with CHO-GHSR62 cells (the fusion library of the first round of selection was directly incubated with CHO-GHSR62 cells without preincubation with CHO cells). The cells were washed with ice-cold selection buffer and incubated with 0.1 M glycine-HCl (pH 3.5) for 10 min at room temperature. The supernatant was collected, and 1 M Tris-HCl (pH 8.9) was added to the supernatant to *neutralize the pH*. The collected sample was concentrated with butanol, precipitated with ethanol, and purified using a QIA-quick PCR purification column (Qiagen). The cDNA portion of the purified sample was amplified using PCR to create the DNA library for the next round of selection. These rounds of selection were performed with a change of reaction volumes and several conditions to enhance gradually the selection pressure. The selection conditions of each round are presented in Table S1. The PCR products of the molecules selected in the fifth round of selection were cloned into pGEM-T Easy vectors (Promega) and analyzed by means of general sequencing. Detailed procedures are provided in *SI Materials and Methods*.

Intracellular Calcium Mobilization Assay. Ghrelin was synthesized as previously reported (66), and the G5-1, G5-2, and G5-1-N8 peptides were custom synthesized (SCRUM Inc.). [D-Lys-3]-GHRP-6 was purchased from Bachem, and human atrial natriuretic peptide (hANP) was synthesized using standard Fmoc chemistry. Ghrelin (final concentration, 5 nM) and selected concentrations of the selected or control peptides were loaded onto CHO-GHSR62 cells, which were pretreated with FLIPR Ca 3 (Molecular Devices), and the intracellular calcium concentration change was determined by measuring the change in fluorescence intensity. Detailed procedures are provided in *SI Materials and Methods*.

Binding Assay. Ligand binding to cell membranes from CHO-GHSR62 cells was investigated using [^{125}I]-ghrelin as a tracer ligand. The IC_{50}, K_d, and K_i values of the ligands were determined by fitting the competition curves. Detailed procedures are provided in *SI Materials and Methods*.

Organ Bath Assay. Organ bath assay was performed as previously described (53, 54) using isolated stomachs of fasting female adult *Suncus*. Contractile activities of the stomachs were monitored using an isometric force transducer (UM-203; Iwashiya Kishimoto Medical Industrials). The stomachs were stabilized in an organ bath and control contraction was measured using acetylcholine (ACh; 10 μM) treatment. Ghrelin (10 pM–100 nM) was added cumulatively to the organ bath from 30 s after adding 100 pM of motilin. Either [D-Lys-3]-GHRP-6 (1 μM) or G5-1 (1 μM) was treated in conjunction with motilin before cumulative administration of ghrelin. Contractions evoked by ghrelin were expressed as a percentage of the control contraction induced by ACh. Detailed procedures are provided in *SI Materials Methods*. All procedures used in this experiment were approved and performed in accordance with the institutional guidelines for animal care at Saitama University.

Feeding Tests. Male mice of the *ddy* strain (7 weeks old) were purchased and individually housed under controlled conditions (21 ± 2 °C, lights on from time 0800 to 2000) for a week. Mice (8 weeks old, 26–33 g) were fasted for 16 h (time 1800–1000) before being provided free access to water. G5-1 and [D-Lys-3]-GHRP-6 were administrated into the subclavian vein under anesthesia with 3% isoflurane. Immediately after administration, the incision site was *closed with super glue*. Mice were then reintroduced into the cage, in which food (which had been weighed) and water were served. Food intake was measured at 5, 10, 15, 30, 60, 120, and 240 min after administration. All procedures used in this experiment were performed in accordance with the institutional guidelines for animal care at Saitama University.

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