Interrogation of Modulators of GPCR and small GTPase Activity: Screening GAPs and GEFs with the Transcreener® GDP Assay

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Overview

GTPases control diverse cellular processes by cycling between inactive GDP- and active GTP-bound conformations. Though they are clearly involved in many diseases (e.g., K-Ras in cancer) development of GTP-competitive inhibitors is extremely challenging for a number of reasons. Targeting the effector proteins that control and regulate the activation state of GTPases is a logical alternative approach. Toward this end, we have developed HTS-compatible assay methods for detection of GTPase-accelerating proteins (GAPs) and guanine nucleotide exchange factors (GEFs) based on their ability to stimulate steady state GTPase activity. The Transcreener® GDP Assay, a homogenous, fluorescent assay for direct detection of GDP is used to detect GTPase activity. Members of the "regulator of G-protein signaling" (RGS)-protein superfamily act as GAPs for heterotrimeric G-protein alpha subunits, thereby attenuating GPCR signaling. Their selectivity for specific GPCRs and downstream signaling components makes them attractive as potential therapeutic targets, with the potential to modulate the effects of existing GPCR agonists. We used kinetically altered Gα subunits of heterotrimeric G proteins in combination with the Transcreener® GDP Assay to provide a robust HTS method for identifying inhibitors of RGS protein GAP activity: from initial screen to profiling hits. Rho family GTPases control cell growth, movement and gene expression and are often misregulated in cancer pathways, especially cell migration and invasion. However Rho GTPases are infrequently mutated, and their tumorigenic functions are often mediated by overexpression of GEFs which positively regulate Rho GTPases by accelerating GDP dissociation to form the active, GTP-bound complex. We developed a mechanistically unbiased biochemical assay platform for measuring Rho GEF catalytic activity based on stimulation of steady state GDP formation, detected using the Transcreener® GDP Assay. We demonstrate detection of two DOCK family Rho GEFs, Dbl and P-Rex1 with their respective Rho GTPase substrates, RhoA, CDC42 and Rac1. A pilot screen and hit validation with an Orthogonal Pooled Screening (OPS™) library was demonstrated with Rac1 and P-Rex1 to find GEF inhibitors. These assays are agnostic with respect to whether an inhibitor binds to the GTPase or its effector protein and whether they bind at the active site or act allosterically. They are more sensitive than the traditional fluor-GTP binding assays, as they rely on multiple catalytic cycles for the GTPase readout, and should be broadly applicable to diverse GTPases and their GAPs and GEFs.

Transcreener® GDP Assay

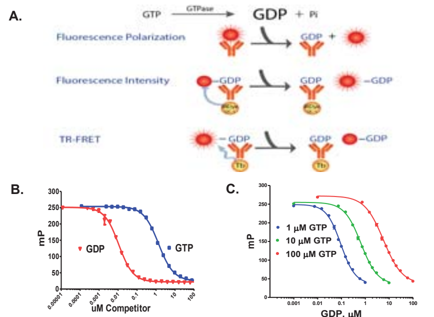


Figure 1. Selective immunodetection of GDP enables homogenous detection of GTPase activity. A. The Transcreener® GDP Assay relies on highly selective antibodies and far red fluorescent tracers to allow homogenous detection of GDP with FP, FI or TR-FRET readouts. **B.** Greater than 100-fold selectivity enables detection of GDP in the presence of excess GTP. **C.** The dynamic range of the assay can be tuned for different initial GTP concentrations.

Transcreener® RGScreen™ GAP Assay

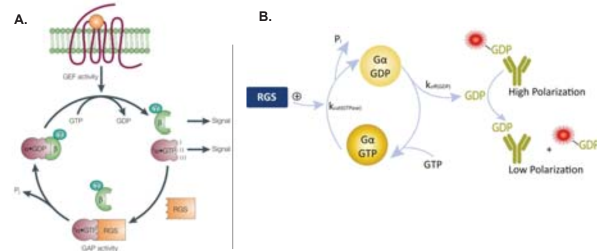


Figure 2. Coupling Kinetically Altered Gα Proteins With the Transcreener® GDP Assay Enables Direct Detection of RGS Protein GAP Activity. A) RGS proteins act catalytically on Gα-GTP complexes to stimulate GTP hydrolysis rates as much as 100-fold, thereby attenuating GPCR agonist signals. **B)** Detection of GDP using the Transcreener® GDP Assay, a competitive fluorescence polarization immunoassay, offers an HTS compatible approach for directly measuring RGS GAP effects. However GDP dissociates from isolated Gα proteins very slowly, so RGS GAP effects are not detectable using steady state GTPase assays. To overcome this kinetic limitation, we combined Gα mutations that increased GDP dissociation and decreased GTPase activity to measure RGS GAP activity.

Pilot Screen Utilizing RGScreen™

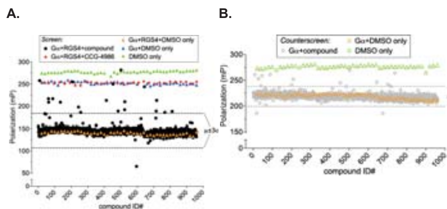
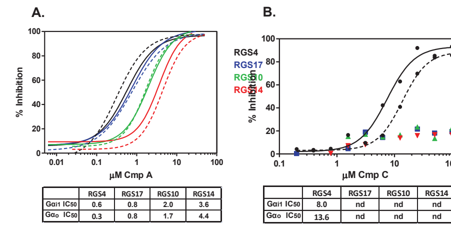


Figure 3. Pilot Screen with Doubly-Mutated (DM) $G\alpha_{i1}$ +/- RGS4. Assays were run with 50 nM $G\alpha_{i1}$ DM with (A) or without (B) 250 nM RGS4 protein and 10 µM compound, 150 µM of reactive RGS4 inhibitor CCG-4986, or DMSO controls. The Z' in **A** at 120 minutes was 0.83, which reflects only the RGS4-dependent increase in GTPase activity of the controls. The Z factor for the GenPlus library screen was 0.73. Of the 17 hits in **A**, 10 were excluded as GTPase inhibitors by **B**, thus the RGS4-specific hits rate was 0.7%.

Profiling Compounds with RGS Proteins



A.

	RGS4	RGS17	RGS10	RGS14
$G\alpha_{i1}$ IC_{50}	0.6	0.8	2.0	3.6
$G\alpha_o$ IC_{50}	0.3	0.8	1.7	4.4

B.

	RGS4	RGS17	RGS10	RGS14
$G\alpha_{i1}$ IC_{50}	8.0	nd	nd	nd
$G\alpha_o$ IC_{50}	13.6	nd	nd	nd

Figure 4. Profiling of compounds. Compounds A and C were profiled with $G\alpha_{i1}$ (solid line) and $G\alpha_o$ (dashed lines) with RGS4 (black), RGS17 (blue), RGS10 (red) and RGS14 (green). **A)** Compound A resulted in mostly similar IC_{50} values for $G\alpha_{i1}$ versus $G\alpha_o$, the exception with RGS4 where $G\alpha_o$ showed a 2 fold selectivity over $G\alpha_{i1}$. Selectivity across RGSs varied up to 7 fold. **B)** Compounds C produced a 1.7X selectivity for $G\alpha_{i1}$ over $G\alpha_o$ with RGS4, while exhibiting no selectivity for RGS17, RGS10 or RGS14.

GEF Assay Principle

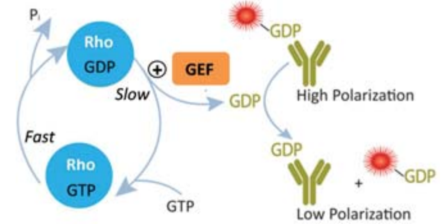
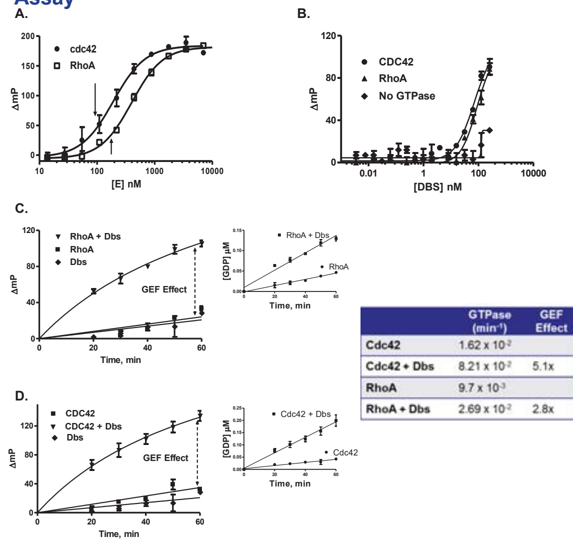


Figure 5. GEFs accelerate steady state GTP hydrolysis rates. By accelerating the rate limiting step of the GTPase catalytic cycle, GEFs enhance the steady state rates of GDP formation by GTPases, which can be detected using the Transcreener® GDP Assay.

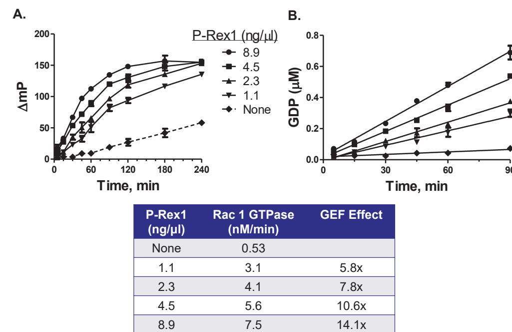
Development of GEF assays with Transcreener® GDP Assay



	GTPase (min⁻¹)	GEF Effect
Cdc42	1.62×10^{-2}	
Cdc42 + Dbs	8.21×10^{-2}	5.1x
RhoA	9.7×10^{-3}	
RhoA + Dbs	2.69×10^{-2}	2.8x

Figure 6. Development of GEF assays for Dbs/RhoA and Dbs/Cdc42. A. GTPases are first titrated to identify the concentration that produced 20% of the maximal signal: 39 nM Cdc42 and 78 nM RhoA. **B.** Titration of Dbs into limiting GTPase to determine optimal Dbs concentration (125 nM). **C, D.** Continuous assays are used to determine rates of GTPase activity in the presence and absence of Dbs and polarization data is converted to GDP formation using a standard curve as in Fig. 1C. **Table:** The ratio of rates in the presence and absence of DBS is used to determine the GEF effect.

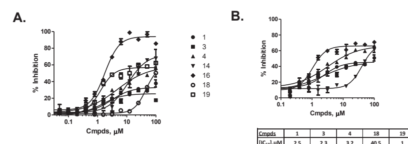
Detection of P-Rex1 GEF activity with Rac1



P-Rex1 (ng/µl)	Rac 1 GTPase (nM/min)	GEF Effect
None	0.53	
1.1	3.1	5.8x
2.3	4.1	7.8x
4.5	5.6	10.6x
8.9	7.5	14.1x

Figure 7. P-Rex1 stimulates Rac1 GTPase activity. Reactions containing a limiting concentration of Rac1 (50 nM), 10 µM GTP, and Transcreener® GDP detection reagents were titrated with P-Rex1 (DH-PH domain) and read at intervals. **A.** Time courses for P-Rex1 dependent stimulation of Rac1 GTPase activity. **B.** Polarization data from A. was converted to GDP formation using a standard curve as in Fig. 1C. **Table:** Rates of GDP formation by Rac1 and the effect of GEF stimulation.

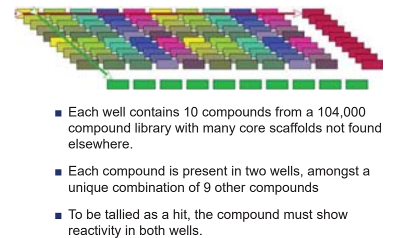
Hit Validation from P-Rex1/Rac1 OPS Screen



Cmpds	1	3	4	18	19
IC_{50} µM	2.5	2.3	3.2	40.5	1

Figure 8. Hit Validation:. A) Excluding artifacts, 7 compounds exhibited a dose response in the complete reaction. Of them, 2 were excluded as GTPase inhibitors and the 5 remaining hits are shown **B)** along with $[IC_{50}]$ values.

Orthogonal Pooled Screening (OPS™)



- Each well contains 10 compounds from a 104,000 compound library with many core scaffolds not found elsewhere.
- Each compound is present in two wells, amongst a unique combination of 9 other compounds
- To be tallied as a hit, the compound must show reactivity in both wells.

P-Rex1/Rac1 Pilot OPS™ Screen

- **6400 compounds (N=2) in 4 pre-dispensed plates**
- **Start reaction with addition of substrates**
- **Read plate after 1 hr at RT**
- **Data sent to LCGC for deconvolution**

Screen Statistics	
Z'-From DMSO Controls	0.8
Z-factor of Entire Screen	0.6
Hits > 3 Std Devs	21

Conclusions

- The Transcreener® GDP Assays can detect GAP and GEF activity of heterotrimeric Gα proteins and small GTPases
- The Transcreener® GDP Assay can be used for screening and profiling for RGS inhibitors with multiple Gα proteins
- The Transcreener® GDP-based GEF assay was demonstrated for Dbs with RhoA and Cdc42 and for P-Rex1 with Rac1
- Both GAP and GEF assays are HTS compatible

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