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ORIGINAL ARTICLE

Novel bioluminescent receptor-binding assays for peptide hormones: using ghrelin as a model

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Abstract Peptide hormones perform important biological functions by binding specific cell membrane receptors. For hormone–receptor interaction studies, receptor-binding assays are widely used. However, conventional receptor-binding assays rely on radioactive tracers that have drawbacks. In recent studies, we established novel non-radioactive receptor-binding assays for some recombinant protein hormones based on the ultrasensitive bioluminescence of a newly developed nanoluciferase (NanoLuc) reporter. In the present work, we extended the novel bioluminescent receptor-binding assay to peptide hormones that have small size and can be conveniently prepared by chemical synthesis. Human ghrelin, a 28-amino acid peptide hormone carrying a special O-fatty acid modification, was used as a model. To prepare a bioluminescent ghrelin tracer, a chemically synthesized ghrelin analog with a unique cysteine residue at the C-terminus was site-specifically conjugated with an engineered NanoLuc with a unique exposed cysteine residue at the C-terminus via a reversible disulfide linkage. The NanoLuc-conjugated ghrelin retained high binding affinity with the ghrelin receptor GHSR1a ($K_d = 1.14 \pm 0.13$ nM, $n = 3$) and was able to sensitively monitor the receptor-binding of various GHSR1a ligands. The novel bioluminescent receptor-binding assay will facilitate the interaction studies of ghrelin with its receptor. We also proposed general procedures for convenient conjugation of other peptide hormones with NanoLuc for novel bioluminescent receptor-binding assays.

Keywords Peptide hormone · Receptor-binding · Ghrelin · Bioluminescence · NanoLuc

Introduction

Peptide hormones are a large group of endogenous signaling molecules that play important biological functions through binding and activating specific cell membrane receptors. For hormone–receptor interaction studies, there are two kinds of assays, receptor-binding assays and functional assays. The receptor-binding assays directly measure binding of ligands with their receptors based on sensitive tracers, conventionally radioisotope-labeled radioligands. The functional assays measure the downstream signaling of activated receptors, such as concentration change of the intracellular secondary messengers. For examples, the intracellular Ca^{2+} concentration change caused by activation of some G protein-coupled receptors (GPCRs) can be sensitively monitored using some fluorescent probes; the intracellular cAMP concentration change can be conveniently monitored by a cAMP-response element-controlled reporter gene. The receptor-binding assays and functional assays are two kinds of complementary approaches for ligand–receptor interaction studies and both of them are widely used in laboratories. Through receptor-binding assays, receptor-binding potencies of various ligands and receptor densities on various cell lines and tissues can be accurately quantified; through functional assays, agonistic

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or antagonistic effects of various ligands and downstream signaling pathways of various receptors can be quantitatively measured.

Conventionally, receptor-binding assays rely on radioactive tracers labeled by radioisotopes, typically iodine-125 (^{125}I) for peptide hormones. Use of the radioactive tracers has drawbacks, such as their short shelf lives and radioactive hazards to operators and environments. In recent studies, we established novel non-radioactive receptor-binding assays for some recombinant protein hormones based on the ultrasensitive bioluminescence of a nanoluciferase (NanoLuc) reporter (He et al. 2014; Zhang et al. 2013). NanoLuc is a newly developed luciferase reporter with several advantages, such as the brightest bioluminescence reported to date, small size (171 amino acid, 19 kDa), and high stability (Hall et al. 2012). In the present study, we attempted to extend the novel bioluminescent receptor-binding assay to peptide hormones that have small size and can be conveniently prepared by chemical peptide synthesis.

In the present work, ghrelin was used as a model to develop novel bioluminescent receptor-binding assays for peptide hormones. The mature human ghrelin has 28 amino acids and carries a special n-octanoyl moiety at the side-chain of a serine residue at the third position. Ghrelin was identified in 1999 by screening the endogenous agonist of the growth hormone secretagogue receptor GSHR1a (Howard et al. 1996; Kojima et al. 1999). Ghrelin is known to be involved in multiple biological functions, such as stimulation of growth hormone secretion, increase of appetite and food intake, and regulation of glucose homeostasis and cardiovascular functions, mediated via the known G protein-coupled receptor GSHR1a or other as yet unknown receptors (Delporte 2013; Gahete et al. 2013; Heppner and Tong 2014; Labarthe et al. 2014; Pradhan et al. 2013; Rak-Mardyla 2013). To prepare a novel bioluminescent ghrelin tracer for non-radioactive receptor-binding assays, a chemically synthesized ghrelin analog (ghrelin-Cys) with a unique cysteine residue at the C-terminus was site-specifically conjugated with an engineered NanoLuc (Luc-Cys) carrying a unique exposed cysteine residue at the C-terminus via a reversible disulfide linkage. We also proposed general procedures for site-specific conjugation of other peptide hormones with the NanoLuc reporter for novel bioluminescent receptor-binding assays in future studies.

Materials and methods

Chemical synthesis and purification of human ghrelin and its analogs

Mature human ghrelin and its analogs were chemically synthesized by GL Biochem Ltd (Shanghai, China) using the standard Fmoc methodology. Crude peptides were purified to homogeneity by high performance liquid chromatography (HPLC) using a C18 reverse-phase column (Zorbax 300SB-C18, 9.4 × 250 mm, from Agilent Technologies, Santa Clara, CA, USA). The sample was eluted from the C18 reverse-phase column by acidic acetonitrile gradient containing 0.1 % trifluoroacetic acid (TFA), manually collected and lyophilized. Their molecular masses were measured by electrospray mass spectrometry. The peptidyl GHSR1a agonist GHRP-2 (purity over 98 %) was purchased from GL Biochem Ltd (Shanghai, China). The nonpeptidyl GHSR1a agonist MK-0677 (ibutamoren mesylate) was from MedChem Express (Monmouth Junction, NJ, USA).

Overexpression and purification of Luc-Cys

The expression construct of the engineered Luc-Cys was generated by insertion of a chemically synthesized DNA linker into the previously generated pNLuc vector (He et al. 2014) that was pretreated with restriction enzymes EcoRI and NotI, resulting in the expression construct pET/Luc-Cys. The coding region of Luc-Cys was confirmed by DNA sequencing. This construct was transformed into *Escherichia coli* strain BL21 (DE3) and transformants were cultured in liquid LB medium at 37 °C to OD_{600} of ~1.0. Thereafter, the stock solution of isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the final concentration of 1.0 mM and the *E. coli* cells were continuously cultured at 25 °C for 6–8 h. After harvesting by centrifugation (5000×*g*, 10 min), the cell pellet was resuspended in lysis buffer (20 mM phosphate, 0.5 M NaCl, pH 7.5) and cells were lysed by sonication. After a further centrifugation (10,000×*g*, 20 min), supernatant of the cell lysate was applied to an immobilized metal ion affinity chromatography (Ni^{2+} column). After washing, bound Luc-Cys was eluted from the Ni^{2+} column by lysis buffer containing 250 mM imidazole. The eluted Luc-Cys fraction was dialyzed against 20 mM phosphate buffer (pH 7.5) overnight, treated by 10 mM dithiothreitol (DTT) at room temperature for 30 min, and then loaded onto a DEAE ion-exchange column (TSKgel DEAE-5PW, 7.5 × 75 mm, from Sigma-Aldrich, St. Louis, MO, USA). The bound Luc-Cys was eluted using a linear gradient of sodium chloride (in 20 mM phosphate buffer, pH 7.5), manually collected, and analyzed by SDS-PAGE and bioluminescent assay.

Activation of the purified Luc-Cys by 2,2′-dipyridyl disulfide

For activation of the unique exposed cysteine of Luc-Cys, a stock solution of 2,2′-dipyridyl disulfide (50 mM dissolved

in acetonitrile) was added into the above purified Luc-Cys fraction (~200 μM) to the final concentration of 10 mM. The activation reaction was carried out at 30 °C for 15 min. Thereafter, the reaction mixture was first diluted fivefold with 20 mM phosphate buffer (pH 7.5) containing 20 % (v/v) acetonitrile and loaded onto a DEAE ion-exchange column (TSKgel DEAE-5PW, 7.5 × 75 mm, from Sigma-Aldrich). The activated Luc-Cys fraction was eluted from the ion-exchange column by a linear gradient of sodium chloride in 20 mM phosphate buffer (pH 7.5) containing 20 % (v/v) acetonitrile, manually collected, and analyzed by SDS-PAGE and bioluminescent assay.

Site-specific conjugation of the activated Luc-Cys with ghrelin-Cys

The purified ghrelin-Cys (dissolved in 1.0 mM aqueous hydrochloride, ~300 μM) was mixed with the activated Luc-Cys (~25 μM, eluted from the DEAE column) at a 1:1 molar ratio. The conjugation reaction was carried out at 30 °C for 30 min. Thereafter, the reaction mixture was diluted fivefold with 20 mM phosphate buffer (pH 7.5) and loaded onto a DEAE ion-exchange column (TSKgel DEAE-5PW, 7.5 × 75 mm, from Sigma-Aldrich). The fractions eluted from the ion-exchange column by a linear gradient of sodium chloride in 20 mM phosphate buffer (pH 7.5) were manually collected, and analyzed by SDS-PAGE and bioluminescent assays. For mass spectrometry analysis, aliquot of the conjugate fraction eluted from the ion-exchange column was loaded onto a gel filtration column (TSKgel G2000SW$_{XL}$, 7.8 × 300 mm, from Sigma-Aldrich) and eluted by 0.1 % aqueous TFA containing 20 % (v/v) acetonitrile. The eluted peak was manually collected, lyophilized and subjected to MALDI-TOF mass spectrometry.

Saturation and competition receptor-binding assays

HEK293T cells were transiently transfected with a human GHSR1a expression construct pENTER/GHSR1a (Vigene Bioscience, Rockville, MD, USA) using the transfection reagent Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Next day, the transfected HEK293T cells were detached with 1 mM of ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline, washed with serum-free DMEM medium, and collected by centrifugation (1000×g, 2 min). The cell pellet was resuspended in assay solution (serum-free DMEM medium containing 1 % bovine serum albumin) and added into a 96-well filtration plate (50 μl/well). Thereafter, 50 μl of serially diluted sample solution (diluted in assay solution), containing either varied concentrations of ghrelin-Luc (for saturation assays) or a constant concentration of ghrelin-Luc and varied concentrations of chemically synthesized human ghrelin or its analog (for competition assays), were added. After incubation at 20–21 °C for 1 h, assay solution was removed by centrifugation (400×g, 2 min) and cells were washed twice with ice-cold phosphate-buffered saline (200 μl/well) by centrifugation (400×g, 2 min). Cell pellets were finally resuspended in phosphate-buffered saline (100 μl/well) and transferred to a white opaque 96-well plate (50 μl/well). Thereafter, 100 mM DTT solution (dissolved in Lysis solution from Promega, Madison, WI, USA) was added into the cell suspension (25 μl/well). After incubation at room temperature for 10 min, diluted substrate solution (25 μl/well, diluted in Lysis solution from Promega) was added and bioluminescence was immediately measured using a SpectroMax M5 plate reader (Molecular Devices, Sunnyvale, CA, USA). The measured binding data were expressed as mean ± SE ($n = 3$) and fitted to a one-site receptor-binding model using SigmaPlot 10.0 software. Nonspecific binding data were obtained by competition with 1.0 μM of human ghrelin.

Results and discussion

A strategy for site-specific conjugation of ghrelin with NanoLuc

To prepare a bioluminescent ghrelin tracer for non-radioactive receptor-binding assays, we proposed a strategy for site-specific conjugation of ghrelin with NanoLuc via a reversible disulfide linkage (Fig. 1). For conjugation with the NanoLuc reporter, we introduced a unique cysteine residue at the C-terminus of human ghrelin and the resultant ghrelin analog was designated as ghrelin-Cys (Fig. 1a). Previous studies have demonstrated that the C-terminal residues are not required for receptor-binding of ghrelin (Bednarek et al. 2000; Matsumoto et al. 2001), thus we deduced that the introduced cysteine had no detriments to the receptor-binding potency of ghrelin. For convenient conjugation with chemically synthesized peptide hormones, we engineered a NanoLuc analog (designated as Luc-Cys) carrying a long flexible hydrophilic arm and a cysteine residue at the C-terminus and a 6 × His-tag at the N-terminus (Fig. 1b and its complete nucleotide and amino acid sequence in supplementary Fig. s1). The introduced C-terminal cysteine residue provides an ideal handle for site-specific conjugation with peptide hormones because it is a unique exposed cysteine in the engineered Luc-Cys. As shown in Fig. 1c, site-specific conjugation of ghrelin-Cys and Luc-Cys was carried out in a two-step procedure. First, the unique exposed cysteine residue of Luc-Cys was activated by 2,2′-dipyridyl disulfide, resulting in an active disulfide bond that is ready to react with the sulfhydryl

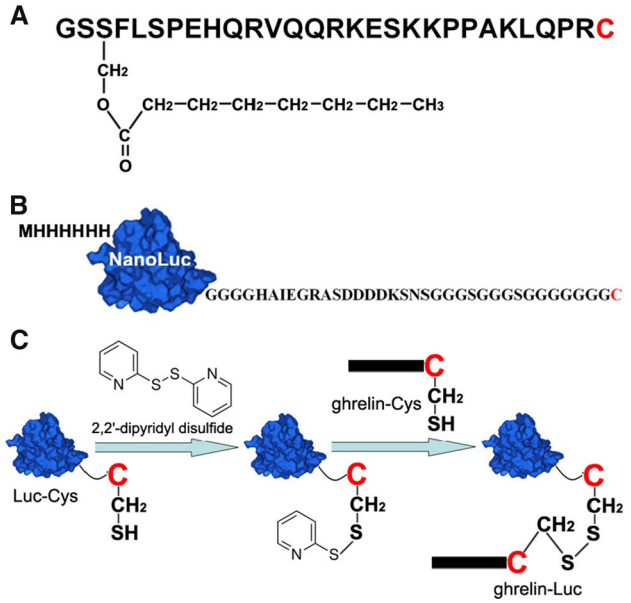

Fig. 1 A strategy for site-specific conjugation of ghrelin with NanoLuc. **a** Amino acid sequence and post-translational modification of ghrelin-Cys carrying a unique cysteine residue at C-terminus. **b** Schematic representation of the engineered Luc-Cys showing the flexible arm and the unique exposed cysteine at the C-terminus and the 6 × His-tag at the N-terminus. **c** Schematic representation of the site-specific conjugation of ghrelin-Cys with Luc-Cys in a two-step procedure

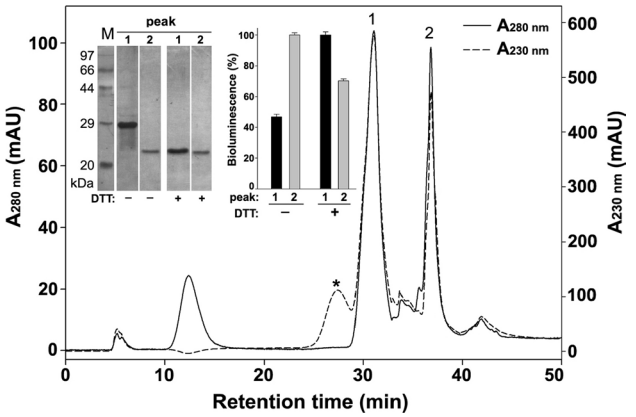

Fig. 2 Purification of the ghrelin-Luc conjugate by ion-exchange chromatography. The conjugation reaction mixture was loaded onto a DEAE ion-exchange column and two major peaks (labeled as *1* and *2*) were eluted by a linear gradient of sodium chloride as monitored by absorbance at both 280 and 230 nm. The minor peak indicated by an asterisk was the unreacted ghrelin-Cys as confirmed by mass spectrometry. *Left inner panel* SDS-PAGE analysis with or without DTT treatment. Aliquot of the collected peak 1 fraction or peak 2 fraction was treated with DTT or not and then loaded on a 15 % SDS–polyacrylamide gel that was silver stained after electrophoresis. *Right inner panel* bioluminescence measurement. Luciferase activity of the collected peak 1 fraction and peak 2 fraction was measured under non-reducing or reducing conditions

side-chain of an exposed cysteine. Second, mixing the chemically synthesized ghrelin-Cys with the activated Luc-Cys would lead to formation of a ghrelin-Luc conjugate through reaction of the cysteine residue of ghrelin-Cys with the active disulfide bond of the activated Luc-Cys. Using this strategy, chemically synthesized ghrelin could be conveniently conjugated with the engineered NanoLuc in a site-specific manner via a reversible disulfide linkage.

Preparation of NanoLuc-conjugated ghrelin

The engineered Luc-Cys was efficiently overexpressed in *E. coli* in soluble form and was purified to homogeneity by immobilized metal ion affinity chromatography and ion-exchange chromatography (data not shown). From one liter of *E. coli* culture, typically ~60 mg of purified Luc-Cys was obtained, providing sufficient Luc-Cys for conjugation with various peptide hormones. Bioluminescence assays showed that the engineered Luc-Cys was fully active when compared with 6 × His-NanoLuc prepared in our previous work (Zhang et al. 2013): the specific activity of both enzymes was ~1.5 × 10^5 counts/fmol when measured with a Spectromax M5 plate reader using white opaque 96-well plates. Thus, the introduced C-terminal arm and the unique exposed cysteine residue had no detrimental effects on the enzymatic activity of NanoLuc.

For conjugation with ghrelin-Cys, purified Luc-Cys was first treated with excess 2,2′-dipyridyl disulfide to activate its unique exposed cysteine by formation of an active disulfide bond. Activation reaction was carried out in a solution containing 20 % (v/v) of acetonitrile because 2,2′-dipyridyl disulfide has a low solubility in water. After removal of the excess modification reagent by ion-exchange chromatography, the activated Luc-Cys retained full enzymatic activity. Thereafter, the activated Luc-Cys was reacted with the chemically synthesized ghrelin-Cys that was purified to homogeneity by C18 reverse-phase HPLC (Fig. s2) and displayed correct molecular mass (measured value 3475.0; theoretical value 3474.0). The conjugation reaction mixture was subjected to ion-exchange chromatography and two major peaks (labeled as 1 and 2) were eluted as monitored by absorbance at both 280 and 230 nm (Fig. 2). The absorbance at 280 nm is originated from side-chain chromofores, including Trp and Tyr residues, while the absorbance at 230 nm is originated from peptide bonds. Thus, the two major peaks probably corresponded to the ghrelin-Luc conjugate and the unreacted Luc-Cys because they have absorbance at both 280 and 230 nm. As analyzed by SDS-PAGE under non-reducing condition (Fig. 2, left inner panel), both peaks contained a single protein band with different molecular weights. Peak 1 seemed to be the expected the ghrelin-Luc conjugate (theoretical molecular weight of 26.5 kDa), while peak 2

seemed to be the unreacted Luc-Cys (theoretical molecular weight of 23.0 kDa). After treatment with the reducing reagent DTT, peak 1 fraction ran as fast as peak 2 fraction on SDS-PAGE (Fig. 2, left inner panel), suggesting that DTT treatment removed the conjugated ghrelin-Cys moiety from Luc-Cys through breakage of their disulfide linkage. The peak 1 fraction was further subjected to MALDI-TOF mass spectrometry analysis (Fig. s3): the measured molecular mass was 26,429 ± 100, consistent with the theoretical value (26,527) of the ghrelin-Luc conjugate, confirming that a single ghrelin moiety was conjugated with Luc-Cys.

Bioluminescence measurement carried out under non-reducing conditions showed that activity of peak 1 was lower than that of peak 2 (Fig. 2, right inner panel), even though peak 1 was higher than peak 2. We deduced that the covalently attached ghrelin moiety probably slightly impaired the NanoLuc activity. To confirm this deduction, bioluminescence measurement was carried out after DTT treatment. As expected, peak 1 showed higher luciferase activity than peak 2 after breakage of the disulfide linkage between ghrelin-Cys and Luc-Cys (Fig. 2, right inner panel). To increase the detection sensitivity, the luciferase activity of the receptor-bound ghrelin-Luc was measured after DTT treatment in subsequent receptor-binding assays. The minor peak eluted around 28 min (indicated by an asterisk) had only absorbance at 230 nm, we deduced it was the unreacted ghrelin-Cys because it had no absorbance at 280 nm. Subsequent mass spectrometry analysis confirmed it really was the unreacted ghrelin-Cys (measured value 3476.0, theoretical value 3674.0). Another minor peak eluted around 12 min had only absorbance at 280 nm, thus it was not a peptide or protein fraction, it might be an impurity from the solution.

Using ghrelin-Luc as a novel bioluminescent tracer for receptor-binding assays

To test whether ghrelin-Luc retained high binding affinity with the ghrelin receptor GHSR1a, a saturation receptor-binding assay was carried out. As shown in Fig. 3a, ghrelin-Luc bound the living HEK293T cells transiently overexpressing human GHSR1a in a typical saturation manner, with a calculated dissociation constant (K_d) of 1.14 ± 0.13 nM ($n = 3$), which was only slightly higher than the measured K_d (~0.5 nM) of the radioactive ^{125}I-labeled ghrelin tracer (Katugampola et al. 2001; Muccioli et al. 2001). Thus, ghrelin-Luc retained high binding affinity with the ghrelin receptor GHSR1a despite the attached large NanoLuc moiety. Additionally, nonspecific binding of ghrelin-Luc was reasonably low due to the highly hydrophilic nature of the NanoLuc reporter. The measured maximal binding capacity (B_{max}) was 24,900 ± 1100 counts per 25,000 transfected HEK293T cells, equal to approximately 4000 receptors/cell in average, calculated from the Luc-Cys specific activity of 1.5×10^5 counts/fmol. A Scatchard plot of the specific binding data was linear (Fig. 3b), indicating one-site binding of ghrelin-Luc to receptor GHSR1a. Luc-Cys itself showed no detectable specific binding with HEK293T cells overexpressing GHSR1a (Fig. 3c), confirming that the measured specific binding of ghrelin-Luc with the transfected cells arose from the attached ghrelin moiety. Furthermore, ghrelin-Luc did not bind to un-transfected HEK293T cells (Fig. 3c), suggesting that HEK293T cells did not express detectable endogenous ghrelin receptor. In summary, the bioluminescent ghrelin-Luc retained high and specific binding affinity with the ghrelin receptor GHSR1a.

As ghrelin-Luc retained high binding affinity with the ghrelin receptor GHSR1a, we used it as a novel bioluminescent tracer in competition receptor-binding assays (Fig. 3d). Mature human ghrelin competed with ghrelin-Luc in a typical sigmoidal manner, with a calculated IC_{50} value of 4.7 ± 0.4 nM ($n = 3$), when 1.0 nM of ghrelin-Luc was used as tracer. In contrast, unacylated ghrelin did not compete with ghrelin-Luc, consistent with the fact that unacylated ghrelin cannot bind receptor GHSR1a (Bednarek et al. 2000; Matsumoto et al. 2001). The peptidyl agonist GHRP-2 (amino acid sequence D-Ala-D-2-Nal-Ala-Trp-D-Phe-Lys-NH_2) bound the overexpressed GHSR1a with less potency ($IC_{50} = 21.4 \pm 2.8$ nM, $n = 3$) compared with human ghrelin. In contrast, the nonpeptidyl agonist MK-0677 (ibutamoren mesylate) bound receptor GHSR1a as effective as ghrelin ($IC_{50} = 5.6 \pm 0.6$ nM, $n = 3$). Thus, ghrelin-Luc could sensitively monitor binding potencies of various ligands with receptor GHSR1a and represented a novel sensitive bioluminescent tracer for non-radioactive receptor-binding assays to study the interaction of ghrelin with its receptor in future work.

Application of the bioluminescent receptor-binding assay to others peptide hormones

In the present study, we prepared a novel bioluminescent ghrelin tracer for non-radioactive receptor-binding assays through site-specific conjugation of an engineered Luc-Cys with a chemically synthesized ghrelin analog. Other peptide hormones could also be conjugated with Luc-Cys to prepare bioluminescent tracers for non-radioactive receptor-binding assays. For peptide hormones without a natural cysteine, a single cysteine residue could be introduced into an appropriate position by chemical synthesis for site-specific conjugation with Luc-Cys according to the procedure shown in Fig. 1c. For peptide hormones with natural cysteines that typically form disulfide bond(s), an L-propargylglycine residue could be introduced into an appropriate position by chemical synthesis. After formation of the correct disulfide

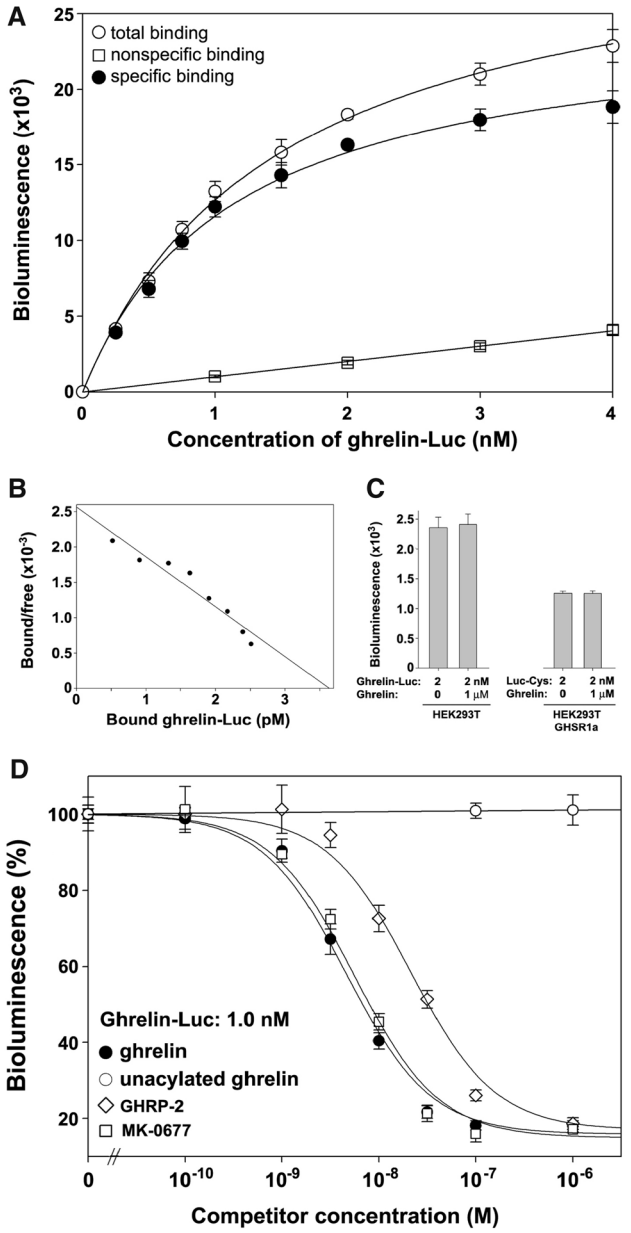

Fig. 3 Novel bioluminescent ghrelin receptor-binding assays. **a** Saturation binding of ghrelin-Luc with the ghrelin receptor GHSR1a. Nonspecific binding data were obtained by competition with 1.0 μM of human ghrelin. The measured bioluminescence data were expressed as mean ± SE ($n = 3$). Total binding data were fitted to $Y = B_{max}X/(K_d + X) + N_sX$, specific binding data to $Y = B_{max}X/(K_d + X)$, and nonspecific binding data to a linear curve, using the SigmaPlot10.0 software. **b** Scatchard plot of specific binding data. **c** Binding of ghrelin-Luc with non-transfected HEK293T cells and binding of Luc-Cys with HEK293T cells overexpressing GHSR1a. The measured bioluminescence data were expressed as mean ± SE ($n = 3$). **d** Competition receptor-binding assays using ghrelin-Luc as a novel bioluminescent tracer. The measured relative bioluminescence data were expressed as mean ± SE ($n = 3$). The competition data of unacylated ghrelin were fitted to a linear curve and those of other ligands were fitted to sigmoidal curves using the SigmaPlot10.0 software

bond(s) via in vitro oxidative refolding, an active disulfide bond could be covalently attached to the folded peptide hormone via reaction with a bifunctional reagent carrying an active disulfide bond and an azide moiety through click chemistry. Thereafter, purified Luc-Cys could be site-specifically conjugated with the modified peptide hormone via a disulfide linkage through reaction of its unique exposed C-terminal cysteine with the introduced active disulfide bond of the peptide hormone. Thus, novel bioluminescent tracers for other peptide hormones could also be conveniently prepared according to the proposed procedures for non-radioactive receptor-binding assays in future studies.

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Conflict of interest The authors declared that they have no conflict of interest.

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