Assessing GPCR activation using protein complementation: a novel technique for HTS

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Abstract

GPCRs (G-protein-coupled receptors) are critical targets in drug discovery. Although most HTS (high-throughput screening) assays are routinely used to identify functional agonism or antagonism, they are sub-optimal as methods to screen for modulators of other, novel, aspects of GPCR function. Indeed, it is now evident that GPCRs are highly complex proteins that interact with RAMPs (receptor-activity-modifying partners), β-arrestins, G-proteins, as well as functioning in potential homo- or hetero-meric complexes. Consequently, novel HTS technologies are now required that would facilitate interrogation of GPCRs in terms of their cellular protein–protein interactions. One approach is oligomerization-assisted complementation of monomeric protein fragments and detection of fragment reassembly. Notably, the use of enzymes has advantages in this regard, since complementation results in catalytically competent protein. The assay signal generated in this fashion results in assays of high sensitivity, thereby enabling protocols to be developed in HTS systems that require extremely low fluid volumes. The use of complementing proteins that generate a luminescent signal also provides assays that are markedly free from artefactual interferences.

HTS (high-throughput screening) of GPCRs (G-protein-coupled receptors)

Historically, evaluation of large numbers of compounds at GPCRs employs highly automated liquid-dispensing systems, accompanied by detection instruments that rapidly and sensitively detect signals generated in radiometric, fluorometric or luminescent assays [1]. Such HTS assays generally use techniques that measure second messenger accumulation, and have been widely adopted by the pharmaceutical industry owing to their simplicity and robustness [1]. However, as knowledge of the complexity of GPCR function increases, particularly when studied in a cellular environment, it becomes clear that novel approaches are needed to identify molecules that modulate the GPCR activity. Consequently, HTS approaches are now required to detect inverse agonists, allosteric regulators, as well as compounds that modulate GPCR function in a manner that is specific to a defined cellular pathway. A detailed review of this subject can be found in a recent paper by Eglen et al. [2]

Classically, the stoichiometry of GPCR–G-protein coupling is considered to be 1:1. However, they may also function in assemblies, by formation of dimers, or even higher-order assemblies [3]. Although the functional significance of this phenomenon is unclear, and controversial, it does suggest a means by which GPCRs exhibit tissue-dependent pharmacologies [3]. GPCRs also interact with a range of proteins with roles in membrane trafficking, signalling, desensitization, internalization and recycling [3]. Such ancillary proteins include GRKs (GPCR kinases), second-messenger-dependent kinases, arrestins, molecular chaperones that promote protein folding and insertion at the plasma membrane, as well as RAMPs (receptor-activity-modifying proteins) and PDZ domain-containing proteins [3]. This diversity of cellular interactions has important implications for drug discovery at GPCRs in general, and the development of HTS assays in particular. A related consideration is that assessment of these phenomena requires functional interrogation of GPCR activity, i.e. analysis in a cellular context. Consequently, the widespread adoption of cell-based assays for GPCR screening has driven the development of many new techniques for GPCR HTS assays, as well as an improved understanding that the function of GPCRs, and thus the pharmacology of novel ligands, is best evaluated in a cellular context [2]. Currently, it is a widely held assumption that such assays will promote better assessment of ligand pharmacologies and thus provide better leads in drug discovery programmes.

Protein complementation assays and GPCR screening

A promising approach developed to assess the function of GPCR–protein interaction in a cell is that of oligomerization-assisted complementation of monomeric protein fragments and detection of fragment reassembly or protein complementation, i.e. it will be referred to below [4]. In the last decade or so, protein complementation has been developed rapidly in the area of GPCR screening assays. This is due to a range of inherent advantages that facilitate the development of cell-based...
assays for HTS. This article provides an overview of the uses of protein complementation in GPCR screening and highlights those assays which have found most widespread use, i.e. BRET (bioluminescence resonance energy transfer), FRET (fluorescence resonance energy transfer) and EFC (enzyme fragment complementation) assays [5–7]. An excellent review of this area has also been published recently [3].

Generally, two-hybrid protein complementation assays have been limited to protein interactions in the nucleus. Consequently, interactions that involve proteins anchored at the plasma membrane, such as GPCRs, have been inaccessible to this approach. This caveat was resolved by extending the two-hybrid approach to protein complementation assays [4]. Initially, implemented using a split ubiquitin technique, the approach was subsequently extended to other proteins, including enzymes. To monitor protein interactions in living cells, several protein complementation assays with direct read-outs have been developed, and several employ a reporter protein split into two fragments and genetically fused to the interaction proteins. Upon the protein–protein interaction, the reporter proteins complement so that the function of the fragmented protein is restored [4]. Enzyme reporter proteins such as β-galactosidase, DHFR (dihydrofolate reductase) or lactamase have been utilized, all of which can convert chromogenic or fluorogenic substrates. Luminescent signals can be generated where the enzyme reporter is either luciferase or β-galactosidase. The use of enzyme complementation notably has the advantage that the signal is generated catalytically, and thus the assay can exhibit high sensitivity [4]. However, as split enzyme-based reporters require substrate incubation, HTS assays often require optimization with respect to concentration and incubation time, each of which depends upon the protein under investigation. Moreover, the substrate can be a source of background signal, which can also compromise assay sensitivity [6].

Consequently, for assays where rapid detection of interactions is required, particularly for assays with low background signals, complementation assays with direct read-outs were developed using split FPs (fluorescent proteins). Here the signal can be detected by microscopy and scanning spectroscopy instruments. These FRET-based assays can be limited by a high background of cellular autofluorescence, as well as by the direct excitation of the fluorescence acceptor [8]. Some of these drawbacks are avoided by BRET assays, which makes use of bioluminescence as the ‘energy donor’, typically with Renilla luciferase as the BRET donor and FPs as the acceptors. BRET has been widely used to study oligomerization of GPCRs often in assay protocols suitable for HTS, particularly when used in conjunction with sensitive detectors [9]. The use of BRET as a means to characterize GPCR oligomerization is controversial, with at least one group recently proposing that a more “rigorous” treatment of BRET data is needed in order to distinguish “random” (non-specific) from true oligomeric protein interactions” [10]. A discussion of these contrasting views can be found in [11].

BRET has also been widely used as an assay format to study the interaction of GPCRs with other cellular proteins, including interaction of the GPCRs with arrestins or with GRKs. The measurement of the GPCR–β-arrestin interaction was developed as a universal assay to assess GPCR activation. Until recently, this interaction was studied using confocal imaging techniques that measured the redistribution of β-arrestin recombinantly fused to an FP [12]. However, a mutated β-arrestin has now been employed in assays in order to sustain the interaction for both Family A and Family B GPCRs, thereby prolonging the BRET assay signal [13].

At least two other assay formats have been developed to measure GPCR–β-arrestin interactions via protein complementation. One of these measures the association by means of β-galactosidase fragments, one of which is a small peptide derived from the α peptide and which is fused to the C-terminus of the GPCR. The second fragment is fused to the β-arrestin [14,15]. This EFC format allows for a homogenous assay to be conducted in a microtitre plate format, and, upon GPCR activation, generates a luminescent signal [15]. Although little information has been published to date comparing the pharmacology of this approach with that observed using classical second messenger accumulation assays, it appears to be able to accommodate a large range of GPCRs. A third method to assess GPCR–β-arrestin association consequent to GPCR activation involves complementation of FP fragments [16]. This approach measures the redistribution of the GPCR–β-arrestin complex either by using a plate reader or by using confocal microscopy. The latter permits assessment of interactions occurring at a cellular or subcellular level [16]. This approach contrasts with BRET and EFC approaches in which the spatial resolution is limited, particularly at the subcellular level [3]. This format also has the advantage that other GPCR–protein interactions can be analysed, such as the interaction between the receptor and RAMPs as well as GRKs [17].

Summary

Many protein complementation techniques appear to be able to provide generic homogenous assays for GPCR HTS and are consequently now entering mainstream use in several HTS campaigns. However, several differences are emerging between the pharmacology of GPCRs characterized by this approach in comparison with classical second messenger accumulation assays [18]. These disparities may relate to a low ‘receptor reserve’ associated with the modified β-arrestin response, in comparison with assays that measure the accumulation of cAMP. As a result, it is possible that protein complementation assays aimed at assessing GPCR–β-arrestin may detect only those agonists of high efficacy [18]. It is probable that both assays measuring GPCR–β-arrestin interaction as well as second messenger accumulation could be undertaken in the same cell, allowing ‘multiplexed’ assays to be developed, thereby enabling direct comparison of ligand pharmacologies to be made. Consequently, it is perhaps surprising that few reports have been published comparing the pharmacology of ligands assessed using complementation approaches with those of classical second messenger accumulation assays.
Collectively, the potential for completely novel approaches for GPCR assays to be developed using BRET/FRET techniques in general and protein complementation in particular now exists. These techniques should enable assays in which compounds can be assessed for actions at perturbing the GPCR–G-protein interface, as well as the interaction of the GPCR with other proteins such as GRKs or ion channels. Moreover, these assays are likely to be suitable for automated screening systems using either high-throughput microtitre plate readers or confocal imaging instruments. Nonetheless, emerging data suggest that care needs to be exercised in terms of the pharmacology of the data obtained, so that one has a good understanding of the apparent potency and efficacy of the compounds estimated in the assay and consequently the validity of the leads obtained.

References

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