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Method for rapid optimization of recombinant GPCR protein expression and stability using virus-like particles

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ABSTRACT

Recent innovative approaches to stabilize and crystallize GPCRs have resulted in an unprecedented breakthrough in GPCR crystal structures as well as application of the purified receptor protein in biophysical and biochemical ligand binding assays. However, the protein optimization process to enable these technologies is lengthy and requires iterative overexpression, solubilization, purification and functional analysis of tens to hundreds of protein variants. Here, we report a new and versatile method to screen in parallel hundreds of GPCR variants in HEK293 produced virus-like particles (VLPs) for protein yield, stability, functionality and ligand binding. This approach reduces the time and resources during GPCR construct optimization by eliminating lengthy protein solubilization and purification steps and by its adaptability to many binding assay formats (label or label-free detection). We exemplified the robustness of our VLP method by screening 210 GALR3-VLP variants in a radiometric agonist-based binding assay and a subset of 88 variants in a label-free antagonist-based assay. The resulting GALR3 agonist or antagonist stabilizing variants were then further used for recombinant protein expression in transfected insect cells. The final purified protein variants were successfully immobilized on a biosensor chip and used in a surface plasmon resonance binding assay.

1. Introduction

G-protein-coupled receptors (GPCRs) are seven-transmembrane domain receptors that mediate signal transduction initiating downstream cell signaling events triggered by a variety of small molecules, hormones, peptides and neurotransmitters. GPCRs regulate many biological processes and are linked to a wide range of disease areas [1]. As a result, the GPCR superfamily is the largest family of drug targets in the human body with an estimated 30–50% of marketed drugs acting directly on GPCRs or through GPCR-associated mechanisms [2]. Novel small-molecule hit identification has traditionally relied on screening ligands against GPCR overexpressing heterologous cells or homogenized cell-membrane preparations. No biophysical or biochemical assays that use purified protein material were readily available because of the lack of tools to keep GPCRs functionally intact and stable outside the cell membrane. In the past few years however, considerable progress has been made in the development of innovative approaches to

Abbreviations: A2A, Adenosine A2A Receptor; eGFP, enhanced Green Fluorescent Protein; ESI, Electrospray Ionization; GALR3, Galanin Receptor Type 3; GPCR, G-Protein Coupled Receptor; HA, Hemagglutinin; HEK, Human Embryonic Kidney; MS, Mass Spectrometry; SEC/LC-MS, Size-Exclusion Chromatography/Liquid Chromatography Mass Spectroscopy; SPR, Surface Plasmon Resonance; VLP, Virus-Like Particle; WT, Wild-Type.

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express and purify these receptors in low milligram quantities [3,4].

Despite the great progress made, the process of protein engineering to overexpress and stabilize GPCRs outside of the cell membrane is tedious, costly and time-consuming. It is complicated by low protein yields and a mixed population of various conformational and functional forms of the native form of the receptor. Although this conformational flexibility is necessary for receptor coupling to multiple effectors and signaling, it hampers the formation of well-ordered crystals or the biophysical analysis of a specific functional form of the receptor. To reduce some of the structural motions, putative flexible regions have been deleted, mutations have been added and/or soluble proteins of about 15–20 kDa have been fused to promote crystal contacts and stabilization [3,4]. Published processes to attain the most appropriate protein construct for biochemical, biophysical and/or structural studies can be lengthy and intricate, depending on the GPCR, and typically includes iterative recombinant overexpression, purification and analysis of detergent-solubilized protein variants [3,4].

Here, we propose a new technology platform to express and characterize GPCRs constituted *in situ* in VLPs produced from HEK293 cell cultures. VLPs are multiprotein nanostructures assembled from viral structural proteins and contain a cellular lipid bilayer necessary to maintain the structural integrity of a GPCR. VLPs containing membrane or non-membrane proteins have been used for diverse applications in vaccination, targeted drug delivery, gene therapy and immune therapy [5]. The chemically stabilizing environment of the phospholipid bilayer in a VLP has also enabled *in vitro* biophysical assay development for GPCRs such as surface plasmon resonance to study antibody binding [6,7], confocal fluorescence microscopy for high-throughput ligand screening [8] and NMR to study ligand interactions with viral membrane proteins [9]. In addition, GPCR-VLPs aided in the selection of stabilizing mutants for the agonist-bound free-fatty acid receptor 1 using a label-free binding assay [10]. In our study, we demonstrate that GPCRs in VLPs are properly folded, functionally active and can be used to study receptor protein behavior through a wide array of biochemical and biophysical technologies without the need for time consuming protein purification or detergent-solubilization. Most importantly, we provide experimental evidence that GPCR-VLP protein characteristics are similar to detergent solubilized and/or purified protein from baculovirus mediated insect cell expression or from other homogenized membrane preparations thereby making it a useful pre-screen to predict protein quality. Selection of the most appropriate detection method to study GPCR-VLP protein characteristics or to perform a ligand binding screen will depend on particular project needs and objectives, but the potential applications are numerous. Several examples are provided herein demonstrating the versatility of this technology and its applications in GPCR drug discovery and structural studies.

2. Materials and methods

2.1. VLP production and analysis

2.1.1. DNA plasmid generation

The native DNA sequence coding for the 502 amino acids of HIV-1SF2 p55Gag (GenBank accession no. K02007) was codon-optimized for mammalian cell expression (DNA 2.0, Neward, CA, USA). The resulting modified HIV-1SF2 gag encoded a p55Gag protein with three amino acid changes (Asn377Thr, Ile403Thr, and Lys405Arg); the resulting amino acid sequence conformed to the sequences for other HIV-1 subtype B Gag proteins in the HIV sequence database (Los Alamos National Laboratory (GenBank accession no. AF201927)). The gene encoding for HIV-1SF2 p55Gag was subcloned into pcDNA™ 3.3-TOPO® vector (Thermo Fisher Scientific, Waltham, MA, USA) which employs the cytomegalovirus (CMV) immediate-early enhancer/promoter resulting in the plasmid pCMV-Gag (Fig. S1). The DNA sequence coding for the full-length protein of the human Galanin Receptor Type 3 (GALR3) (2-368) and flanked by a hemagglutinin (HA) signal sequence/FLAG tag at the N-terminus and a Tobacco Etch Virus (TEV)-10xHis tag at the C-terminus was codon-optimized for mammalian cell expression and subcloned into the pEF6/V5-His TOPO vector (Thermo Fisher Scientific, Waltham, MA, USA). The resulting plasmid is called pEF6-GALR3 (Fig. S1). The DNA sequence encoding the Adenosine A2A receptor (A2A) was codon-optimized for mammalian cells expression (DNA 2.0, Neward, CA, USA) and subcloned into pcDNA™ 3.3-TOPO® vector (Thermo Fisher Scientific, Waltham, MA, USA). A HA signal sequence/FLAG N-terminal tag and a TEV-eGFPA206K-8xhis C-terminal tag were added and the resulting plasmid is called pCMV-A2A (Fig. S1). All subsequent A2A constructs contain the same bordering sequences in addition to the coding sequences for the A2A (with/without fusions partners) as reported in Jokoola et al. and Liu et al. [11,12]. A2A and GALR3 point mutants were generated using site-directed mutagenesis.

2.1.2. VLP generation

DNA encoding T4-Lysozyme or A23–L128 of apocytochrome b562RIL (BRIL, PDB: 1M6T) were synthesized as gBlocks (IDT, San Diego, CA, USA) and inserted into A2A constructs using Gibson assembly (NEB, Waltham, MA, USA). Plasmid DNA for mammalian cell transfection was prepared using PureLink HiPure Plasmid Midiprep Kits (Thermo Fisher Scientific, Waltham, MA, USA). 15 ml of a 293FT cell line (Thermo Fisher Scientific, Waltham, MA, USA) suspension culture of 2.0×10^6 cells/ml was infected with 9 μg of pEF6-GALR3 or pCMV-A2A plasmid and 3 μg of pCMV-Gag plasmid using X-TremeGENE HP transfection reagent (Roche), according to manufacturer's instructions. Following transfection, supernatants were harvested 48 h later and clarified by centrifugation at 3000 rpm for 10 min at 4 °C. The supernatants were treated with MembranePro precipitation reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's instructions. The final VLP pellets were suspended in 50 μl of 1× Phosphate buffered saline (PBS), 25–30% Glycerol, 1× Protease Inhibitor (Roche). Samples were frozen at −80 °C until further use.

2.1.3. A2A-eGFP VLP fluorescence measurement

50 μl wild-type (WT) or mutant A2A-VLP were centrifuged, washed once with 1xPBS buffer and then resuspended back into 50 μl 1xPBS. VLP samples were loaded onto a 96-well plate for GFP fluorescence measurement (Perkin Elmer EnVision 2104 Multilabel Reader). The same sample was recovered from each well and applied to a bicinchoninic acid assay (BCA) assay to determine the total protein concentration of the individual VLPs (Pierce™ BCA Protein Assay Kit). The expression level of A2A was calculated as GFP Relative Fluorescence Units (RFU) per ng VLP.

2.2. Radioligand binding assays

2.2.1. Materials

Porcine galanin (GWTLNSAGYLLGPHAIDNHRSFHDKYGLA-NH2) was synthesized by Abgent (WuXi AppTec). Spexin NPQ (cat# 023-81, lot# 430195) was purchased from Phoenix Pharmaceuticals. SNAP 37889 was synthesized at Dart Neuroscience, LLC. [^{125}I]-porcine galanin (cat# NEX243) was obtained from Perkin Elmer. ZM241385 (cat # 1036) was purchased from Tocris Bioscience and [^{3}H]-ZM241385 (cat# ART0884) was obtained from American Radiolabeled Chemicals.