Biochimica et Biophysica Acta 1848 (2015) 688–694

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Quantitative measurement of cell membrane receptor internalization by the nanoluciferase reporter: Using the G protein-coupled receptor RXFP3 as a model

Yu Liu [a,b], Ge Song [a], Xiao-Xia Shao [a], Ya-Li Liu [b], Zhan-Yun Guo [a,b,*]

[a] Institute of Protein Research, College of Life Sciences and Technology, Tongji University, Shanghai 200092, China
[b] Central Laboratory, Shanghai East Hospital, Tongji University School of Medicine, Shanghai 200120, China

ARTICLE INFO

Article history:
Received 24 July 2014
Received in revised form 14 November 2014
Accepted 17 November 2014
Available online 28 November 2014

Keywords:
Receptor
Internalization
Bioluminescence
Relaxin-3
RXFP3
GPCR

ABSTRACT

Nanoluciferase (NanoLuc) is a newly developed small luciferase reporter with the brightest bioluminescence to date. In the present work, we developed NanoLuc as a sensitive bioluminescent reporter to measure quantitatively the internalization of cell membrane receptors, based on the pH dependence of the reporter activity. The G protein-coupled receptor RXFP3, the cognate receptor of relaxin-3/INSL7, was used as a model receptor. We first generated stable HEK293T cells that inducibly coexpressed a C-terminally NanoLuc-tagged human RXFP3 and a C-terminally enhanced green fluorescent protein (EGFP)-tagged human RXFP3. The C-terminal EGFP-tag and NanoLuc-tag had no detrimental effects on the ligand-binding potency and intracellular trafficking of RXFP3. Based on the fluorescence of the tagged EGFP reporter, the ligand-induced RXFP3 internalization was visualized directly under a fluorescence microscope. Based on the bioluminescence of the tagged NanoLuc reporter, the ligand-induced RXFP3 internalization was measured quantitatively by a convenient bioluminescent assay. Coexpression of an EGFP-tagged inactive [E141R]RXFP3 had no detrimental effect on the ligand-binding potency and ligand-induced internalization of the NanoLuc-tagged wild-type RXFP3, suggesting that the mutant RXFP3 and wild-type RXFP3 worked independently. The present bioluminescent internalization assay could be extended to other G protein-coupled receptors and other cell membrane receptors to study ligand–receptor and receptor–receptor interactions.

1. Introduction

Nanoluciferase (NanoLuc) is a newly developed small luciferase reporter (171 amino acids, 19 kDa) with the brightest bioluminescence to date [1]. It emits a long half-life glow-type bioluminescence using furimazine and molecular oxygen as substrates, in an adenosine triphosphate-independent manner. NanoLuc has several advantages over conventional luciferase reporters, such as high specific activity, small size, high stability and lack of posttranslational modifications, rendering it an ideal bioluminescent reporter in biological sciences [2]. In a recent study, we used NanoLuc as a sensitive reporter to monitor the internalization of the iron efflux transporter ferroportin based on the pH dependence of the NanoLuc activity [3]. In the present work, we tested whether NanoLuc can be used as a general quantitative reporter to monitor the internalization of other cell membrane receptors. A G protein-coupled receptor (GPCR), RXFP3, was used as a model receptor, because GPCRs are the largest group of cell membrane receptors and play critical biological functions.

The relaxin family peptide receptor RXFP3 is the cognate receptor of relaxin-3 (also known as INSL7), a neuropeptide belonging to the insulin/relaxin superfamily [4–7]. The relaxin-3/RXFP3 system is involved in the regulation of food intake, stress response, as well as arousal and exploratory behaviors [8–14]. In vitro, relaxin-3 can also bind and activate the relaxin-family peptide receptor RXFP1 [15] and RXFP4 [16] whose endogenous ligands are relaxin [17] and INSL5 [18], respectively.

In the present work, we generated stable HEK293T cell that inducibly coexpressed a NanoLuc-tagged RXFP3 and an EGFP-tagged RXFP3 and demonstrated that ligand-induced RXFP3 internalization could be measured quantitatively based on the bioluminescence of the tagged NanoLuc reporter. We also demonstrated that an inactive RXFP3 mutant had no detrimental effect on the ligand-binding and internalization of the wild-type RXFP3, suggesting that the mutant and the wild-type RXFP3s worked independently.

2. Materials and methods

2.1. DNA manipulation

The coding region of the C-terminally NanoLuc-tagged human RXFP3 (RXFP3-Luc) was PCR-amplified, using the previously generated

* Corresponding author at: Institute of Protein Research, College of Life Sciences and Technology, Tongji University, 1239 Siping Road, Shanghai 200092, China. Tel.: +86 21 65988634; fax: +86 21 65988403.
E-mail address: zhan-yun.guo@tongji.edu.cn (Z.-Y. Guo).

http://dx.doi.org/10.1016/j.bbamem.2014.11.026
0005-2736/

pcDNA6/RXFP3-Luc construct [3] as the template, and ligated into a pGM-T vector (Transgene, Beijing, China). After confirmation by DNA sequencing, the coding region of RXFP3-Luc was released from the pGM-T vector by digestion with restriction enzymes ApaI and BamHI, and then ligated into the multiple cloning site 1 of vector pTRE3G-BI (Clontech, Mountain View, CA, USA) pretreated with the same restriction enzymes, resulting in the construct pTRE3G-BI/RXFP3-Luc. The coding region of human RXFP3 (without the stop codon) was PCR-amplified using pcDNA6/RXFP3 [19] as the template, digested by restriction enzymes KpnI and AgeI, and then ligated into a previously generated pGM-T/EGFP vector, resulting in the construct pGM-T/RXFP3-EGFP. After confirmation by DNA sequencing, the coding region of the C-terminally EGFP-tagged RXFP3 (RXFP3-EGFP) was released from pGM-T/RXFP3-EGFP by digestion with restriction enzymes KpnI and NdeI, and then ligated into the multiple cloning site 2 of pTRE3G-BI/RXFP3-Luc, resulting in a coexpression construct of pTRE3G-BI/RXFP3-Luc:RXFP3-EGFP. Site-directed mutagenesis of RXFP3-EGFP was carried out using the QuikChange approach based on the construct pGM-T/RXFP3-EGFP. After confirmation by DNA sequencing, the coding region of [E141R]RXFP3-EGFP was released by digestion with restriction enzymes KpnI and NdeI, and then ligated into multiple cloning site 2 of the construct pTRE3G-BI/RXFP3-Luc, resulting in a coexpression construct of pTRE3G-BI/RXFP3-Luc:[E141R]RXFP3-EGFP. Site-directed mutagenesis of RXFP3-Luc was carried out using the QuikChange approach based on the construct pGM-T/RXFP3-Luc. After confirmation by DNA sequencing, the gene of [E141R]RXFP3-Luc was released from the pGM-T vector by restriction enzymes ApaI and BamHI, and then ligated into the multiple cloning site 1 of the construct pTRE3G-BI/RXFP3-Luc:RXFP3-EGFP in which the gene of RXFP3-Luc had been removed from the multiple cloning site 1 by ApaI and BamHI, resulting in the coexpression construct of pTRE3G-BI/[E141R]RXFP3-Luc:RXFP3-EGFP. To generate the construct of pTRE3G-BI/RXFP3-HA-Luc, a small nucleotide linker encoding the HA-tag was inserted into the construct pTRE3G-BI/RXFP3-Luc after it was treated by restriction enzyme AgeI, and the correct colony was identified by DNA sequencing. A small nucleotide linker encoding the Flag-tag was inserted into the construct of pGM-T/RXFP3-EGFP after it was treated by restriction enzyme AgeI. After confirmation by DNA sequencing, the gene of RXFP3-Flag-EGFP was released from the pGM-T vector by restriction enzymes KpnI and NdeI, and then ligated into the multiple cloning site 2 of the construct pTRE3G-BI/RXFP3-HA-Luc, resulting in the coexpression construct pTRE3G-BI/RXFP3-HA-Luc:RXFP3-Flag-EGFP.

2.2. Generation of stable HEK293T cells

For transient transfection, HEK293T cells in a 35-mm dish were cotransfected with the RXFP3 expression construct and an expression control vector pCMV-Tet3G (Clontech), using the Xfect transfection reagent (Clontech) according to the manufacturer's instructions. The transiently transfected cells were cultured for approximately 10 days with normal passages. To induce expression of RXFP3-EGFP and RXFP3-Luc, the cells were treated by doxycycline (50 ng/ml) overnight. Thereafter, the cells, in two 10-cm dishes, were trypsinized and subjected to fluorescence-activated cell sorting (FACS) according to the fluorescence of the tagged EGFP reporter. The positive cells were pooled together and continuously cultured for approximately 10 days with normal passages. Thereafter, the cells were treated with doxycycline (50 ng/ml) overnight and subjected to a second round of FACS selection according to the fluorescence of the tagged EGFP reporter. The positive cells were pooled together, continuously cultured and used as stable cells for subsequent experiments.

2.3. Recombinant preparation and chemical labeling of relaxin-3

A fully active, easily labeled relaxin-3 was prepared through recombinant expression of a single-chain precursor in *Escherichia coli* and subsequent enzymatic conversion, according to our previous procedure [19]. The mature easily labeled relaxin-3 was purified to homogeneity by C18 reverse-phase high-performance liquid chromatography (HPLC) and its molecular mass was measured by mass spectrometry. For chemical modification with 5-carboxytetramethylrhodamine succinimidyl ester (Invitrogen, Carlsbad, CA, USA), one volume of the easily-labeled relaxin-3 solution (1.5 mM in 100 mM phosphate buffer, pH 7.4) was mixed with 1/3 volume of the labeling reagent (20 mM in dimethyl sulfoxide). After incubation at room temperature for 1–2 h, the reaction mixture was acidified to pH 3.0 using trifluoroacetic acid and subjected to HPLC. The labeled relaxin-3 was eluted from a C18 reverse-phase column by an acidic acetonitrile gradient, manually collected, lyophilized and analyzed by mass spectrometry.

2.4. Fluorescent microscopic observation

The stable HEK293T cells inducibly coexpressing RXFP3-Luc and RXFP3-EGFP were seeded into 35-mm glass-bottomed dishes and grown to approximately 60% confluence. Thereafter, doxycycline was added to a final concentration of 20 ng/ml and the cells were continuously cultured overnight. Thereafter, the cells were changed into the assay solution (serum-free DMEM medium plus 1% bovine serum albumin) containing 50 nM of the fluorescent dye-labeled relaxin-3. After continuous culture for 1–2 h, the cells were washed with the assay solution and observed under a confocal fluorescence microscope.

2.5. Ligand-binding assays

The stable HEK293T cells inducibly coexpressing RXFP3-Luc and RXFP3-EGFP were seeded into a 96-well plate and grown to approximately 60% confluence. Thereafter, doxycycline was added into the medium to a final concentration of 50 ng/ml and the cells were continuously cultured overnight. For the saturation binding assays, the induction medium was removed and the binding solution (serum-free DMEM medium plus 1% bovine serum albumin, 200 μl/well) containing different concentrations of DTPA/Eu^{3+}-labeled R3/I5 tracer [20] was added. For the competition assays, the binding solution (serum-free DMEM medium plus 1% bovine serum albumin, 200 μl/well) containing 4.0 nM of DTPA/Eu^{3+}-labeled R3/I5 tracer and different concentrations of competitor was added. After binding at 21–22 °C for 2 h, the binding solution was removed and the cells were washed twice with cold washing solution (serum-free DMEM medium, 200 μl/well). Thereafter, inducer solution (from PerkinElmer, Waltham, MA, USA, 100 μl/well) was added to dissociate Eu^{3+} from the DTPA chelator. After shaking for 15–30 min, the solutions (80 μl/well) were transferred to a white opaque 384-well plate for time-resolved fluorescence measurement using a Spectramax M5 plate reader (Molecular Devices, Sunnyvale, CA, USA). Nonspecific binding was obtained by competition with 1.0 μM of relaxin-3. The saturation binding data were fitted to hyperbolic curves, $Y = B_{max}X / (K_d + X)$, and the competition binding data were fitted to sigmoidal curves, $Y = 100 / (1 + 10^{X-\log IC50})$, using SigmaPlot 10.0 software.

2.6. Quantitative receptor internalization assays

The stable HEK293T cells inducibly coexpressing RXFP3-Luc and RXFP3-EGFP were seeded into a 96-well plate and grown to approximately 60% confluence. Thereafter, doxycycline was added to a final concentration of 3.5 ng/ml and the cells were continuously cultured overnight. Thereafter, the induction medium was removed and the assay solution (serum-free DMEM medium plus 1% bovine serum albumin) containing different concentrations of peptide was added (100 μl/well). After continuous culture at 37 °C for 4–5 h if not otherwise mentioned, the assay solution was removed and the cells were detached

by 1.0 mM ethylenediaminetetraacetic acid (EDTA) solution (in PBS, 100 μl/well). The living cell suspension (50 μl/well) was then transferred to a white, opaque 96-well plate. After addition of diluted substrate solution (50 μl/well, diluted in phosphate-buffered saline), the bioluminescence was immediately measured using a SpectroMax M5 plate reader (Molecular Devices). The measured bioluminescence data were expressed as mean ± SE and fitted to sigmoidal curves, $Y = \min + (100 - \min) / (1 + 10^{X - \log EC50})$, using SigmaPlot 10.0 software.

3. Results and discussion

3.1. Generation of stable HEK293T cells inducibly coexpressing RXFP3-Luc and RXFP3-EGFP

To test whether the newly developed NanoLuc reporter could be used for quantitative measurement of the internalization of RXFP3, we generated a RXFP3-Luc fusion protein in which the NanoLuc reporter was fused at the C-terminus of human RXFP3. To visualize the internalization of RXFP3 in the transfected cells, we coexpressed a C-terminally EGFP-tagged RXFP3 (RXFP3-EGFP) with RXFP3-Luc using a Tet-On inducible bi-directional promoter that insures equivalent coexpression of RXFP3-Luc and RXFP3-EGFP in a controllable manner. The tagged EGFP reporter was also used as a selection marker to generate stable HEK293T cells coexpressing both RXFP3-Luc and RXFP3-EGFP using FACS selection. After the coexpression construct and an expression control vector were transiently cotransfected into HEK293T cells, the resultant stable cells were generated by two rounds of FACS selection according to the fluorescence of tagged EGFP reporter and cultured as a stable cell pool. After these stable cells were induced by doxycycline, most of them had green fluorescence (from the tagged EGFP reporter) (Fig. 1); while no green fluorescence was observed without induction (data not shown), indicating that these cells expressed RXFP3-EGFP in a controllable manner. The observed green fluorescence was predominantly located on the cell membrane (Fig. 1), suggesting that the C-terminal tag had no detrimental effect on the structural integrity and intracellular trafficking of RXFP3. To test whether the tagged RXFP3 could bind relaxin-3 and undergo internalization after ligand binding, we treated these cells with a red fluorescent dye-labeled relaxin-3: most of the green fluorescence on the cell membrane disappeared after treatment with the labeled ligand, meanwhile many intracellular green dots appeared (Fig. 1), suggesting that the cell membrane RXFP3-EGFP was internalized into intracellular compartments. Red intracellular dots were also observed in these cells, suggesting that the labeled relaxin-3 was internalized together with the receptor. After the green fluorescent image and the red fluorescent image were merged, many yellow dots were observed (Fig. 1), suggesting that these internalized dots contained the green RXFP3-EGFP and the red labeled relaxin-3. Some red dots were also observed in the merged image (Fig. 1), suggesting that these internalized dots contained the colorless RXFP3-Luc and the red labeled relaxin-3. Our later bioluminescent assay also confirmed the inducible expression of RXFP3-Luc in these stable cells (Fig. 3A). Thus, we successfully generated stable HEK293T cells inducibly coexpressing both RXFP3-Luc and RXFP3-EGFP, based on transient transfection and subsequent FACS selection. This approach could also be used to generate other stable cells quickly.

3.2. Ligand-binding potency of the C-terminally tagged RXFP3

Our above results demonstrated qualitatively that the C-terminally tagged RXFP3 retained the ligand binding ability. Next, its ligand binding potency was measured quantitatively via receptor-binding assays using a DTPA/Eu^{3+}-labeled R3/I5 tracer [20]. As shown in Fig. 2A,

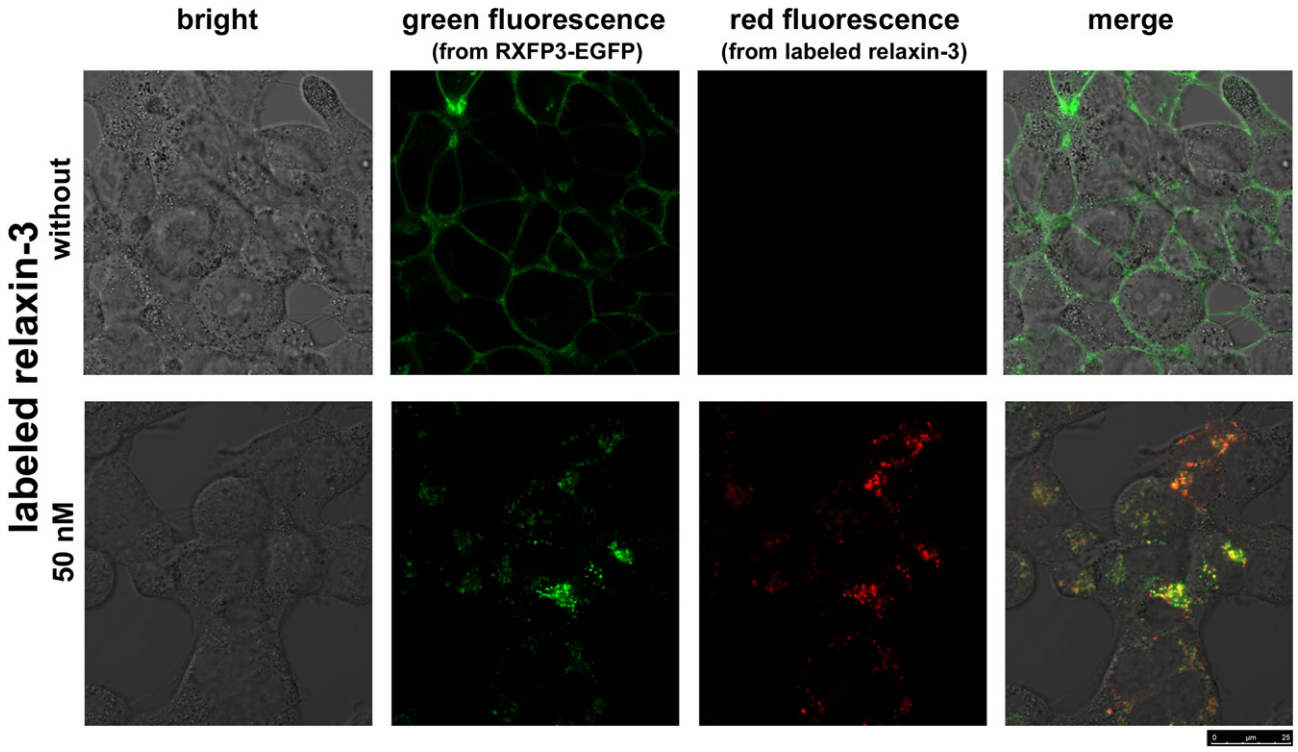

Fig. 1. Fluorescent microscopic observation of the coexpression of RXFP3-EGFP and RXFP3-Luc in the stable HEK293T cells. The stable HEK293T cells were induced by doxycycline overnight and then either treated with red fluorescent dye-labeled relaxin-3 or not, and observed under a confocal microscope.

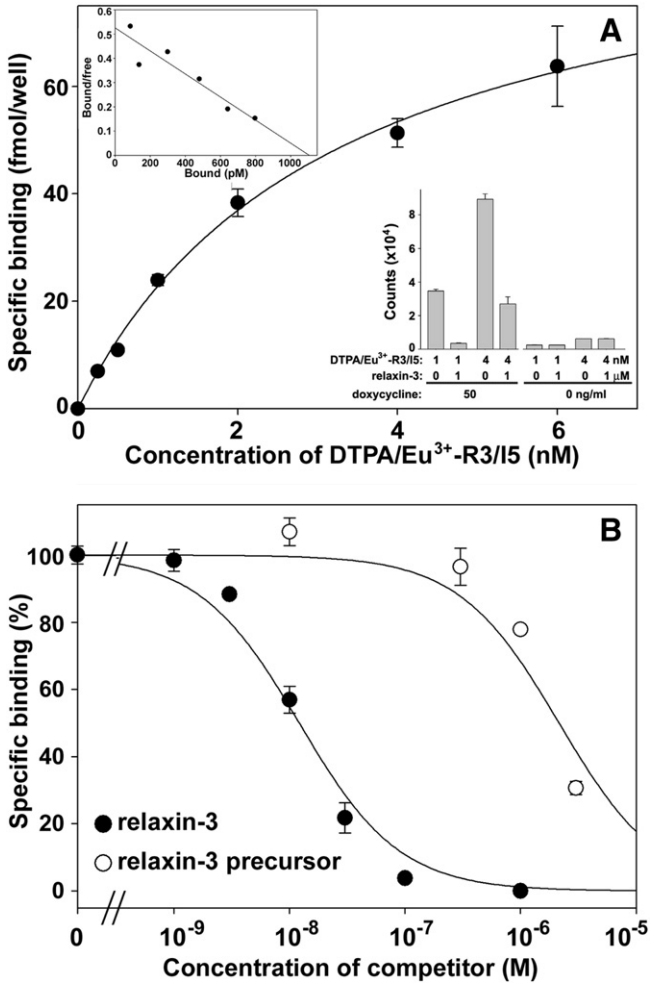

Fig. 2. (A) Saturation binding of the DTPA/Eu^{3+}-labeled R3/I5 to the stable HEK293T cells after doxycycline induction. Nonspecific binding was obtained by competition with 1.0 μM of relaxin-3. The specific binding data were expressed as means ± SE ($n = 3$) and fitted to a one-site saturation binding curve $Y = B_{max}X / (K_d + X)$ using SigmaPlot 10.0 software. Upper left inner panel, the Scatchard plot of the specific binding. Lower right inner panel, the total binding and nonspecific binding of the tracer with the stable cells with or without doxycycline induction. (B) Competition receptor-binding of mature and single-chain relaxin-3s to the stable HEK293T cells after doxycycline induction. The DTPA/Eu^{3+}-labeled R3/I5 concentration used in this assay was 4.0 nM. The normalized binding data were expressed as means ± SE ($n = 3$) and fitted to a sigmoidal curve $Y = 100 / (1 + 10^{X - \log IC50})$ using SigmaPlot 10.0 software.

a typical saturation binding curve was obtained with a calculated dissociation constant (K_d) of 3.2 ± 0.8 nM ($n = 3$) after the stable HEK293T cells were induced by doxycycline. The present K_d value was similar to our previous value measured using the same tracer and the untagged RXFP3 [20], suggesting that the C-terminal tag had no detrimental effect on the ligand-binding potency of RXFP3. For the stable HEK293T cells, the calculated maximum ligand-binding capacity (B_{max}) reached 96 ± 11 fmole/well ($n = 3$), suggesting that the C-terminally tagged RXFP3 could be efficiently trafficked to the cell membrane. For the cells without doxycycline induction, no specific binding was detected (Fig. 2A, the lower left inner panel), suggesting that the expression of the tagged RXFP3 was under a controllable manner. As the stable HEK293T cells efficiently overexpressed active RXFP3, we also used them as a receptor source for competition receptor-binding assays: typical sigmoidal curves were obtained for both two-chain mature relaxin-3 and its single-chain precursor, with different pIC_{50} values: 7.92 ± 0.04 for mature relaxin-3 and 5.67 ± 0.09 for the precursor (Fig. 2B). Thus, the stable HEK293T cells also provided a convenient receptor source to study the interaction of RXFP3 with various ligands.

3.3. Quantitative RXFP3 internalization assay using the tagged NanoLuc reporter

To confirm the inducible coexpression of RXFP3-Luc, we measured the NanoLuc activity after the stable HEK293T cells were induced by different concentrations of doxycycline overnight (Fig. 3A). The measured bioluminescence increased as the concentration of the inducer increased, suggesting that RXFP3-Luc was expressed in these stable cells in a controllable manner. To confirm that the differently tagged RXFP3s had similar expression levels, we coexpressed RXFP3-HA-Luc and RXFP3-Flag-EGFP in which an HA-tag and a Flag-tag were inserted between RXFP3 and the reporter, respectively. As analyzed by Western blot (Fig. 3A, the inner panel), the bands of RXFP3-Flag-EGFP and RXFP3-HA-Luc were detected only after doxycycline induction using the antibodies against the Flag-tag and the HA-tag, respectively. However, two bands were detected for both RXFP3-Flag-EGFP and RXFP3-HA-Luc, which might be caused by partial degradation of the fusion proteins since the apparent molecular weights of the lower bands were slightly smaller than the expected values (~79 kDa for RXFP3-Flag-EGFP and ~72 kDa for RXFP3-HA-Luc). We used the C-terminally HA-Flag-10 × His-tagged cell membrane iron efflux transporter ferroportin (~68 kDa) transiently overexpressed in HEK293T cells as a loading control to assess the expression levels of the differently tagged RXFP3s. The band densities of RXFP3-Flag-EGFP and RXFP3-HA-Luc were comparable with that of the control protein (equal loading amount in both Western blots), confirming that the differently tagged RXFP3s had similar expression levels. Subsequently, we tested whether RXFP3 internalization could be measured quantitatively according to the tagged NanoLuc reporter. After removal of the inducer doxycycline, the measured bioluminescence of the intact living cells decreased gradually (Fig. 3B), suggesting slow spontaneous internalization of RXFP3-Luc in the absence of ligand binding. After the addition of relaxin-3, the measured bioluminescence decrease was accelerated significantly (Fig. 3B), suggesting that relaxin-3 binding induced fast internalization of the cell membrane RXFP3-Luc into intracellular compartments. NanoLuc had a much lower activity under an acidic environment; thus, the measured bioluminescence using intact living cells would be significantly decreased after the tagged NanoLuc reporter was sorted into acidic lysosomes [3]. Our previous work demonstrated that the NanoLuc substrate furimazine can efficiently permeate the cell membrane and enter intracellular compartments; thus, the bioluminescence could be measured using intact living cells [3]. Thereafter, we measured the dose response of RXFP3-Luc internalization versus relaxin-3 concentration (Fig. 3C). The fully active two-chain relaxin-3 induced RXFP3 internalization in a typical sigmoidal manner, with a calculated pEC50 value of 8.32 ± 0.04 ($n = 3$) (Fig. 3C). In contrast, the low activity single-chain precursor had almost no effect on RXFP3 internalization. These results demonstrated that the ligand-induced internalization of RXFP3 could be measured quantitatively using the tagged NanoLuc reporter.

3.4. Effect of an inactive RXFP3 mutant on ligand-binding and internalization of wild-type RXFP3

There are reports that some GPCRs form dimers to exert their biological functions [21–24]. There is no report of RXFP3 forming dimers. In the present work, we generated stable HEK293T cells coexpressing a NanoLuc-tagged wild-type RXFP3 (RXFP3-Luc) and an EGFP-tagged mutant RXFP4, [E141R]RXFP3-EGFP, in which the highly conserved extracellular Glu141 was replaced by an oppositely charged Arg residue. The mutant [E141R]RXFP3 cannot be activated by wild-type relaxin-3,

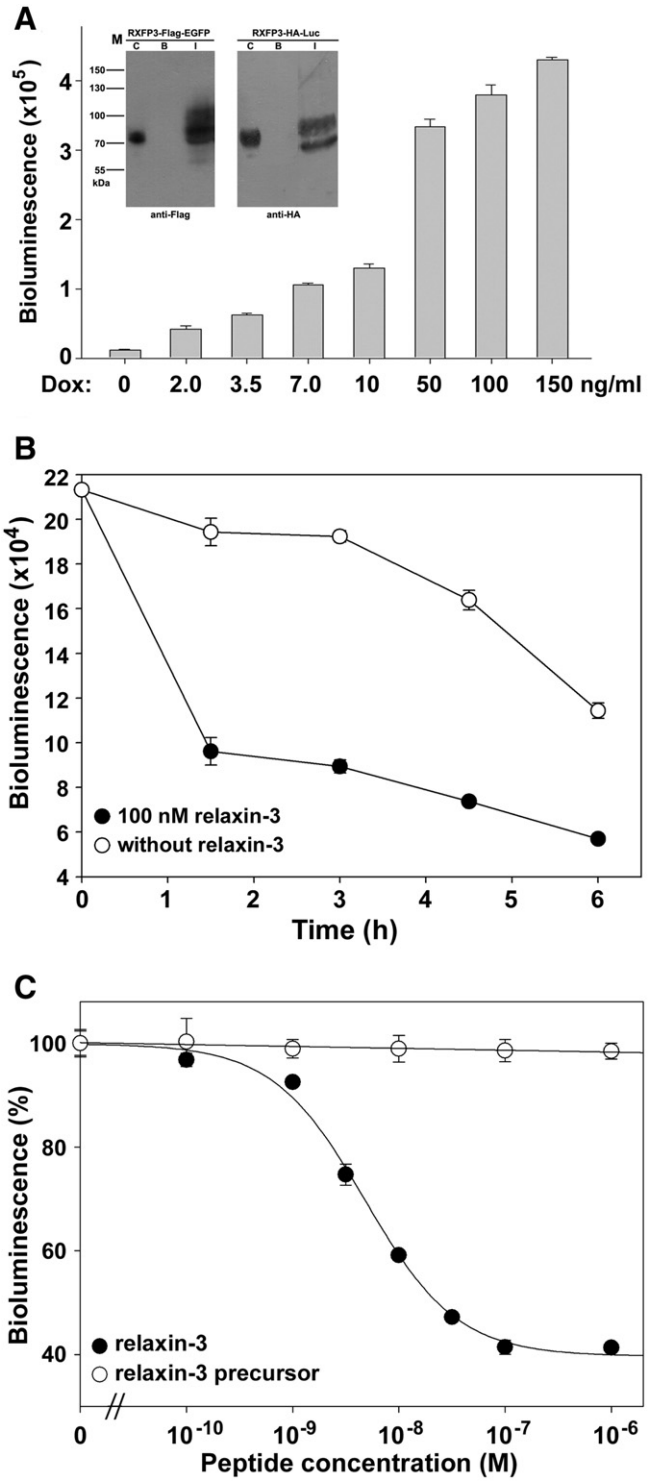

Fig. 3. (A) Doxycycline-dependent expression of RXFP3-Luc in the stable HEK293T cells. The measured bioluminescence data using intact living cells were expressed as means ± SE ($n = 3$). Inner panel, Western blot analysis of RXFP3-HA-Luc and RXFP3-Flag-EGFP in transiently transfected HEK293T cells. C, control protein: human ferroportin carrying a tandem HA-Flag-10 × His-tag at the C-terminus (~68 kDa); B, transfected HEK293T cells without doxycycline induction; and I, transfected HEK293T cells induced by 50 ng/ml doxycycline overnight. (B) Time course of RXFP3-Luc internalization in the presence or absence of relaxin-3. The measured bioluminescence data were expressed as means ± SE ($n = 3$). (C) Dose response of RXFP3-Luc internalization induced by relaxin-3 or single-chain relaxin-3 precursor. The relative bioluminescence data were expressed as means ± SE ($n = 3$) and fitted by a sigmoidal curve (relaxin-3) $Y = \min + (100 - \min) / (1 + 10^{X-\log EC50})$ or a linear curve (relaxin-3 precursor) using SigmaPlot 10.0 software.

as demonstrated in our previous work [25]. As observed by fluorescence microscopy (Fig. 4A), [E141R]RXFP3-EGFP was normally trafficked to the cell membrane after inducible expression in HEK293T cells, suggesting that replacement of Glu141 by Arg had no serious effect on the structural integrity and intracellular trafficking of RXFP3. After the stable HEK293T cells were treated with the red fluorescent dye-labeled relaxin-3 (Fig. 4A), [E141R]RXFP3-EGFP still stayed on the cell membrane and the internalized dots were predominantly red, suggesting that the labeled relaxin-3 could induce internalization of the colorless RXFP3-Luc, but could not induce internalization of the green [E141R]RXFP3-EGFP. Thus, we deduced that the inactive [E141R]RXFP3 had no detrimental effects on the ligand-induced internalization of the wild-type RXFP3. To confirm this deduction, we generated transiently transfected HEK293T cells that coexpressed the inactive [E141R] RXFP3-Luc and the wild-type RXFP3-EGFP (Fig. 4B). After these cells were treated by the red fluorescent dye-labeled relaxin-3, the internalized green dots (from RXFP3-EGFP) and the internalized red dots (from the labeled relaxin-3) were well co-localized, suggesting that only the wild-type RXFP3-EGFP was internalized with the labeled relaxin-3. Thus, the presence of the inactive mutant RXFP3 seemed to have no effect on the ligand-binding and internalization of the wild-type RXFP3.

To test quantitatively whether the inactive mutant RXFP3 affected the ligand-binding potency of the wild-type RXFP3, we carried out a saturation receptor-binding assay using DTPA/Eu^{3+}-labeled R3/I5 as a tracer. As shown in Fig. 5A, a typical saturation binding curve was obtained, with a calculated K_d of 2.8 ± 0.7 nM ($n = 3$), which was similar to the measured K_d value (3.2 ± 0.8 nM, $n = 3$) without the presence of the mutant RXFP3. Thus, the presence of the inactive mutant RXFP3 had no effect on the ligand-binding potency of the wild-type RXFP3. To test quantitatively whether the inactive RXFP3 mutant affected internalization of the wild-type RXFP3, we measured the ligand-induced RXFP3 internalization using the tagged NanoLuc reporter (Fig. 5B). The internalization curves of RXFP3-Luc induced by relaxin-3 in the presence of the inactive [E141R]RXFP3-EGFP or the wild-type RXFP3-EGFP were quite similar, with calculated pEC50 values of 8.34 ± 0.05 (presence of [E141R]RXFP3-EGFP) and 8.32 ± 0.04 (presence of RXFP3-EGFP), respectively. In contrast, no bioluminescence decrease could be measured for the cells coexpressing [E141R]RXFP3-Luc and RXFP3-EGFP (Fig. 5B, inner panel), confirming that the inactive RXFP3 could not undergo ligand-induced internalization. Thus, the presence of the inactive mutant RXFP3 had no effect on the ligand-induced internalization of the wild-type RXFP3, suggesting that the wild-type RXFP3 and the mutant RXFP3 worked independently.

3.5. Application of the NanoLuc-based internalization assay to other cell membrane receptors

In the present work, we demonstrated that the ligand-induced internalization of RXFP3 could be measured quantitatively using the tagged NanoLuc reporter. Once the internalized receptor was sorted into acidic lysosomes, the measured bioluminescence of the tagged NanoLuc reporter was significantly decreased because of its pH sensitivity when assayed using the intact living cells. GPCRs are the largest group of cell membrane receptors and play important biological functions. So far, there are over one hundred orphan GPCRs whose endogenous ligands remain unknown. Deorphanization of these orphan GPCRs is a critical step to elucidate their biological functions. Ligand-binding induced internalization is a general phenomenon to GPCRs and is independent of the knowledge of intracellular signaling pathways that are unknown for orphan GPCRs. Thus, the present NanoLuc-based quantitative internalization assay could be applied to deorphanization of some orphan GPCRs. The present assay could be easily adapted to high throughput screening and is thus suitable for screening large compound libraries. For GPCRs with known ligands, the present quantitative

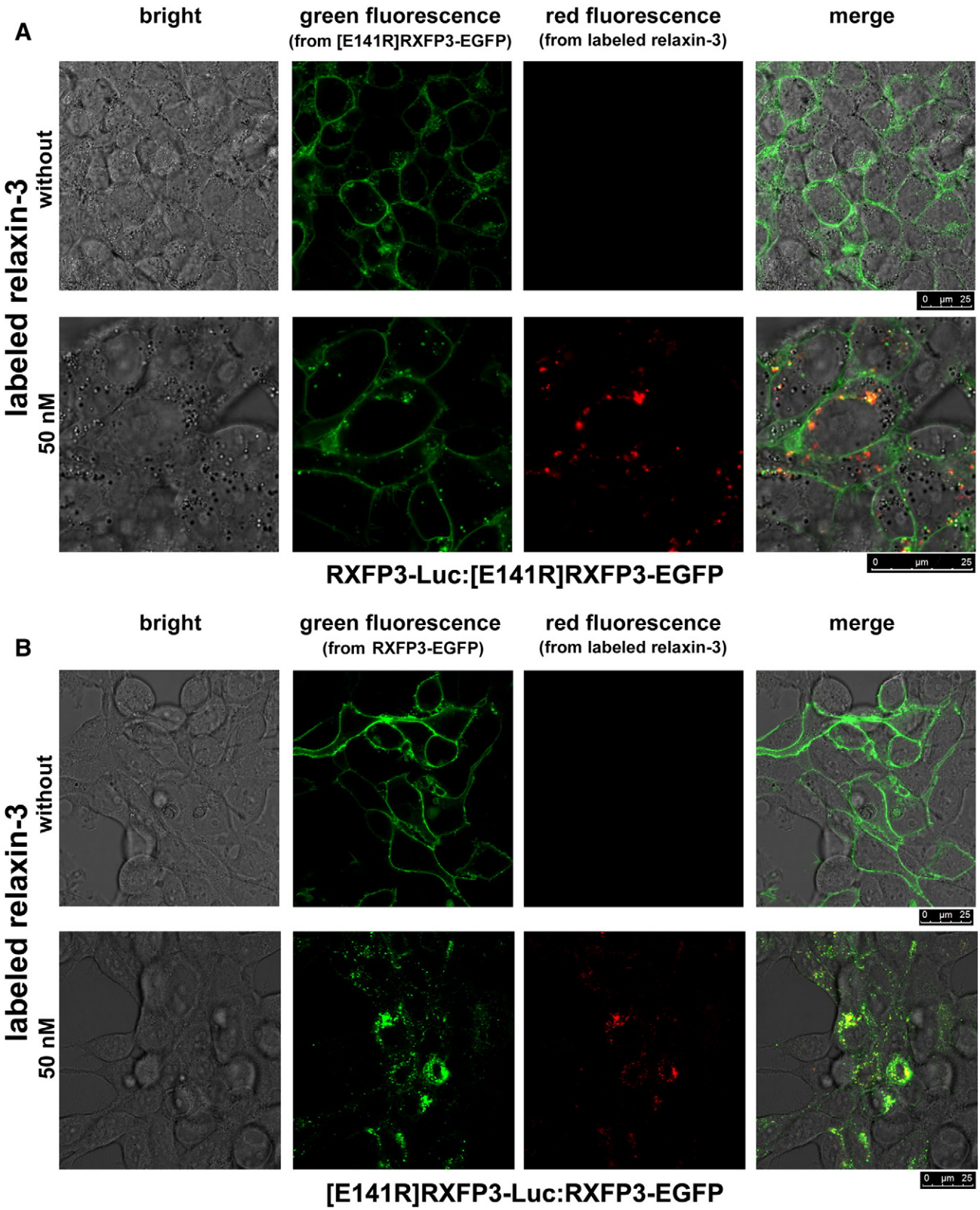

Fig. 4. (A) Fluorescent microscopic observation of the inducible coexpression of [E141R]RXFP3-EGFP and RXFP3-Luc in the stable HEK293T cells. The stable HEK293T cells were induced by doxycycline overnight and then either treated with red fluorescent dye-labeled relaxin-3 or not and observed under a confocal microscope. (B) Fluorescent microscopic observation of the inducible coexpression of RXFP3-EGFP and [E141R]RXFP3-Luc in the transiently transfected HEK293T cells. The transiently transfected HEK293T cells were induced by doxycycline overnight and then either treated with red fluorescent dye-labeled relaxin-3 or not and observed under a confocal microscope.

internalization assay could be used to study the ligand–receptor interactions. Internalization of other cell membrane receptors could also be assayed using the NanoLuc-based approach once the internalized receptors are sorted into acidic intracellular compartments.

Acknowledgments

We thank Promega Incorporation for providing the plasmids encoding NanoLuc. This work was supported by grants from the National

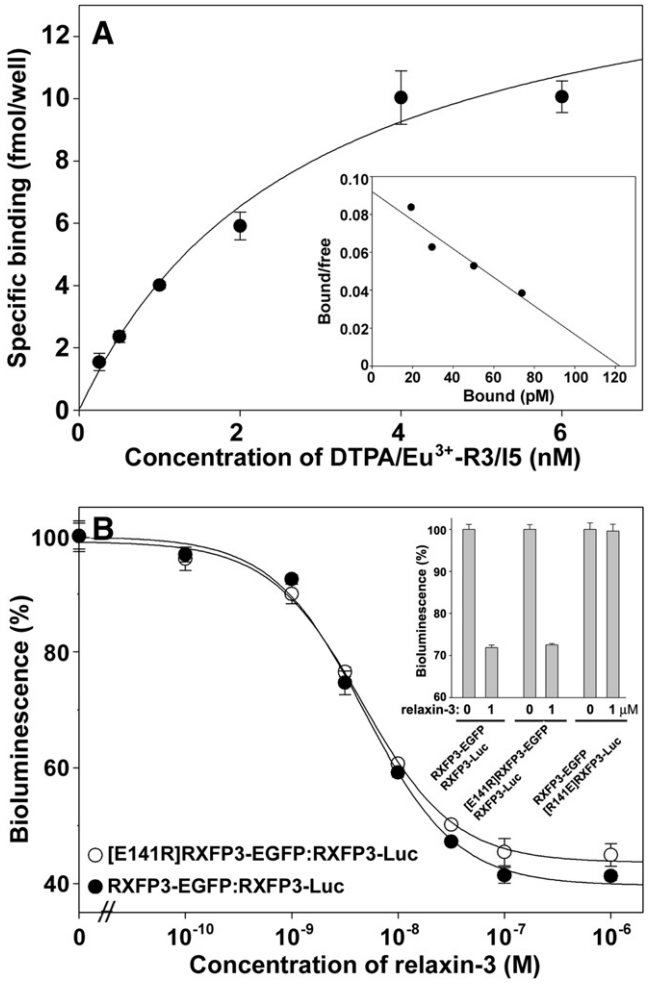

Fig. 5. (A) Saturation binding of the DTPA/Eu^{3+}-labeled R3/I5 to stable HEK293T cells coexpressing RXFP3-Luc and [E141R]RXFP3-EGFP after doxycycline induction. Nonspecific binding was obtained by competition with 1.0 μM of relaxin-3. The specific binding data were expressed as means ± SE ($n = 3$) and fitted to a one-site saturation binding curve $Y = B_{max}X / (K_d + X)$ using the software SigmaPlot 10.0. The Scatchard plot was shown as an inner panel. (B) Ligand-binding induced RXFP3-Luc internalization in HEK293T cells coexpressing RXFP3-EGFP or [E141R]RXFP3-EGFP. The relative bioluminescence data were expressed as means ± SE ($n = 3$) and fitted by sigmoidal curves $Y = \min + (100 - \min) / (1 + 10^{X - \log EC50})$ using SigmaPlot 10.0 software. Inner panel, the ligand-induced internalization of RXFP3-Luc and [E141R]RXFP3-Luc in the transiently transfected HEK293T cells.

Natural Science Foundation of China (31470767, 31270824) and the Fundamental Research Funds from Tongji University (2000219098).

References

[1] M.P. Hall, J. Unch, B.F. Binkowski, M.P. Valley, B.L. Butler, M.G. Wood, P. Otto, K. Zimmerman, G. Vidugiris, T. Machleidt, M.B. Robers, H.A. Benink, C.T. Eggers, M.R. Slater, P.L. Meisenheimer, D.H. Klaubert, F. Fan, L.P. Encell, K.V. Wood, Engineered luciferase reporter from a deep sea shrimp utilizing a novel imidazopyrazinone substrate, ACS Chem. Biol. 7 (2012) 1848–1857.
[2] Z.Y. Guo, Using the newly developed nanoluciferase as an ultrasensitive bioluminescent probe for ligand–receptor interaction studies, Recept. Clin. Invest. 1 (2014) 115–117.
[3] G. Song, Q. Jiang, T. Xu, Y.L. Liu, Z.G. Xu, Z.Y. Guo, A convenient luminescence assay of ferroportin internalization to study its interaction with hepcidin, FEBS J. 280 (2013) 1773–1781.
[4] C. Liu, E. Eriste, S. Sutton, J. Chen, B. Roland, C. Kuei, N. Farmer, H. Jörnvall, R. Sillard, T.W. Lovenberg, Identification of relaxin-3/INSL7 as an endogenous ligand for the orphan G-protein-coupled receptor GPCR135, J. Biol. Chem. 278 (2003) 50754–50764.
[5] R.A. Bathgate, M.L. Halls, E.T. van der Westhuizen, G.E. Callander, M. Kocan, R.J. Summers, Relaxin family peptides and their receptors, Physiol. Rev. 93 (2013) 405–480.
[6] C.M. Smith, P.J. Ryan, I.T. Hosken, S. Ma, A.L. Gundlach, Relaxin-3 systems in the brain — the first 10 years, J. Chem. Neuroanat. 42 (2011) 262–275.
[7] R.A. Bathgate, C.S. Samuel, T.C. Burazin, S. Layfield, A.A. Claasz, I.G. Reytomas, N.F. Dawson, C. Zhao, C. Bond, R.J. Summers, L.J. Parry, J.D. Wade, G.W. Tregear, Human relaxin gene 3 (H3) and the equivalent mouse relaxin (M3) gene: novel members of the relaxin peptide family, J. Biol. Chem. 277 (2002) 1148–1157.
[8] C.M. Smith, P.J. Shen, A. Banerjee, P. Bonaventure, S. Ma, R.A. Bathgate, S.W. Sutton, A.L. Gundlach, Distribution of relaxin-3 and RXFP3 within arousal, stress, affective, and cognitive circuits of mouse brain, J. Comp. Neurol. 518 (2010) 4016–4045.
[9] Y. Watanabe, Y. Miyamoto, T. Matsuda, M. Tanaka, Relaxin-3/INSL7 regulates the stress-response system in the rat hypothalamus, J. Mol. Neurosci. 43 (2011) 169–174.
[10] A. Banerjee, P.J. Shen, S. Ma, R.A. Bathgate, A.L. Gundlach, Swim stress excitation of nucleus incertus and rapid induction of relaxin-3 expression via CRF1 activation, Neuropharmacology 58 (2010) 145–155.
[11] B.M. McGowan, S.A. Stanley, K.L. Smith, N.E. White, M.M. Connolly, E.L. Thompson, J.V. Gardiner, K.G. Murphy, M.A. Ghatei, S.R. Bloom, Central relaxin-3 administration causes hyperphagia in male Wistar rats, Endocrinology 146 (2005) 3295–3300.
[12] B.M. McGowan, S.A. Stanley, J. Donovan, E.L. Thompson, M. Patterson, N.M. Semjonous, J.V. Gardiner, K.G. Murphy, M.A. Ghatei, S.R. Bloom, Relaxin-3 stimulates the hypothalamic–pituitary–gonadal axis, Am. J. Physiol. Endocrinol. Metab. 295 (2008) E278–E286.
[13] C.M. Smith, I.T. Hosken, S.W. Sutton, A.J. Lawrence, A.L. Gundlach, Relaxin-3 null mutation mice display a circadian hypoactivity phenotype, Genes Brain Behav. 11 (2012) 94–104.
[14] Y. Watanabe, A. Tsujimura, K. Takao, K. Nishi, Y. Ito, Y. Yasuhara, Y. Nakatomi, C. Yokoyama, K. Fukui, T. Miyakawa, M. Tanaka, Relaxin-3-deficient mice showed slight alteration in anxiety-related behavior, Front. Behav. Neurosci. 5 (2011) 50.
[15] S. Sudo, J. Kumagai, S. Nishi, S. Layfield, T. Ferraro, R.A. Bathgate, A.J. Hsueh, H3 relaxin is a specific ligand for LGR7 and activates the receptor by interacting with both the ectodomain and the exoloop 2, J. Biol. Chem. 278 (2003) 7855–7862.
[16] C. Liu, J. Chen, S. Sutton, B. Roland, C. Kuei, N. Farmer, R. Sillard, T.W. Lovenberg, Identification of relaxin-3/INSL7 as a ligand for GPCR142, J. Biol. Chem. 278 (2003) 50765–50770.
[17] S.Y. Hsu, K. Nakabayashi, S. Nishi, J. Kumagai, M. Kudo, O.D. Sherwood, A.J. Hsueh, Activation of orphan receptors by the hormone relaxin, Science 295 (2002) 671–674.
[18] C. Liu, C. Kuei, S. Sutton, J. Chen, P. Bonaventure, J. Wu, D. Nepomuceno, F. Kamme, D.T. Tran, J. Zhu, T. Wilkinson, R.A. Bathgate, E. Eriste, R. Sillard, T.W. Lovenberg, INSL5 is a high affinity specific agonist for GPCR142 (GPR100), J. Biol. Chem. 280 (2005) 292–300.
[19] W.J. Zhang, X. Luo, G. Song, X.Y. Wang, X.X. Shao, Z.Y. Guo, Design, recombinant expression and convenient A-chain N-terminal europium-labelling of a fully active human relaxin-3 analogue, FEBS J. 279 (2012) 1505–1512.
[20] W.J. Zhang, Q. Jiang, X.Y. Wang, G. Song, X.X. Shao, Z.Y. Guo, A convenient method for europium-labeling of a recombinant chimeric relaxin family peptide R3/I5 for receptor-binding assays, J. Pept. Sci. 19 (2013) 350–354.
[21] R.S. Kasai, A. Kusumi, Single-molecule imaging revealed dynamic GPCR dimerization, Curr. Opin. Cell Biol. 27 (2014) 78–86.
[22] K. Herrick-Davis, Functional significance of serotonin receptor dimerization, Exp. Brain Res. 230 (2013) 375–386.
[23] B. Taddese, L.M. Simpson, I.D. Wall, F.E. Blaney, N.J. Kidley, H.S. Clark, R.E. Smith, G.J. Upton, P.R. Gouldson, G. Psaroudakis, R.P. Bywater, C.A. Reynolds, G-protein-coupled receptor dynamics: dimerization and activation models compared with experiment, Biochem. Soc. Trans. 40 (2012) 394–399.
[24] F. Fanelli, A. Felline, Dimerization and ligand binding affect the structure network of A(2A) adenosine receptor, Biochim. Biophys. Acta 1808 (2011) 1256–1266.
[25] W.J. Zhang, X.Y. Wang, Y.Q. Guo, X. Luo, X.J. Gao, X.X. Shao, Y.L. Liu, Z.G. Xu, Z.Y. Guo, The highly conserved negatively charged Glu141 and Asp145 of the G-protein-coupled receptor RXFP3 interact with the highly conserved positively charged arginine residues of relaxin-3, Amino Acids 46 (2014) 1393–1402.