An RGS Protein HTS Assay Strategy using the Transcreener™ GDP Assay

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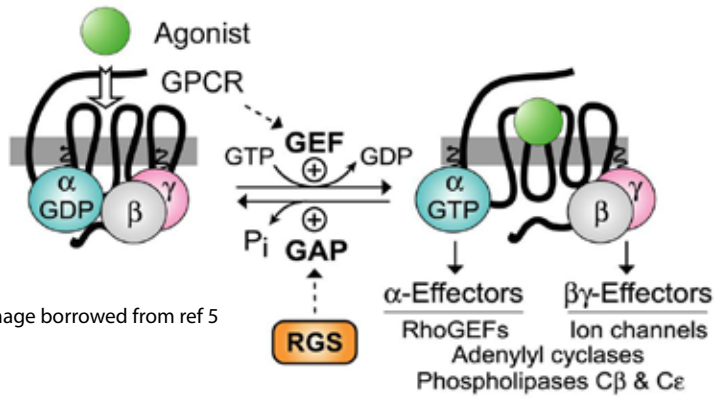
Introduction

GPCRs are by far the most extensively validated class of therapeutic targets, and there remains tremendous potential for targeting new receptors and their downstream effectors [1]. There are over 900 distinct GPCRs encoded in the human genome and over half of existing drugs are GPCR ligands, yet the total number of receptors that they target is less than 30 [2]. The proven clinical utility of modulating GPCR signal transduction has sustained formidable efforts in the pharmaceutical industry to identify new GPCR-ligand pairs that control clinically relevant signaling pathways [3, 4], as well as equally vigorous efforts to discover further components of the GPCR signaling machinery that have the potential to become therapeutic targets. RGS proteins are important components of GPCR signaling regulation.

We have developed a homogenous, fluorescence polarization-based immunodetection assay for GDP which has the potential to serve as a biochemical screening method for identifying RGS modulators. Gα GTPase kinetics make this approach problematic because their GDP dissociation rates are significantly slower than their intrinsic GTP hydrolysis rates. Here, a potential solution to this problem has been explored by increasing GDP dissociation rates and decreasing hydrolysis rates via mutation of the model protein, $G\alpha_{i1}$, with a goal of achieving $k_{off\ (GDP)} / k_{cat\ (GTPase)} \geq 5$. Several $G\alpha_{i1}$ mutants have been constructed and characterized. Some of these demonstrate kinetics amenable for use in an HTS assay format using GDP detection designed to monitor GTPase and GAP activity. Here we present the strategy and initial results for the development of an HTS RGS assay relying on detection of changes in Gα GTPase rates.

Figure 1

RGS Proteins as GTPase-accelerating Proteins



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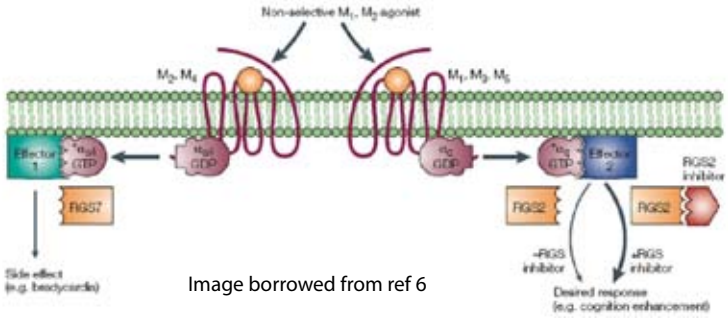
Agonist activation of the GPCR results in conformational changes transmitted to the Gαβγ heterotrimer resulting in the release of GDP from Gα and subsequent exchange for GTP (thus serving as guanine nucleotide exchange factors, or GEFs), release of Gβγ, and signal commencement [5]. Activated Gα•GTP and liberated Gβγ modulate the activity of several downstream effectors responsible for cellular responses to extracellular ligands. The intrinsic GTPase activity of the Gα subunit hydrolyzes bound GTP to GDP, resulting in reassociation of the G-protein heterotrimer. However, the intrinsic GTPase activity of the Gα subunit is generally insufficient to correlate with physiological rates of G-Protein inactivation. RGS proteins catalytically facilitate signal termination by increasing the intrinsic GTP hydrolysis rate of Gα•GTP, thus serving as GTPase-accelerating proteins (GAPs).

Figure 2.

RGS Proteins as Targets for Drug Discovery

There are over 30 mammalian RGS proteins divided into several subfamilies based on sequence homology and the presence of familiar structure domains. All RGS proteins contain an ~120 amino acid RGS domain which mediates binding to Gαproteins. RGS proteins have selectivity for different GPCR mediated signaling pathways which makes them attractive drug targets. An example is shown in figure 2.



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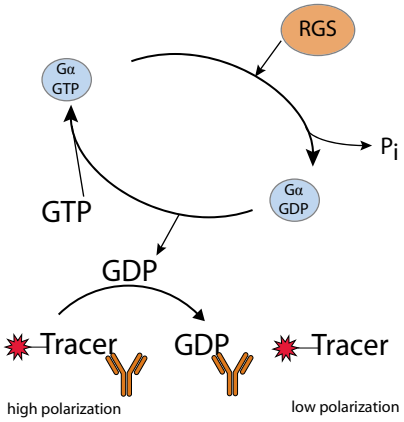
Non-selective muscarinic agonists activate both classes of M receptors leading to side effects such as bradycardia. An inhibitor of the Gq-selective RGS2 could selectively potentiate the cognition enhancing effects of cholinergic agonists and cholinesterase inhibitors in the treatment of Alzheimers disease [6].

Figure 3.

Transcreener™ GDP Assay

Use of Transcreener GDP Assay to monitor RGS GAP activity by immuno-detection of GDP generation in HTS assay format.



The Transcreener GDP Detection Mixture comprises an GDP Alexa633 tracer bound to an GDP antibody, which is displaced by GDP, the product generated by the GTPase activity of Gα . The displaced tracer freely rotates leading to a decrease in fluorescence polarization, relative to the bound tracer. Therefore, GDP production creates a concommitant decrease in polarization values. Normally, GTP dissociates from isolated Gα proteins very slowly, so RGS GAP effects are not detectable using steady state GTPase measurements. To overcome this, we are mutating Gα so that GDP dissociation is rapid enough for detection.

Advantages of using a Transcreener GDP assay for steady state GTPase activity:

- Overcomes the cumbersome protocol of a radioactive GTPase and RGS GAP assay and obviates the need to reconstitute recombinant GPCRs and Gα subunits for steady state GTPase measurements.
- Ratiometric FP-based immunodetection of GDP with a far red tracer overcomes the signal-to-noise limitations of phosphate detection and minimizes compound interference.
- Enables detection of both allosteric and competitive RGS inhibitors.
- Homogenous "mix-and-read" assay fits ideally into an automated HTS infrastructure.

Development of an HTS Assay to Measure RGS GAP Activity

Development of an HTS assay for RGS GAP activity measuring steady state Gα GTPase activity with a fluorescence polarization immunoassay (FPIA) for GDP.

- To allow detection of RGS GAP activity using steady state GTPase assays, we will alter the relative rates of GTP hydrolysis and GDP dissociation of human $G\alpha_{i1}$ protein using a combination of mutagenesis and optimization of extrinsic conditions.
- The goal is to increase $k_{off\ (GDP)}$ / $k_{cat\ (GTPase)}$ from the wild type level of 0.03 to at least 5 to allow steady state enzymatic assays for monitoring changes in GTPase activity by eliminating GDP dissociation as the rate limiting step.
- Our strategy has been guided by well-characterized mutant variants (Table 1) of the $G\alpha_{i1}$ protein and other Gα proteins that have significantly different GTPase and GDP dissociation rates *without affecting functional interaction of* $G\alpha_{i1}$ *with RGS proteins* [7, 8].
- Initial characterization will be done via a steady state Transcreener GDP Assay followed by thorough characterization of GDP dissociation and GTPase rates using well established radioassays.

Table 1.

Mutations that will be tested for altering $G\alpha_{i1}$ GTP hydrolysis and GDP dissociation rates

Mutation	Gα protein mutated	Decrease in $k_{cat\ (GTPase)}$	Increase in $k_{off\ (GDP)}$	Equivalent Site in $G\alpha_{i1}$
A326S [7]	$G\alpha_{i1}$	none	25x	A326
D55G/G56S [9]	$G\alpha_{t}$	none	10x	A50/G60
R144A [10]	$G\alpha_{i1}$	ND	5x	R144
C325A [11]	$G\alpha_{o}$	ND	10x (Kd)	C325
R178C [12]	$G\alpha_{i1}$	>100x	Reported (not quantified)	R178
T182A [13]	$G\alpha_{i2}$	>100x	2x	T181

Figure 4.

10 μM GTP/GDP Standard Curve

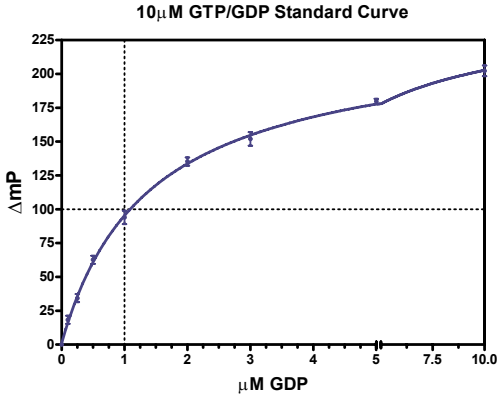
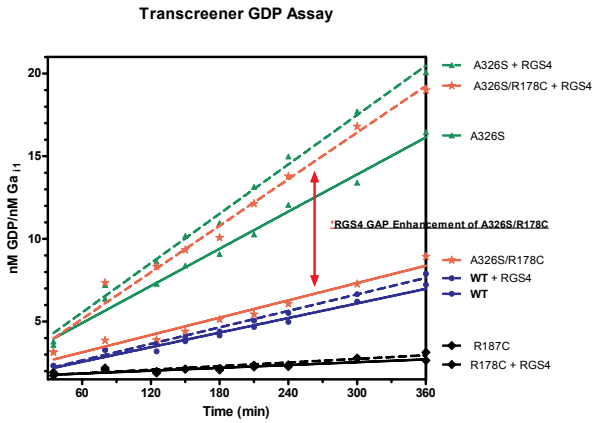


Figure 4 Transcreener GDP Assay data plotted from an 10 μM GTP/GDP standard curve performed concurrently with reactions from Figure 5 using the same assay conditions. This mimics a typical enzyme reaction (as GDP is produced, GTP is depleted) by keeping the total nucleotide concentration constant. This data can be used to quanitfy product formation. ΔmP = mP $[GDP]_{zero}$ - mP $[GDP]_{\mu M}$.

Figure 5.

Proof of concept for HTS RGS GAP Assay



100nM $G\alpha_{i1}$ wild type (blue) $G\alpha_{i1}$ A326S Dissociation mutant (green), $G\alpha_{i1}$ R178C GTPase mutant (black), or $G\alpha_{i1}$ A326S/R178C Double Mutant (Red) protein was added to reaction mix (20 mM Tris 7.5 pH, 1 mM EDTA, 10 mM $MgCl_2$, 10 μM GTP, 40 μg/mL GDP Ab, 2 nM GDP Tracer) with(solid line) or without (dashed line) 2 μM RGS4 in a 384 well plate and incubated at 30C. FP was measured on a Tecan Ultra-Ex/Em 612/670. mP data was converted to GDP produced using the standard curve (fig 4).

Results demonstrate an initial proof of concept for the development of an HTS RGS GAP assay

- A326S, the "GDP-dissociation-rate-accelerating" mutation caused an increase in the observed GTPase rate compared with WT $G\alpha_{i1}$; addition of RGS4 caused a slight acceleration.
- R178C, the "intrinsic-GTPase-rate-decreasing" mutation caused a decrease in the GTPase rate relative to WT and there was no stimulation by RGS4.
- The double mutant, A326S/R178C, exhibited basal GTPase activity similar to WT which was stimulated more than 2-fold by RGS4.

Future Direction

- Develop additional $G\alpha_{i1}$ mutants in order to improve k_{off}/k_{cat}, therefore improve observable GAP activity and assay window.
- Characterize GDP dissociation rates, GTPase rates, and specific activity of the $G\alpha_{i1}$ proteins with radioassays to verify mechanism of action.
- Interrogate RGS4 with known inhibitors and a compound library using final optimized assay.

References

1 Roth, B.L. and Kroeze, W.K., Screening the receptorome yields validated molecular targets for drug discovery. Curr Pharm Des, 2006;12(14):1785-95.
2 Esbenshade, T., G Protein-Coupled Receptors as Targets for Drug Discovery. G Protein-Coupled Receptors in Drug Discovery, 2005. Taylor and Francis: p. 15-36.
3 Mertens, I., et al., Postgenomic characterization of G-protein-coupled receptors. Pharmacogenomics, 2004; 5(6):657-72.
4 Armbruster, B.N. and Roth, B.L., Mining the receptorome. J Biol Chem, 2005; 280(7):5129-32.
5 Siderovski, D.P. and Willard, F.S., The GAPs, GEFs, and GDIs of heterotrimeric G-protein alpha subunits. Int J Biol Sci. 2005;1(2):51-66.
6 Neubig, R.R. and Siderovski, D.P., Regulators of G-protein signalling as new central nervous system drug targets. Nat Rev Drug Discov, 2002; 1(3):187-97.
7 Posner, B.A., et al., The A326S mutant Gialpha1 as an approximation of the receptor-bound state. J Biol Chem, 1998; 273(34): 21752-8.
8 Berman, D.M., Wilkie, T.M. and Gilman, A.G., GAIP and RGS4 are GTPase-activating proteins for the Gi subfamily of G protein alpha subunits. Cell, 1996; 86(3): 445-52.
9 Mello, L.V., Millner, P.A., and Findlay, J.B., Biochemical characteristics of guanine nucleotide binding protein alpha-subunit recombinant protein and three mutants: investigation of a domain motion involved in GDP-GTP exchange. J Protein Chem, 2002;21(1):29-34.
10 Remmers, A.E., et al., Interdomain interactions regulate GDP release from heterotrimeric G proteins. Biochemistry, 1999;38(42):13795-800.
11 Thomas, T.C., Schmidt, C.J., and Neer, E.J., G-protein alpha o subunit: mutation of conserved cysteines identifies a subunit contact surface and alters GDP affinity. PNAS, 1993;90(21):10295-8.
12 Coleman, D.E., et al., Structures of active conformations of Gi alpha 1 and the mechanism of GTP hydrolysis. Science, 1994;265(5177):1405-12.
13 Nishina, H., et al., Significance of Thr182 in the nucleotide-exchange and GTP-hydrolysis reactions of the alpha subunit of GTP-binding protein Gi2. J Biochem(Tokyo), 1995;118(5):1083-9.

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