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G Protein-Coupled Receptors in Drug Discovery

Methods and Protocols

Second Edition

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Second Edition

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Preface

G protein-coupled receptors (GPCRs) are one of the largest classes of druggable targets in the human genome. Owing to remarkable recent advances in the structural, biophysical, and biochemical analyses of these receptors, as well as a growing body of evidence hinting at the possible relevance of allosteric modulators, biased agonists, and oligomer-selective ligands as improved therapeutic agents, drug discovery for GPCRs has recently taken a completely new direction.

This book provides an overview of recent techniques employed in the field of GPCRs to screen for new drugs and to derive information about their receptor structure, dynamics, and function for the purpose of developing improved therapeutics. Experts in the field have been contacted to contribute their protocols and views on the impact of these methodologies on modern drug discovery. The book targets a diverse audience from structural and molecular biologists to pharmacologists and drug designers.

New York, NY, USA ***Marta Filizola***

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Chapter 1

Purification of Stabilized GPCRs for Structural and Biophysical Analyses

James C. Errey, Andrew S. Doré, Andrei Zhukov, Fiona H. Marshall, and Robert M. Cooke

Abstract

G protein-coupled receptors (GPCRs) are of particular importance for drug discovery, being the targets of many existing drugs, and being linked to many diseases where new therapies are required. However, as integral membrane proteins, they are generally unstable when removed from their membrane environment, precluding them from the wide range of structural and biophysical techniques which can be applied to soluble proteins such as kinases. Through the use of protein engineering methods, mutations can be identified which both increase the thermostability of GPCRs when purified in detergent, as well as biasing the receptor toward a specific physiologically relevant conformational state. The resultant stabilized receptor (known as a StaR) can be purified in multiple-milligram quantities, whilst retaining correct folding, thus enabling the generation of reagents suitable for a broad range of structural and biophysical studies. Example protocols for the purification of StaR proteins for analysis, ligand screening with the thiol-specific fluorochrome *N*-[4-(7-diethylamino-4-methyl-3-coumarinyl)phenyl]maleimide (CPM), surface plasmon resonance (SPR), and crystallization for structural studies are presented.

Key words GPCR, LCP, SPR, Purification, Membrane protein, G protein, Thermostabilization, StaR, Crystallization

1 Introduction

The biophysical analysis of proteins relies on generating multiple-milligram quantities of pure, correctly folded material. For many years, the lack of suitable protein has represented a major barrier in structural and biophysical analyses of integral membrane proteins such as G protein-coupled receptors (GPCRs). The determination of the structure of rhodopsin in 2000 [1] provided evidence that, when a stable and well-expressed receptor is available, structure determination can be achieved. The development of thermostabilization approaches for GPCRs [2] allows for the creation of receptors that have sufficient stability to allow their purification for a range of biophysical analyses. This has the additional benefit of

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producing proteins that are locked in a specific conformational state, with pharmacology representative of that state [3]. StaR proteins are valuable reagents and can be used for a wide range of biophysical techniques including compound screening [4], kinetic profiling [5], and structural studies [6]. In addition, StaR proteins can be used as antigens for raising conformation-sensitive antibodies [7]. To date stabilized receptors have been generated across the three main GPCR superfamilies, with X-ray structures reported for members from Family A [6, 8–10], Family B [11], and Family C [12].

1.1 Ligand Screening by CPM

Purification of GPCRs is usually performed in the presence of ligands. This is required to add stability to the receptor if this has not been achieved through protein engineering. Even if the receptor has already been stabilized, the additional stability and reduction in entropy provided by the ligand can be beneficial. For membrane proteins such as GPCRs, stability measurements can be measured using a thiol-specific fluorochrome *N*-[4-(7-diethylamino-4-methyl-3-coumarinyl)phenyl]maleimide (CPM) assay [13]. The CPM assay measures the unfolding of the receptor as a function of heating through the exposure of buried Cys residues that the CPM dye can bind to. This is achieved using a purified protein sample that is either ligand-free or contains a low-affinity ligand that can be displaced easily. Like other fluorescence assays, this one can be significantly affected by changes in pH, not only in the ability for the CPM dye to bind to the Cys residues but also in the fluorescence response.

1.2 Surface Plasmon Resonance Assays of GPCRs

Surface plasmon resonance (SPR) is a direct binding technique offering a possibility of real-time label-free monitoring of protein–protein and protein–small molecule interactions. It is widely used at different steps of the drug discovery process from hit identification (e.g. fragment screening) to lead characterization and optimization. Its advantage over endpoint assays such as radioligand binding is that, besides the affinity data, it provides information about interaction kinetics (association and dissociation rates) which are increasingly of interest for correlating with the physiological behaviors of compounds [14]. SPR requires the target protein to be purified to homogeneity, in a form which maintains binding activity for the duration of the experiment, which has proved problematic for GPCRs. Hence, until the advent of stabilization approaches, SPR studies of GPCRs have been limited to very few examples [15, 16]. The development of StaR technology for GPCR stabilization has enabled many more receptors to be suitable for SPR investigations [17], including biophysical mapping of ligand binding sites [18], fragment screening [4], and kinetics of compound binding [5].

The choice of the buffer and detergent for SPR experiments is largely governed by the inherent stability of the receptor, therefore