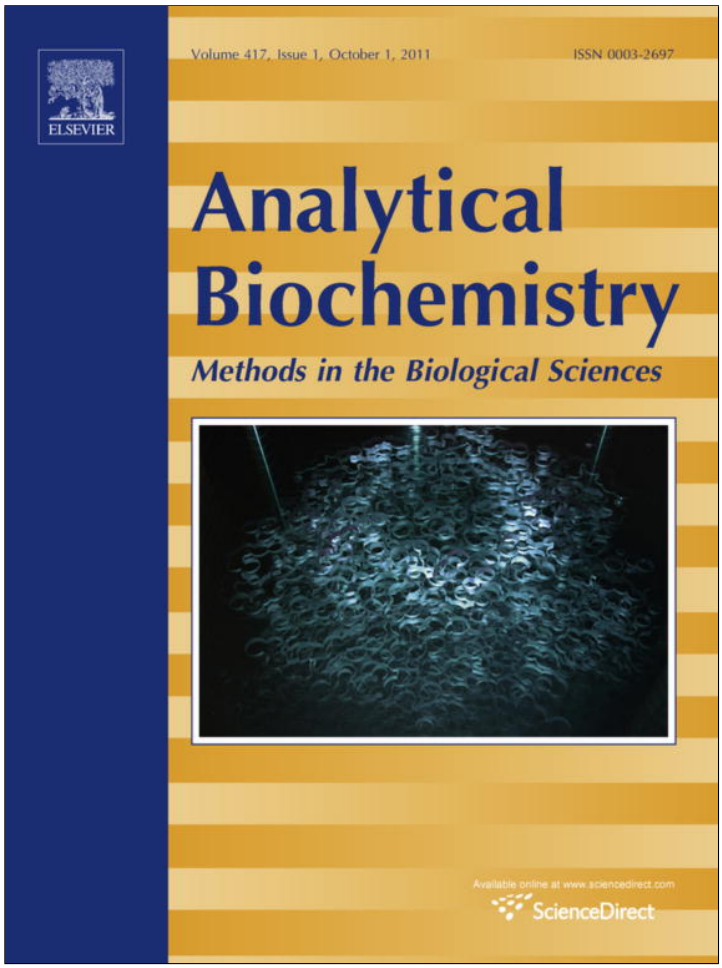

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Development of a novel DnaE intein-based assay for quantitative analysis of G-protein-coupled receptor internalization

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ABSTRACT

G-protein-coupled receptor (GPCR) internalization provides a G-protein-subtype-independent method for assaying agonist-stimulated activation of receptors. We have developed a novel assay that allows quantitative analysis of GPCR internalization based on the interaction between activated GPCRs and β-arrestin2 and on *Nostoc punctiforme* DnaE intein-mediated reconstitution of *Renilla* luciferase fragments. This assay system was validated using four functionally divergent GPCRs treated with agonists and antagonists. The EC_{50} values obtained for the known agonists and antagonists are in close agreement with the results of previous reports, indicating that this assay system is sensitive enough to permit quantification of GPCR internalization. This rapid and quantitative assay, therefore, could be used universally as a functional cell-based assay for GPCR high-throughput screening during drug discovery.

G-protein-coupled receptors (GPCRs),[1] the largest family of cell surface receptors, have seven transmembrane domains and mediate a wide variety of physiological processes, including phototransduction, neurotransmission, metabolism, pain modulation, chemoattraction, and reproduction, making these receptors critical and attractive targets for drug discovery. GPCRs represent major therapeutic targets in drug discovery; nearly half of all currently clinically used drugs target GPCRs. It is well established that on ligand binding, GPCRs undergo conformational changes that lead to the dissociation of the coupled Gα from Gβγ subunit, initiating downstream signaling cascades. Following activation, β-arrestins bind to the carboxyl-terminal end after its phosphorylation by GPCR kinases, resulting in receptor internalization and desensitization [1,2]. Although B1 bradykinin receptor has been found to be disabled in internalization [3], and some GPCRs such as the AT_1 angiotensin receptor can use distinct endocytic pathways to undergo internalization without interaction with β-arrestins [4], the β-arrestin-dependent internalization has been shown to be a common mechanism for most GPCRs [5–9]. In addition, there is increasing evidence that the β-arrestin-mediated internalization is involved not only in GPCR desensitization and resensitization but also in GPCR-mediated activation of mitogen-activated protein kinase (MAPK) pathways [10]. It is desirable to develop new approaches to detect GPCR activity, allowing the identification of novel agonists and antagonists. In addition, agonist-mediated receptor internalization occurs in the same fashion regardless of the type of G protein involved; therefore, β-arrestin-based assay systems should be useful for most GPCRs [11,12].

Numerous GPCR assays have been developed for pharmacological profiling of receptors and identification of novel agonists and antagonists, including ligand-binding, GTP-γS-binding, and second-messenger (cAMP and intracellular Ca^{2+} detection) assays. In addition to these traditional GPCR assays, new assay systems that respond to receptor activation but are independent of G proteins have been created. Recently, several assays that monitor interactions between ligand-activated GPCRs and β-arrestin have been designed, including green fluorescent protein (GFP)-tagged β-arrestin redistribution [13,14], bioluminescence resonance energy transfer (BRET) assay [15], fluorescence resonance energy transfer (FRET) assay [16], and enzyme fragment complementation (EFC) assay [17]. However, these assay systems had some drawbacks, including low sensitivity, the length of time required for data acquisition, the large amount of computing power required to analyze imaging data obtained, and limited usefulness for academic labs due to the requirement for expensive reagents or sophisticated specialized instruments [12].

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[1] *Abbreviations used:* GPCR, G-protein-coupled receptor; GFP, green fluorescent protein; BRET, bioluminescence resonance energy transfer; FRET, fluorescence resonance energy transfer; EFC, enzyme fragment complementation; GRK, G-protein-coupled receptor kinase; AKHR, adipokinetic hormone receptor; AKH, insect adipokinetic hormone; DnaEn (Dn), N-terminal part of DnaE intein; PCR, polymerase chain reaction; DnaE (Dc), C-terminal part of DnaE intein; cDNA, complementary DNA; RlucN (Rn), N-terminal part of *Renilla* luciferase; RlucC (Rc), C-terminal part of *Renilla* luciferase; HM74a (or GPR109A), human nicotinic acid receptor; CB2, cannabinoid receptor 2; H1, histamine H1 receptor; HEK293, human embryonic kidney 293; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; CRE, cAMP response element; ELISA, enzyme-linked immunosorbent assay; TBS, Tris-buffered saline.

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To overcome these deficiencies, we first attempted to express fusion proteins consisting of a GPCR and a β-arrestin coupled to the N-terminal fragment and C-terminal fragment of *Renilla* luciferase, respectively, but no signal was detected in response to agonist stimulation in this system. Therefore, to develop a quantitative assay for monitoring GPCR internalization, we fused a GPCR with the N-terminal half of *Renilla* luciferase and a protein-splicing DnaE intein from *Nostoc punctiforme* and localized the resulting fusion protein at the plasma membrane. At the same time, we fused β-arrestin with the C-terminal half of *Renilla* luciferase and DnaE intein and expressed this in the cytosol. On GPCR activation by agonist binding, it is phosphorylated in the C-terminal domain by G-protein-coupled receptor kinases (GRKs), followed by recruitment of β-arrestin; in our system, this results in reconstitution of full-length *Renilla* luciferase mediated by the trans-splicing DnaE inteins. Using a luciferase-based technique, therefore, GPCR internalization can be easily and sensitively detected by bioluminescence originating from the reconstituted *Renilla* luciferase, and this assay will be useful for those GPCRs that can recruit β-arrestins to the phosphorylated C-terminal tail to undergo internalization.

Materials and methods

Reagents

Cell culture medium was purchased from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum was purchased from Hyclone (South Logan, UT, USA). The forskolin, HM74a receptor agonist nicotinic acid, and histamine receptor H1 agonist histamine were obtained from Sigma (St. Louis, MO, USA). The cannabinoid receptor CB2 agonist Win-55212-2, CP55940, and antagonist AM630 were obtained from Tocris (Ellisville, MO, USA). The adipokinetic hormone receptor (AKHR) agonist insect adipokinetic hormone (AKH) peptide was prepared by solid-phase synthesis (GL Biochem (Shanghai), China).

Construction of expressing vectors

The N-terminal part of the *N. punctiforme* DnaE intein (DnaEn, 1–102 aa) and 3 amino acids (AEY) of the native C-terminal extein sequence of a DnaEn extein fragment were amplified from *N. punctiforme* genomic DNA (American Type Culture Collection [ATCC], Manassas, VA, USA). The polymerase chain reaction (PCR) product obtained was cloned into a pCMV-Flag vector (Sigma) using restriction sites *Bam*HI and *Xba*I, resulting in the fusion protein GSAEY-DnaEn and plasmid pF-Dn. The C-terminal part of the split *N. punctiforme* DnaE intein (DnaEc, 1–36 aa) and 3 amino acids (CFN) of the native N-terminal extein sequence of a DnaEc extein fragment were amplified from the aforementioned genomic DNA. The PCR product obtained was cloned into pCDNA3 using restriction sites *Kpn*I and *Bam*HI, resulting in the fusion protein DnaEc-CFNGT and plasmid pc-Dc.

The complementary DNA (cDNA) encoding the N-terminal domain of *Renilla* luciferase (RlucN, 1–229 aa) [18,19] in pGL4.73 (Promega, Madison, WI, USA) was modified by PCR and fused to pCMV-Flag or pf-DN, resulting in the protein RlucN and plasmid pf-RlucN or the new recombinant fusion protein RlucN-GSAEY-DnaEN and plasmid pf-RnDn. The cDNA encoding the C-terminal domain of *Renilla* luciferase (RlucC, 230–311 aa) was modified by PCR and fused to pCDNA or pcDC, resulting in the protein RlucC and plasmid pc-RlucC or the new recombinant fusion protein DnaEC-CFNGT-RlucC and plasmid pc-DcRc.

Coding sequences for human nicotinic acid receptor (HM74a), cannabinoid receptor 2 (CB2), and AKHR were cloned as described

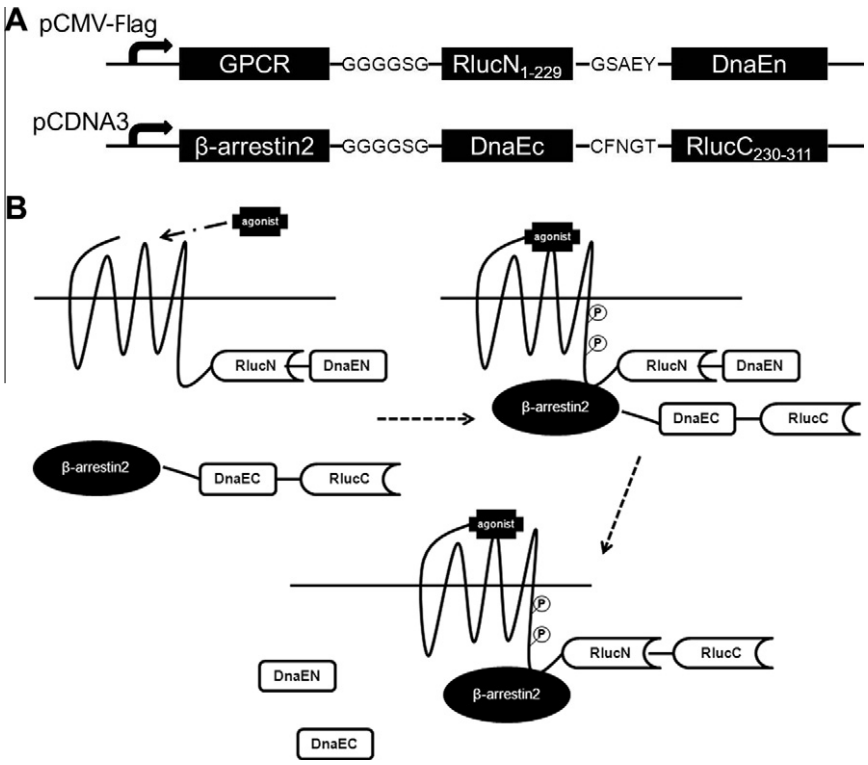

Fig.1. (A) Schematic structures of plasmid constructs. The GPCR of interest is fused to the N terminal of *Renilla* luciferase (RlucN, 1–229 aa) and the N terminal of DnaE inein (DnaEn) with GC linker, whereas β-arrestin2 is fused to the C terminal of DnaE intein (DnaEc) and the C terminal of *Renilla* luciferase (RlucC, 230–311 aa) with GC linker. Sequences encoding GSAEY and CFNGT are inserted between *Renilla* luciferase and DnaE intein in pCMV-Flag and pcDNA3, respectively, for efficient protein splicing. (B) Principle for the intein-mediated reporter gene assay. On agonist binding, activation of the GPCR creates a binding site for arrestin and β-arrestin2 is recruited to the receptor, thereby forcing the intein-mediated reconstitution of split *Renilla* luciferase.

previously [20–22]. Histamine H1 receptor (H1) (GenBank Accession No. NM_000861) and β-arrestin2 (GenBank Accession No. NM_004313) were amplified by PCR from human embryonic kidney 293 (HEK293) cell genomic DNA. The wild-type GPCR PCR products were fused to pCMV-Flag vector and named GPCR wild type. The GPCR PCR products lacking stop codons were cloned to upstream of the RlucN of the vector pf-RlucN or RlucN-GSAEY-DnaEN of the vector pf-RnDn with a 6-amino-acid linker (GGGGSG) that was named GPCR-RlucN or GPCR-RnDn. The β-arrestin2 coding sequence without its stop codon was cloned to upstream of the RlucC of the vector pc-RlucC or DnaEC-VFNGT-RlucC of the vector pC-DcRc with a 6-amino-acid linker (GGGGSG) that was named βarr2-RlucC or βarr2-DcRc (Fig. 1A). The AKHR deletion mutant (Δ343–363 aa) and HM74a deletion mutant (Δ315–326 aa) were constructed with overlap extension PCR strategies. The mutant PCR products lacking stop codons were fused to pf-RnDn expressing the mutant protein AKHRΔ343–363-RnDn and HM74aΔ315–326-RnDn. The HM74a-RnDn and its mutant HM74aΔ315–326-RnDn coding sequences were modified to lack stop codons through PCR and fused to pEGFP-N1-expressing vector (Clontech, Mountain View, CA, USA) and were named HM74a-RnDn-EGFP and HM74aΔ315–326-RnDn-EGFP. All of the primers used can be found in the Supplementary table (see Supplementary material), and all constructs were sequenced to verify the correct sequences and orientations.

Cell culture and transfection

HEK293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS). The plasmid constructs were cotransfected into HEK293 cells using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). Transfected cells were prepared for the assay 48 h after transfection.

cAMP accumulation measurement

The cAMP accumulation was measured through an assay that used a pCRE-fLuc vector (Clontech) consisting of the firefly luciferase coding region under control of a minimal promoter containing cAMP response elements (CREs) [23]. The cells were seeded in a 48-well plate overnight before the beginning of the assay. HEK293 cells cotransfected with a GPCR or GPCR-RnDn and pCRE-fLuc were grown to 90–95% confluence, stimulated with the indicated concentration of agonist (histamine to H1, AKH to AKHR) or 10 μM forskolin with different concentrations of agonist (niacin to HM74a, Win-55212-2 to CB2) in DMEM without FBS, and incubated for 4 h at 37 °C. Firefly luciferase activity was detected using a firefly luciferase assay kit (Ken-real, Shanghai, China) [21]. EC_{50} values were calculated using nonlinear regression with a variable slope sigmoidal dose–response algorithm using Prism 4 software (GraphPad Software, San Diego, CA, USA).

Internalization assay

HEK293 cells expressing the GPCR-RnDn and βarr2-DcRc fusions were seeded in a 48-well plate overnight before the beginning of the assay. Ligand was added for 60 min at 37 °C. *Renilla* luciferase activity was detected using a *Renilla* luciferase assay kit (Promega). The medium was aspirated, and 50 μl of lysis buffer was added to each well. The plates were incubated for 10 to 15 min at room temperature before dilution of 15 μl of the cell suspension with 15 μl of assay buffer (with the substrate diluted 1:100). Diluted samples were then read in a FB12 luminometer (Berthold Technologies, Bad Wildbad, Germany). EC_{50} values were calculated using nonlinear regression with a variable slope sigmoidal dose–response algorithm and Prism 4 software.

Confocal microscopy internalization assay

HEK293 cells expressing HM74a-RnDn-EGFP or HM74aΔ315–326-RnDn-EGFP were seeded in covered glass-bottom 6-well plates. After 24 h, cells were treated with 100 μM niacin for the indicated times at 37 °C. After fixation with 4% paraformaldehyde for 10 min, cells were mounted in mounting reagent (dithiothreitol [DTT]/phosphate-buffered saline [PBS]/glycerol). Confocal images were taken on a Zeiss LSM 510 microscope with an attached Axiovert 200 microscope and LSM5 computer system. Excitation was performed at 488 nm, and fluorescence detection used a 525 ± 25-nm bandpass filter. Images were collected using QED camera software and processed with Adobe Photoshop.

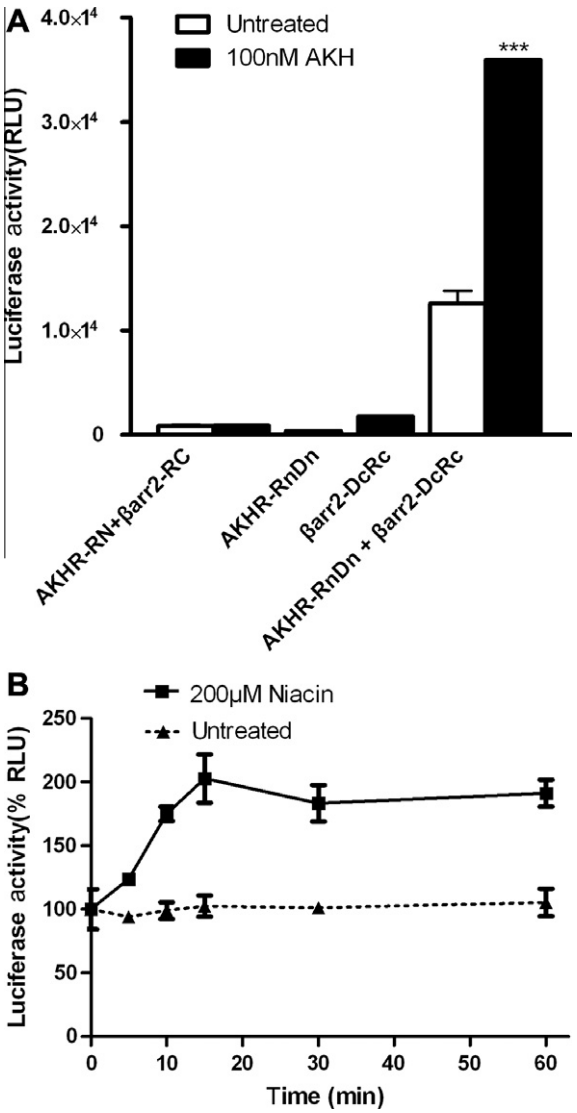

Fig.2. (A) *Renilla* luciferase activities on the AKH-induced internalization of AKHR. HEK293 cells were transiently transfected with AKHR-RlucN + βarr2-RlucC (AKHR-RN + βarr2-RC, complementation), AKHR-RnDn, βarr2-DcRc, and AKHR-RnDn + βarr2-DcRc (reconstitution), respectively. The cells were subject to stimulation with 100 nM AKH or not for 1 h after 48 h, and the luciferase activity was measured. The relative light units (RLU) are represented. Error bars represent standard errors for triplicate measurements. Data were analyzed by using the Student's *t*-test (***$p < 0.001$). (B) Time course of HM74a receptor internalization stimulated with 200 μM niacin. HEK293 cells were transiently transfected with HM74a-RnDn and βarr2-DcRc. The basal luciferase activity was normalized to 100%. The data are means ± standard errors of three independent experiments.

Measurement of cell surface receptors by ELISA

The cell surface expression of AKHR was quantitatively assessed by enzyme-linked immunosorbent assay (ELISA), which was performed as described previously [24]. Briefly, HEK293 cells transfected with the AKHR-RnDn or AKHRΔ343–363-RnDn were seeded in 48-well dishes coated with poly-L-lysine. The next day, the cells were stimulated with 200 nM AKH for the indicated times. The medium was aspirated, and the cells were washed once with Tris-buffered saline (TBS). After fixing the cells for 5 min at room temperature with 3.7% formaldehyde in TBS, the cells were washed three times with TBS and then blocked for 45 min with 1% bovine serum albumin/TBS. Cells were then incubated for 1 h with an alkaline-phosphatase-conjugated monoclonal antibody directed against the Flag epitope (1:1000 dilution). Cells were washed three times, and antibody binding was visualized by adding 0.25 ml of an alkaline phosphatase substrate (nitrophenylphosphate that was dissolved diethanolamine buffer, Bio-Rad, Hercules, CA, USA). Development was stopped by adding 0.1 ml of the substrate to a 96-well microtiter plate containing 0.1 ml of 0.4 M NaOH. Plates were read at 405 nm in a microplate reader (Bio-Rad) using Microplate Manager software.

Data analysis

Statistical significance was determined using Student's *t*-test. Probability values less than or equal to 0.05 were considered significant.

Results

Design of receptor internalization assays

To design a GPCR internalization assay, we exploited protein fragment complementation technology, which is based on the DnaE intein-mediated reconstitution of split *Renilla* luciferase proteins driven by the interaction between two strongly interacting proteins. For the purpose of monitoring the interactions of activated GPCRs with β-arrestin2, the GPCR of interest was fused to RlucN (1–229 aa) and DnaEn with a GC linker (6 aa, GGGGSG), whereas β-arrestin2 was fused to DnaEc and RlucC (230–311 aa) with a GC linker (GGGGSG). Sequences encoding GSAEY and CFNGT were inserted between the Rluc and DnaE fragments in pCMV-Flag and pcDNA3, respectively, for efficient protein splicing (Fig. 1A). On agonist binding, activation of the GPCR creates a binding site for arrestin, and β-arrestin2 is recruited to the receptor, thereby forcing the intein-mediated reconstitution of the fragmented *Renilla* luciferase (Fig. 1B).

As a first test of the system, HEK293 cells transiently expressing AKHR-RnDn and βarr2-DcRc fusion proteins were treated with 100 nM AKH for 1 h, and luciferase activity was measured. Agonist treatment resulted in a robust (~3-fold) induction of luciferase activity compared with baseline (untreated). However, no increase in luciferase activity was detected in 293 cells expressing AKHR-RlucN and βarr2-RlucC fusion proteins (complementation) or in 293 cells expressing only AKHR-RnDn or βarr2-Dc-Rc (Fig. 2A). The time course of HM74a receptor internalization on stimulation with 200 μM nicotinic acid (Fig. 2B) shows that this process reaches a plateau by 1 h after stimulation, in agreement with the previously reported time course of GPCR internalization [20]. These results demonstrate that our intein-based internalization assay was able to measure GPCR internalization.

Agonist-induced internalization assay

To further validate this quantitative internalization assay, we selected four functionally divergent GPCRs—AKHR (a Gs-coupled receptor), HM74a (a Gi-coupled receptor), CB2 (a Gi-coupled receptor), and H1 (a Gq-coupled receptor)—and constructed vectors to express GPCR-RnDn fusion proteins. We first examined

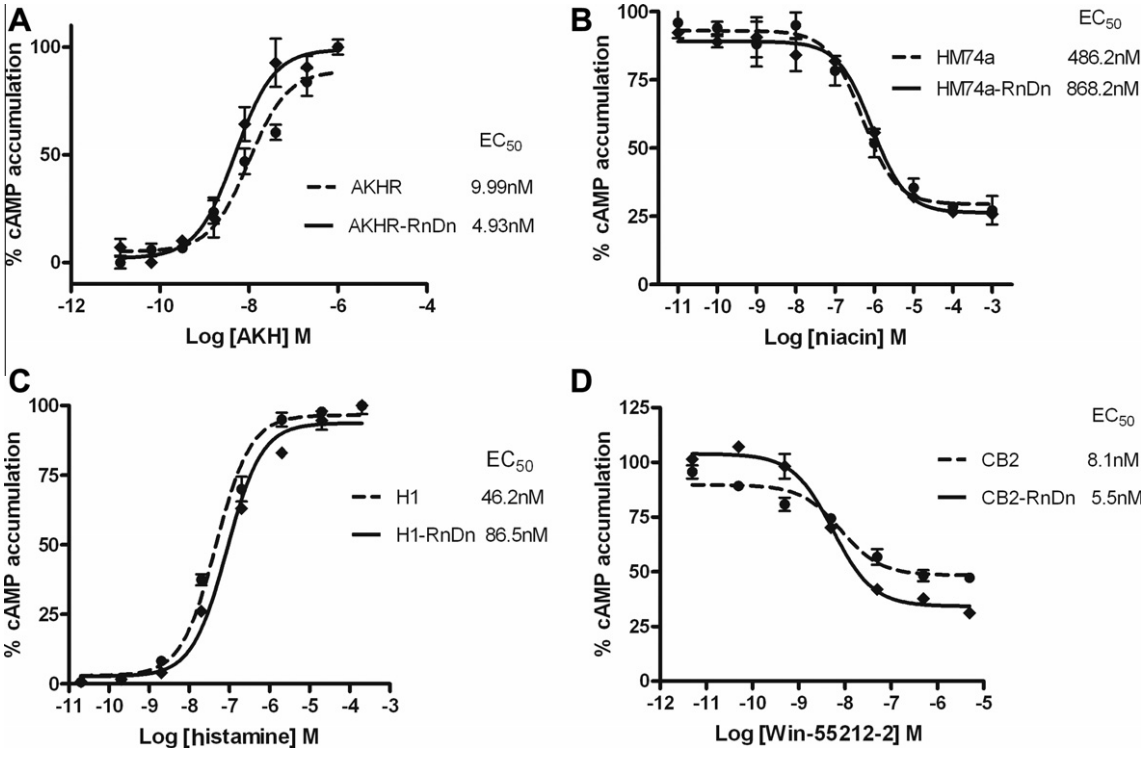

Fig.3. cAMP accumulation in HEK293 cells transiently cotransfected with pCRE-fLuc and GPCR wild type or GPCR-RnDn was determined in response to different doses of agonist for 4 h: (A) AKHR; (B) HM74a; (C) H1; (D) CB2. The dose–response curves were obtained by normalizing the data to 0 (lowest luciferase activity) and 100 (highest luciferase activity). The data are means ± standard errors of three independent experiments.

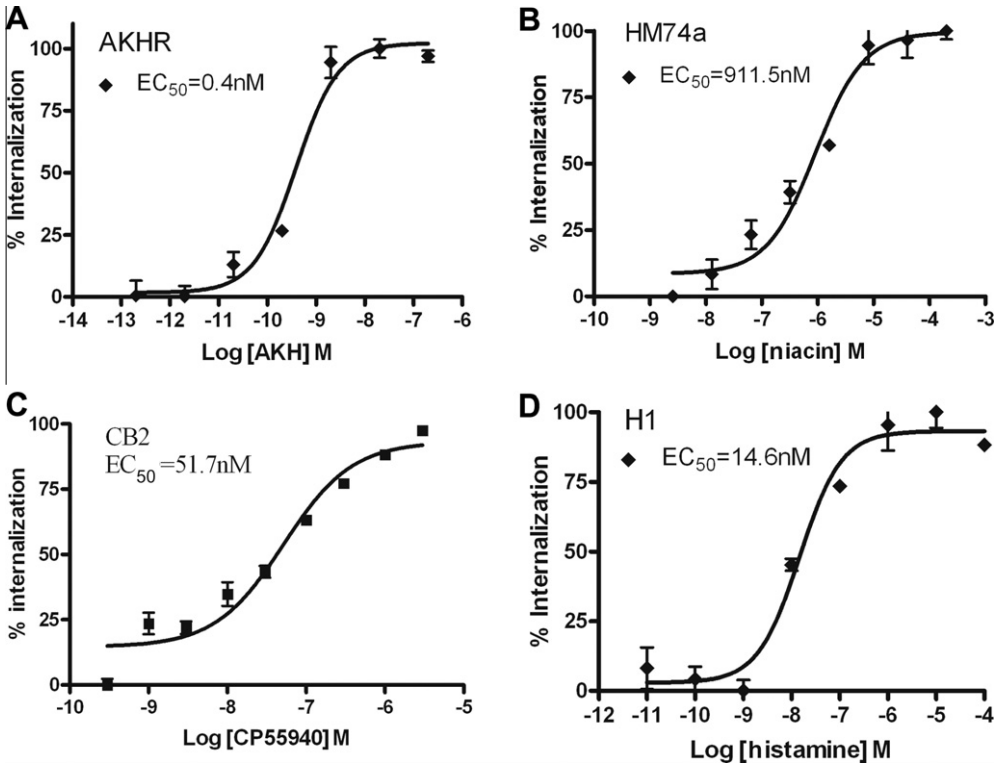

Fig.4. Internalization assays in HEK293 cells transiently cotransfected with GPCR-RnDn and βarr2-DcRc were determined in response to different doses of agonists for 1 h, showing dose–response curves with values normalized to 0 (lowest dose) and 100 (highest dose). (A) AKHR was treated with AKH. (B) HM74a was treated with niacin. (C) CB2 was treated with CP55940. (D) H1 was treated with histamine.

the function of the GPCR-RnDn. As shown in Fig. 3, by cAMP accumulation measurement assay, these four fusion GPCRs function normally compared with wild-type receptors. Then we examined the Rluc activity when the activated GPCR-RnDn recruited the βarr2-DcRc after binding its ligand. We generated the dose–response curve shown in Fig. 4. Luciferase activity was measured after cells treated with agonist for 60 min. The EC_{50} data obtained for known agonists (1 nM for AKH, 911 nM for niacin, 52 nM for CP55940, and 15 nM for histamine) are in close agreement with previously published data using conventional functional assays (6.4 nM for AKH, 700 ± 200 nM for niacin, 52.1 ± 6.2 nM for CP55940, and 69 ± 13 nM for histamine) [24–27], indicating that this assay system has sufficient sensitivity for quantization of GPCR internalization.

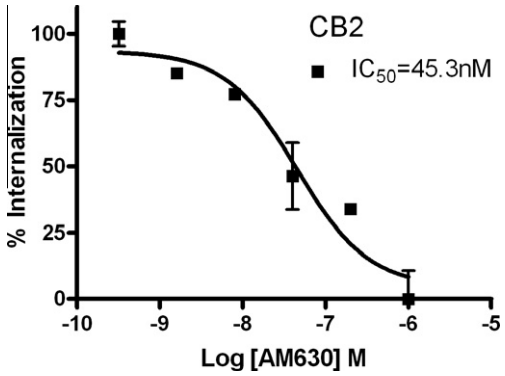

Fig.5. Inhibition of antagonist in internalization assays. HEK293 cells cotransfected with CB2-RnDn and βarr2-DcRc were determined in response to different doses of antagonists AM630 and 4 μM Win-55212–2 for 1 h, and the luciferase activity was measured.

Antagonist-inhibited internalization assay

To determine whether this system could also be used to assess the effects of inhibitors of GPCRs, HEK293 cells expressing CB2-RnDn/βarr2-DcRc fusion proteins were treated with the antagonist AM630. The results show that AM630 had a significant inhibitory effect on the internalization of CB2 with an IC_{50} value of 45 nM in the presence of 4 μM Win-55212-2 (Fig. 5), in agreement with published reports (170 nM) [28]. These results show that our internalization assays can be used to test inhibition of GPCR internalization by antagonists.

Internalization of GPCR mutants

We have described a quantitative internalization assay that monitors the interaction between agonist-activated receptor and β-arrestin2. To determine whether only activated receptors bind β-arrestin2 in our system, the internalization of two GPCR mutants was assayed. The AKHR mutant and HM74a mutant each bore deletions of 21 and 22 amino acids containing Ser and Tyr residues in the C terminal, respectively. This type of mutation ensures that the receptor is not phosphorylated by GRKs and, therefore, cannot bind to or be internalized by β-arrestin2. As shown in Fig. 6A, quantitative analysis by ELISA using HEK293 cells expressing AKHR wild type and mutant revealed that the mutant Flag-AKHRΔ343–363 was found to be significantly impaired in agonist-mediated internalization. The internalization assay shows that luciferase activity did not significantly increase on agonist binding in cells expressing the AKHR mutant compared with the wild type (Fig. 6B). Similarly, confocal microscopy analysis showed that niacin stimulation led to internalization of wild-type HM74a-EGFP from the cell surface to the cytoplasma, whereas the mutant HM74aΔ315–326-EGFP remained in the plasma membrane on activation by niacin (Fig. 6C and D). This shows that

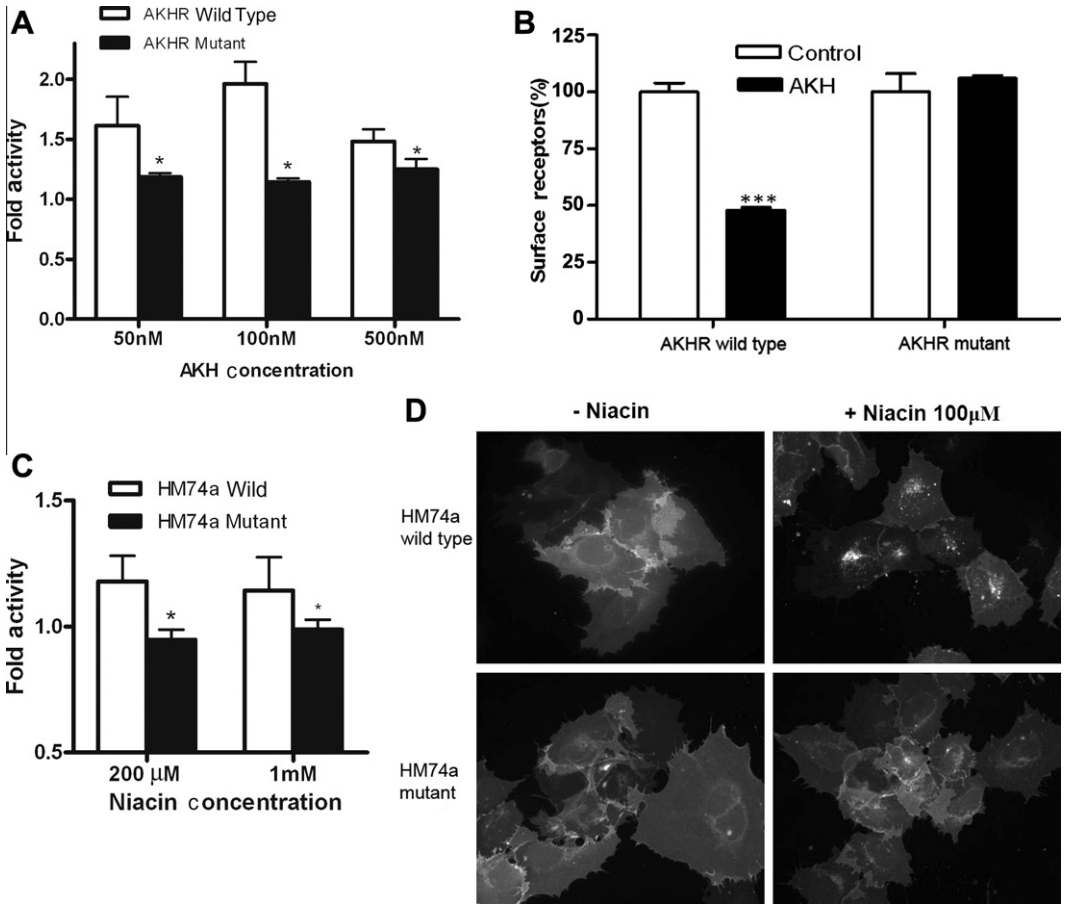

Fig.6. *Renilla* luciferase activities on the agonist-induced internalization of GPCRs. (A) HEK293 cells were transiently transfected with AKHR-RnDn or AKHRΔ343–363-RnDn and βarr2-DcRc. The cells were subject to stimulation with agonist for 1 h after 48 h, and the luciferase activity was measured. (B) Quantification of AKHR-RnDn or AKHRΔ343–363-RnDn internalization by ELISA. (C) HEK293 cells were transiently transfected with HM74a-RnDn or HM74aΔ343–363-RnDn and βarr2-DcRc. The cells were subject to stimulation with agonist for 1 h after 48 h, and the luciferase activity was measured. (D) Observation of HM74a-RnDn-EGFP or HM74aΔ325–326-RnDn-EGFP's translocation under the laser scanning confocal microscopy. Data were analyzed by using the Student's *t*-test ($^{*}p < 0.05$, $^{***}p < 0.001$).

intein-mediated reconstitution of the split *Renilla* luciferase proteins is driven by the interaction between an agonist-stimulated receptor and β-arrestin2.

Discussion

The interaction between agonist-activated GPCRs and β-arrestin has been demonstrated in numerous studies. β-Arrestins are ubiquitously expressed and act to target receptors to clathrin-coated endocytotic vesicles for degradation or recycling [29–32]. On agonist binding, activated GPCRs are first phosphorylated at the carboxyl-terminal tail by GRKs, allowing recruitment of β-arrestins, which bind with high affinity [5–7] and uncouple the receptor from G proteins. This result is not only receptor internalization and desensitization but also G-protein-independent signaling to ERK1/2 [1,8,9]. Therefore, it would be useful to develop cell-based assays for quantitatively monitoring the interaction of GPCRs with β-arrestins; such assays could enable functional characterization of GPCR internalization and discovery of therapeutically valuable compounds. In this article, we have designed and developed a novel assay for detecting interactions of GPCRs with β-arrestins that is based on protein splicing and split *Renilla* luciferase. This system is based on the reconstitution of fragments of *Renilla* luciferase by protein splicing mediated via a DnaE intein (a protein splicing element). The inactive N- and C-terminal fragments of *Renilla* luciferase (fused with the N- and C-terminal DnaE sequences, respectively) were fused to a GPCR of interest and to β-arrestin2, respectively. In cells that stably or transiently expressed these fusion proteins and were treated with agonist, the physical interaction of β-arrestin2 with the phosphorylated C-terminal end of an activated GPCR led to DnaE intein-mediated reconstitution of split *Renilla* luciferase fragments, resulting in measurable enzymatic activity.

Inteins are protein sequences that mediate autocatalytic excision from precursor sequences and concomitant ligation of the two flanking sequences with a peptide bond [33]. Therefore, a naturally occurring split intein can be used in many biotechnological applications such as generation of cyclic peptides, protein semisynthesis, segmental isotopic labeling, site-specific modification of proteins, and detection of protein–protein interactions [34–41]. Among a number of naturally occurring split inteins, we chose to employ DnaE from *N. punctiforme*, which has recently been identified and characterized in an *Escherichia coli* system; so far, however, it has not been demonstrated to function properly in mammalian cells [42,43]. Our previous study indicated that the naturally occurring split *N. punctiforme* DnaE intein can indeed perform protein trans-splicing at a high efficiency in mammalian cells and, therefore, is a useful tool for analyzing protein–protein interactions, although its expression level is low in mammalian cells due to the poor codon usage bias [44]. In the current study, split inteins were placed between a GPCR or a β-arrestin and split *Renilla* luciferase fragments; these constructs showed expression comparable to that of the wild-type GPCR or β-arrestin (data not shown).

Based on recruitment of β-arrestins to the phosphorylated C-terminal end of GPCRs, several cell-based assays have been developed to quantitatively detect GPCR internalization and GPCR–β-arrestin interactions, including FRET and BRET assays [45,46], redistribution assays using receptors [47] or β-arrestins [13,48] tagged with GFP, and EFC assays [49]. In contrast to the GPCR–β-arrestin interaction assays mentioned above, our DnaE intein-based GPCR–β-arrestin interaction assay is easy to manipulate and cost-effective, with no requirements for sophisticated instruments or expensive reagents. A great advantage of the DnaE intein-based *Renilla* luciferase assay is its rapid kinetic profile, which allows signals to be measured within seconds after protein splicing. The *Renilla* luciferase-based assay system offers an opportunity to study living cells and is increasingly used in academia and industry because it provides a safe cheap alternative to radioisotope assays and is compatible with automated high-throughput screening.

In the current study, we selected Gi-coupled HM74a and human CB2, Gs-coupled *Bombyx* AKHR, and Gq-coupled human H1 to validate this DnaE intein-based GPCR–β-arrestin interaction assay. The EC_{50} values obtained from the assays for HM74a, CB2, AKHR, and H1 receptors are in good agreement with previously published data using conventional functional assays [24–27], and the IC_{50} value of CB2 antagonist AM630 obtained in the same assay with stimulation of agonist Win-55212-2 is also similar to that reported previously [28]. As validated in the current study, this assay is also uniquely capable of analyzing the internalization of Gi-, Gs-, and Gq-coupled GPCRs and of identifying both antagonist and agonist compounds in a primary screen.

In summary, we have developed a cell-based functional assay for quantitative analysis of agonist-induced GPCR interaction with β-arrestin and internalization based on a split *Renilla* luciferase and reconstitution by DnaE intein-mediated protein splicing. In the current study, this cell-based assay was demonstrated to be easily manipulated; moreover, it is cost-effective with no requirements for sophisticated instruments or expensive reagents. The sensitivity, specificity, and universality of the assay technology described here provide a means for discovering new therapeutic leads for known GPCRs and for identifying novel ligands for orphan GPCRs.

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Appendix A. Supplementary data

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