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A single-vector EYFP reporter gene assay for G protein-coupled receptors

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ABSTRACT

We here present an improved and simplified assay to study signal transduction of the G_s class of G protein-coupled receptors (GPCRs). The assay is based on a single plasmid combining the genes for any G_s protein-coupled GPCR and the cAMP response element-related expression of enhanced yellow fluorescent protein. On transfection, stable human embryonic kidney 293 (HEK293) cell lines presented high assay sensitivity and an unprecedented signal-to-noise ratio of up to 300, even in the absence of trichostatin A. The robustness of the assay was demonstrated through the cloning of reporter gene cell lines with melanocortin 4 receptor (MC4R), the human type I pituitary adenylate cyclase-activating polypeptide receptor (hPAC1), and the two vasoactive intestinal peptide receptors (VPAC1 and VPAC2).

G protein-coupled receptors (GPCRs)[2] are responsible for a large part of cellular responses to various types of external stimuli, and consequently their malfunction is involved in a range of diseases [1]. The great potential for identifying drugs with either agonist or antagonist activity toward GPCRs renders the members of this receptor class, as well as their downstream kinases, the most pursued targets for drug development [2]. Thus, the development of reliable assays that reflect the true activity of receptor-stimulated gene expression is of great importance.

Binding of an agonist to G_s protein-coupled receptors initiates a signaling cascade starting with activation of the receptor-associated G_s protein. In turn, the activated G_s protein stimulates adenylyl cyclase, which leads to an increase in intracellular cyclic adenosine monophosphate (cAMP). The second messenger cAMP is known to activate the cAMP-dependent protein kinase A (PKA), which subsequently phosphorylates the transcription factor, cAMP response element (CRE) binding protein (CREB). This enables CREB to bind to the CRE sequences present in promoter regions and to consequently initiate transcription of the target gene [3]. Alternatively, transcription might be achieved through β-arrestin-mediated signaling [4,5].

Assays for detecting cAMP concentrations are core to the majority of drug screening platforms targeting G_s protein-coupled GPCR targets [6]. However, regulation of the intracellular level of cAMP is a complex process that is affected by a variety of intracellular events and many cellular components [7]. Reporter gene assays are alternative/supplementary to cAMP assays and measure the downstream CRE-initiated expression of a reporter gene such as an enzyme or a fluorescent protein [8–12]. One can argue that reporter gene assays are more accurate because they measure a signal provided through the activation of the complete range of effectors in the signaling cascades [9]. We here report a GPCR reporter gene assay that circumvents assay problems and provides significantly increased signal-to-noise (S/N) ratios using a single-vector cassette for expression of both GPCRs and EYFP in monoclonal and stable cell lines.

Materials and methods

Materials

Phusion high-fidelity DNA polymerase from New England Biolabs was used for all polymerase chain reactions (PCRs). T4 DNA ligase, T4 polynucleotide kinase, calf intestinal alkaline

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[2] *Abbreviations used:* GPCR, G protein-coupled receptor; cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; CRE, cAMP response element; CREB, CRE-binding protein; S/N, signal-to-noise; PCR, polymerase chain reaction; HEK293, human embryonic kidney 293; NDP–MSH, (Nle4,D-Phe7)-α-MSH; MC4R, melanocortin 4 receptor; CMV, cytomegalovirus; BGH, bovine growth hormone; PEST sequence, proline, glutamic acid, serine, and threonine sequence; EDTA, ethylenediaminetetraacetic acid; hPAC1, human type I pituitary adenylate cyclase-activating polypeptide receptor; VPAC, vasoactive intestinal peptide receptor; FBS, fetal bovine serum; TSA, tricostatin A; FACS, fluorescence-activated cell sorting.

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phosphatase (CIAP), and DH10B cells were obtained from Invitrogen. Adherent human embryonic kidney (HEK293) cells were obtained from the American Type Culture Collection (ATCC). Cell culture media were obtained from Sigma–Aldrich. MC4R-pcDNA3.1(+) plasmid was obtained from UMR cDNA Resource Center (http://www.cdna.org), whereas plasmids of pcDNA3-hPAC1, pCMV.SPORT 6-VPAC1, and pcDNA3-VPAC2 were kindly provided by E.M. Lutz (University of Strathclyde). The α-MSH analog (Nle4, D-Phe7)-α-MSH (cat. no. H-1100), referred to as NDP–MSH throughout this article, was used for melanocortin 4 receptor (MC4R) assays and was purchased from Bachem together with PACAP-38 (cat. no. H-8430). Maxadilan and VPAC1 agonist [(Lys15, Arg16, Leu27)-VIP (1–7)-GRF (8–27)] were kindly provided by Steffen Birk (Rigshospitalet, University of Copenhagen). The 96-well assay microplate was the Packard ViewPlate TM-96 (cat. no. 6005225).

Vector engineering

AsiSI and MluI restriction sites were incorporated into the pPUR vector to form the pPUR-1 vector.

The pPUR vector from Clontech was subjected to PCR amplification using primers 1 and 2 (see Table S1 in online supplementary material), which contains AsiSI and MluI recognition sequence at the 5′ ends. The introduction of AsiSI and MluI restriction sites allowed the subsequent insertion of the fluorescence reporter gene cassette described below. After phosphorylation, the PCR product was submitted to blunt-end ligation and cloning, and the new vector pPUR-1 was characterized by restriction enzyme digestion analysis.

Insertion of GPCR expression cassette to form pPUR-X

A universal GPCR gene expression cassette was constructed to contain a cytomegalovirus (CMV) promoter upstream of a GPCR gene cloning site and a bovine growth hormone (BGH) polyA signal together with f1 origin of replication sequences downstream of it. The two sequences were amplified by PCR from the Invitrogen pcDNA3.1/Zeo(+) vector. Primers 3 and 4 (Table S1) were used for amplification of the CMV promoter sequence incorporating a BamHI site at the 5′ end and two adjacent FseI and BstBI sites at the 3′ end. Primers 5 and 6 (Table S1) were used for amplification of the BGH polyA and the f1 origin DNA sequence, comprising adjacent BstBI and AscI sites at the 5′ end and an EcoRI site at the 3′ end. The two PCR fragments were digested with BstBI and ligated with T4 DNA ligase. The ligation product was then PCR amplified using primers 3 and 6 to obtain the GPCR expression cassette comprising FseI and AscI as GPCR gene cloning sites flanking the upstream CMV promoter and the downstream BGH polyA–f1 origin. The PCR product of the GPCR expression cassette was ligated into the pPUR-1 vector through the BamHI and EcoRI sites. Several clones were identified by restriction enzyme digestion analysis as well as PCR. The obtained intermediate plasmid was designated pPUR-X.

Fluorescence reporter gene cassette pX-3CRE-EYFP

The CRE sequence (3 CRE binding sequences) was chosen as part of a promoter sequence for directing the expression of fluorescent reporter genes. The promoter containing 3 CRE binding sequences was amplified by PCR from the Clontech vector pCRE-d2EGFP using primers 7 and 8 (Table S1). This enabled the introduction of the MluI restriction site at the 5′ end and the XhoI site at the 3′ end of the obtained DNA amplicon. The gene encoding fluorescent protein d2EYFP was amplified from the Clontech plasmid pd2EYFP-1 using primers 9 and 10 (Table S1). Besides the EYFP gene, the amplified sequence also included Kozak consensus translation initiation site, mouse ornithine decarboxylase PEST (proline, glutamic acid, serine, and threonine) sequence, SV40 early mRNA polyadenylation signal, and f1 single-strand DNA origin. Primers 9 and 10 introduced the XhoI restriction site at the 5′ end and the AsiSI site at the 3′ end of the amplified sequence. The PCR products for CRE and d2EYFP were digested with XhoI and ligated. The obtained fluorescence reporter gene expression cassette of CRE-d2EYFP was PCR amplified using primers 7 and 10 and was further verified by XhoI digestion. The CRE-d2EYFP cassette was ligated into pPUR-X through the AsiSI and MluI sites. The clones were identified by restriction enzyme digestion analysis and various PCRs. The obtained plasmid construct was designated pX-3CRE-EYFP.

Fluorescence reporter gene cassette pX-9CRE-d2EYFP

Because the 3 CRE sequences and TATA-like promoter are connected by the BglII recognition site, more copies of the CRE sequence could be inserted to enhance reporter expression through the site. A DNA fragment containing 3 CRE sequences flanking "cut" BglII restriction sites in both ends was obtained from an annealing reaction of two 81-nt oligonucleotides (primers 11 and 12; see Table S1) The annealing reaction mixture (50 μl) containing the sense oligo (5 μg) and the antisense oligo (5 μg) in 1× annealing buffer (10 mM Tris–HCl [pH 8.0], 50 mM NaCl, and 1 mM ethylenediaminetetraacetic acid [EDTA]) was maintained in a heating block at 95 °C for 3 min and gradually cooled down to room temperature. The annealed 81-bp DNA (4 μg) was phosphorylated using T4 Polynucleotide Kinase in a 50-μl reaction and directly subjected to self-ligation in 100 μl using T4 DNA ligase (1 U) for 15 min at room temperature. The ligated product was separated by 10% TBE PAGE (Tris–borate–EDTA polyacrylamide gel electrophoresis; Invitrogen), and the 162-bp band that contained six copies of the CRE sequence was isolated and purified. Subsequently, it was ligated with BglII digested and dephosphorylated pX-3CRE-EYFP and transformed into DH10B cells. The positive clones with 9 CRE sequences were identified by colony PCR using primers 7 and 8 for obtaining an expected 428-bp fragment. Eight plasmids isolated from positive clones were sequenced using the sequencing primer 5′-GGC CTT TTG CTC ACA TGT TC-3′. The plasmids containing 9 CRE sequences in the correct direction were named pX-9CRE-d2EYFP.

GPCR reporter gene assay vector pGPCR-9CRE-d2EYFP

Any GPCR gene can be inserted into pX-9CRE-d2EYFP through the two cloning sites of FseI and AscI. The open reading frames of GPCR genes were amplified using the template plasmids and the forward and reverse primer pairs 11 and12 (human type I pituitary adenylate cyclase-activating polypeptide receptor, hPAC1), 13 and 14 (vasoactive intestinal peptide receptor 1, VPAC1), 15 and 16 (VPAC2), and 17 and 18 (MC4R) listed in Table S1. Each primer pair was designed to amplify the corresponding GPCR gene and to introduce FseI and AscI recognition sites at the 5′ and 3′ ends, respectively. The correct GPCR sequences as listed with the GenBank accession number in Table S1 were validated through sequencing.

Monoclonal cell lines

The adherent HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) or Ham's nutrient mixture F-12 supplemented with heat-inactivated fetal bovine serum (FBS, 10%, v/v), penicillin (100 U/ml), and streptomycin (100 μg/ml) in a humidified carbon dioxide atmosphere (5% CO_2) at 37 °C. The