Engineering G alpha Proteins for Detection of RGS Protein GAP Activity with the Transcreener® GDP Assay

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Introduction

Recently, members of the "regulator of G-protein signaling" (RGS)-protein superfamily have emerged as critical modulators of specific GPCR signal transduction pathways. Via their conserved RGS domain that confers "GTPase-accelerating protein" (GAP) activity, RGS proteins attenuate GPCR signaling by deactivating heterotrimeric G-protein α subunits. The steady-state GTPase activity of isolated Gα proteins is limited by GDP dissociation, so steady-state GTP hydrolysis cannot be used to measure GAP activity in simple biochemical assays. To overcome this hurdle, we developed mutated Gα proteins with decreased GTP hydrolysis rates and increased GDP dissociation rates. The GTPase activities of the mutated Gα proteins were stimulated 6-10 fold by known interacting RGS domains, demonstrating the ability to directly measure RGS domain GAP activity using steady state GTP hydrolysis assays. Importantly, there was no stimulation of GTPase activity by non-interacting RGS domains, demonstrating that the selectivity of the Gα/RGS domain interaction was not altered by the mutations. Moreover, independent binding studies confirmed that the mutated Gα proteins interacted with RGS proteins with the expected specificity. The mutated Gα proteins and RGS domains were coupled with the homogenous, fluorescence-based Transcreener® GDP Assay to demonstrate an HTS-compatible assay system for applying a novel approach to selective modulation of GPCRs.

Mutation	Gα protein mutated	Decrease in $k_{cat\ (GTPase)}$	Increase in $k_{off\ (GDP)}$	Equivalent Site in $G\alpha_{i1}$	Mutation
A326S	$G\alpha_{i1}$	none	25x	A326	A326S
D55G/G56S	$G\alpha_t$	none	10x	A50/G60	D55G/G56S
R144A	$G\alpha_{i1}$	ND	5x	R144	R144A
R178C	$G\alpha_{i1}$	>100x	ND	R178	R178C

Table 1. Previously reported mutations that were used to develop strategy for altering $G\alpha_{i1}$ GTP hydrolysis and GDP dissociation rates. 11 single mutants and 7 double mutants were constructed, including alternative amino acid substitutions at the positions shown. Mutants were screened by the ability to detect RGS 4 GAP activity using the Transcreener GDP Assay.

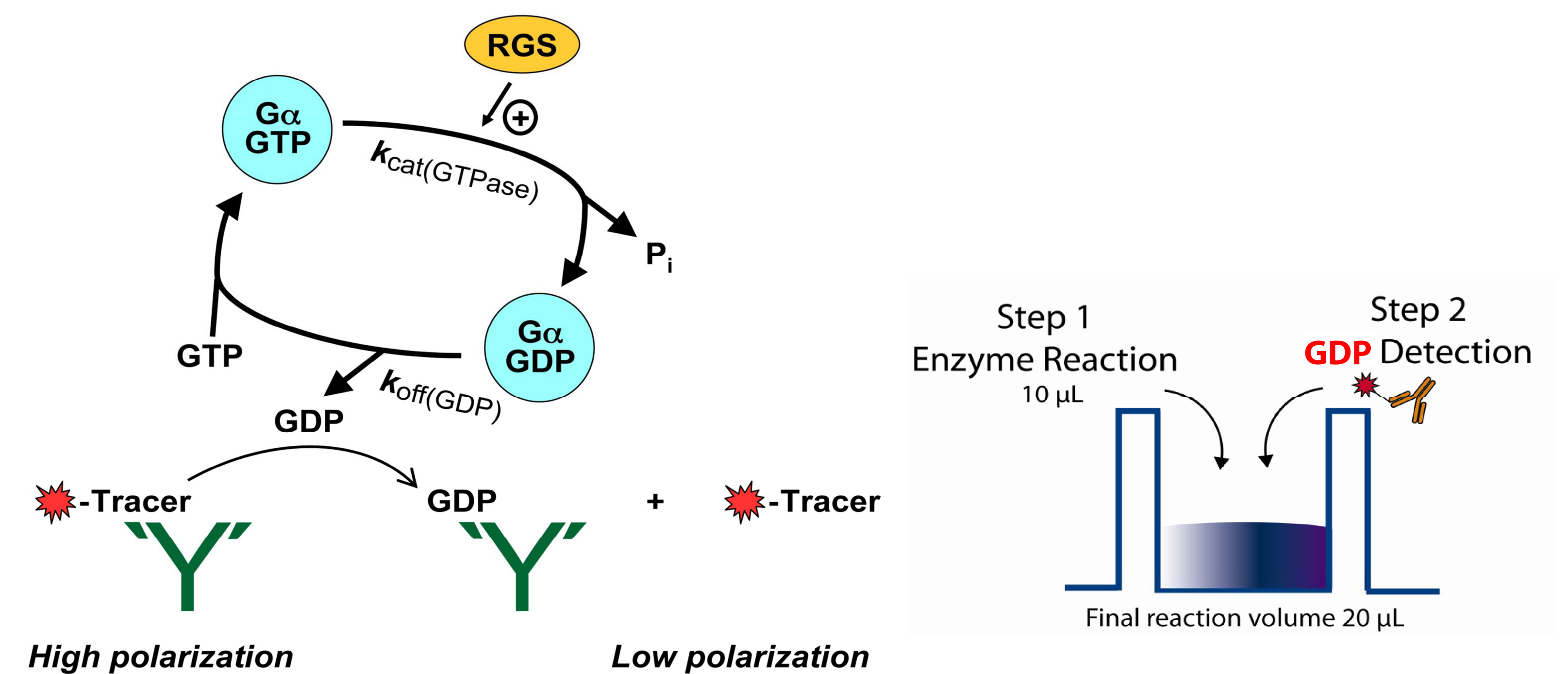
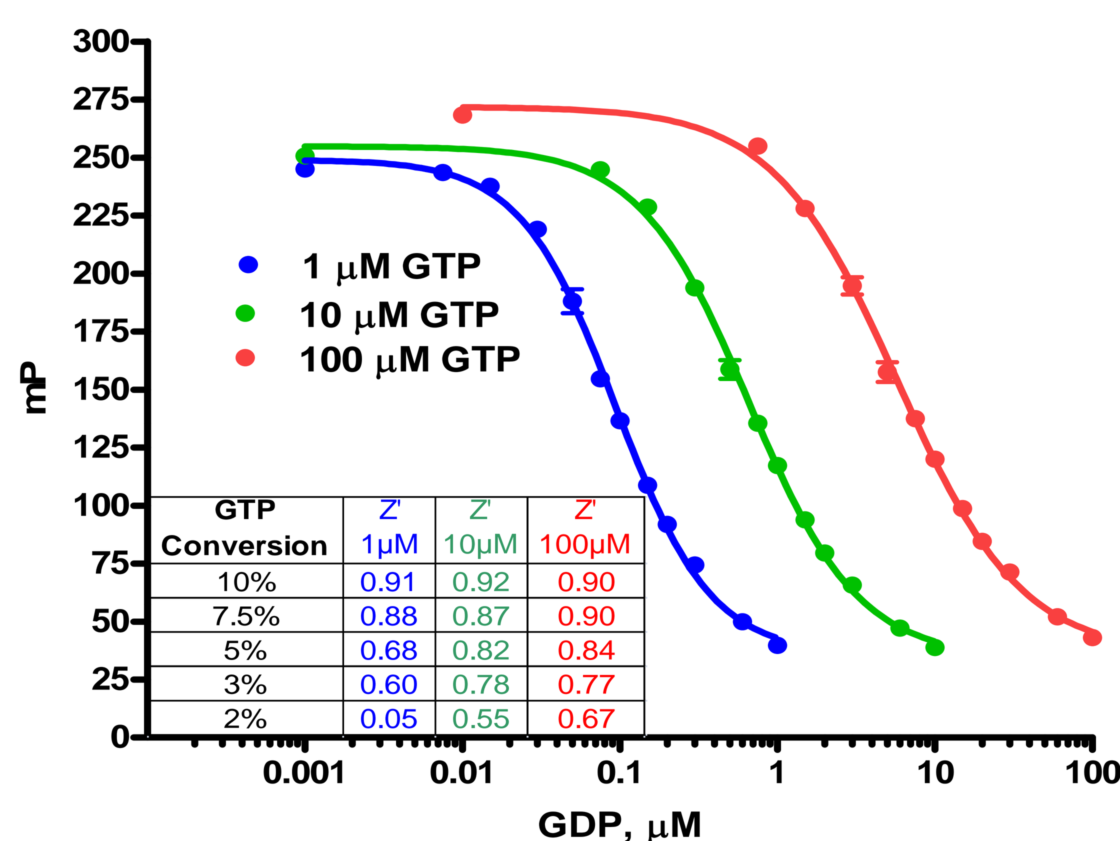


Figure 1. RGS protein HTS assay based on immunodetection of GDP produced by stimulated Gα protein. A) RGS proteins act catalytically on Gα-GTP complexes to stimulate GTP hydrolysis rates as much as 100-fold. Normally, GDP dissociates from isolated Gα proteins very slowly, so RGS GAP effects are not detectable using steady state GTPase assays. We mutated $G\alpha_{i1}$ so that GDP dissociation was no longer rate limiting, yet the protein still served as a functional substrate in RGS GAP reactions. B) GDP was detected using the Transcreener GDP Assay, a homogenous immunoassay with a fluorescence polarization readout.



GTP Conversion	Z' 1µM	Z' 10µM	Z' 100µM
10%	0.91	0.92	0.90
7.5%	0.88	0.87	0.90
5%	0.68	0.82	0.84
3%	0.60	0.78	0.77
2%	0.05	0.55	0.67

Figure 2. Standard curves for conversion of GTP to GDP using the Transcreener GDP Assay. Standard curves mimicking enzyme reactions were constructed for conversion of GTP to GDP using initial GTP concentrations of 1 µM, 10 µM, 100 µM, and 1,000 µM GTP; as GDP was add, GTP was decreased proportionately. Antibody was present at its EC_{85} concentration for each initial GTP concentration; Alexa-Fluor® 633-ADP tracer was present at 2 nM (n=24). Fluorescence polarization assays were performed in black Corning® 384 Well Black Flat Bottom Microplates (Part #3654). Assays were read in a Tecan Safire2™ multiwell reader. The free tracer reference was set to 20 mP, and buffer containing antibody was used as a blank for sample and reference wells. All points were done in 16 replicates so that Z' values could be determined (inset table).

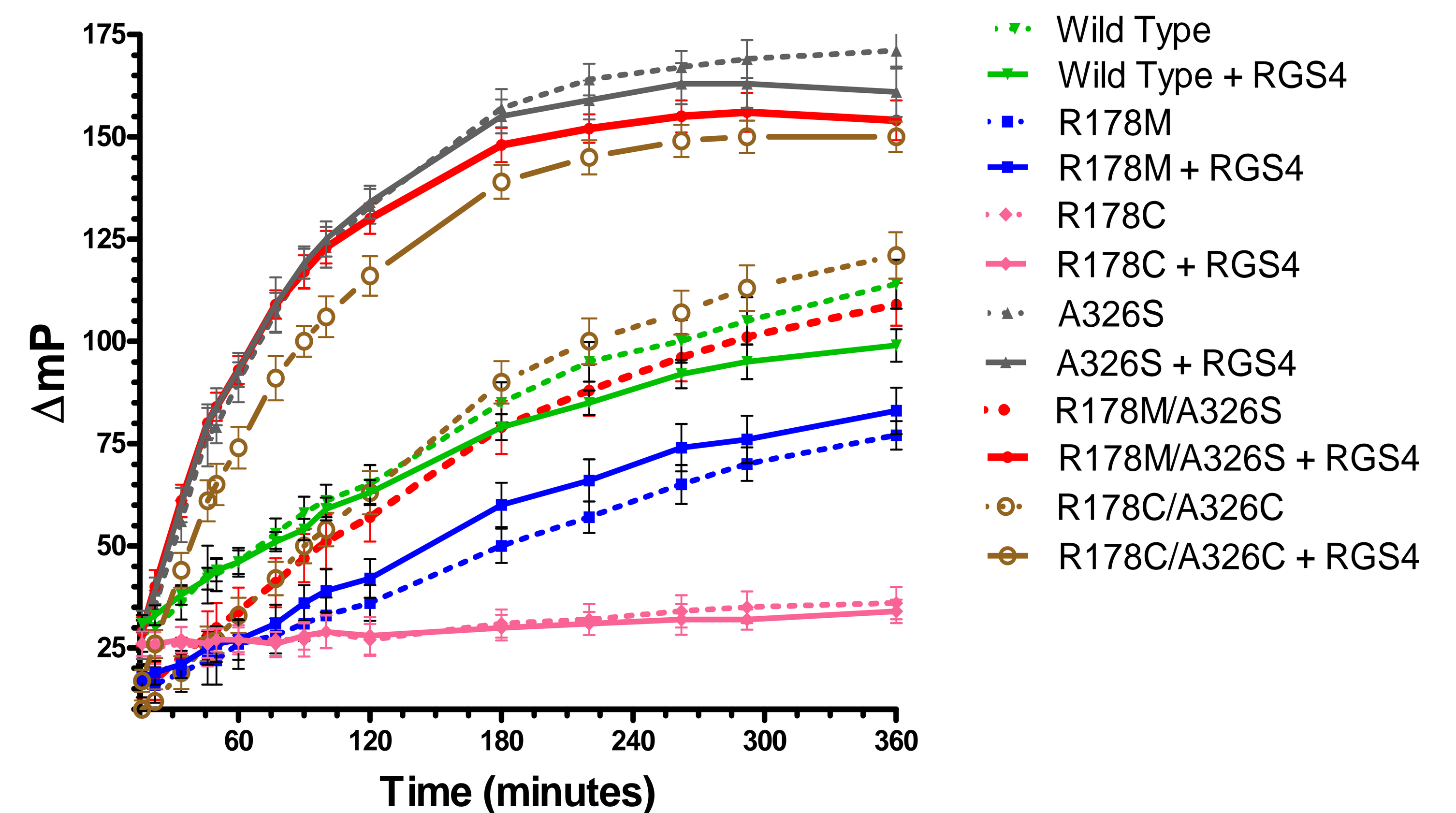


Figure 3. Effect of RGS4 on steady state GTPase activity of WT and mutated Gαi1 proteins shown as A) change in polarization and B) GDP produced. Dashed lines are in the absence and solid lines in the presence of 250 nM RGS4. Transcreener GDP assay reagents were added to enzyme reactions at time zero and plates were read at intervals starting at 15 min. All $G\alpha_{i1}$ proteins were present at 50 nM except R178C, which was at 100 nM. Control reactions lacked $G\alpha_{i1}$ protein. Reactions were run in 20 mM Tris 7.5 pH, 1 mM EDTA, 10 mM $MgCl_2$, 10 µM GTP, 8 µg/ml GDP mAb, and 2 nM ADP Alexa-633 Tracer in a final 20µl volume.

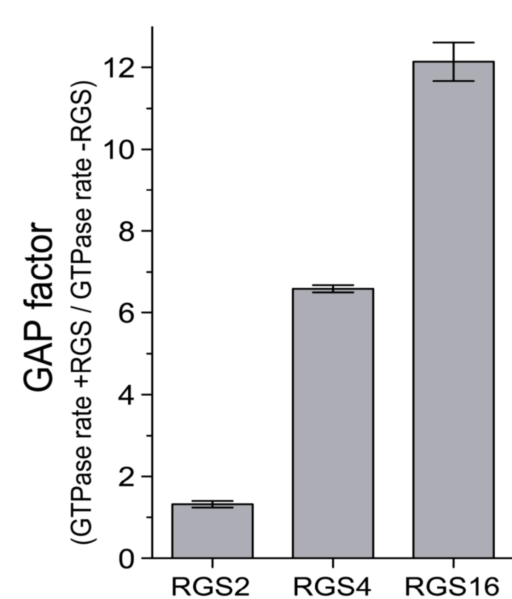
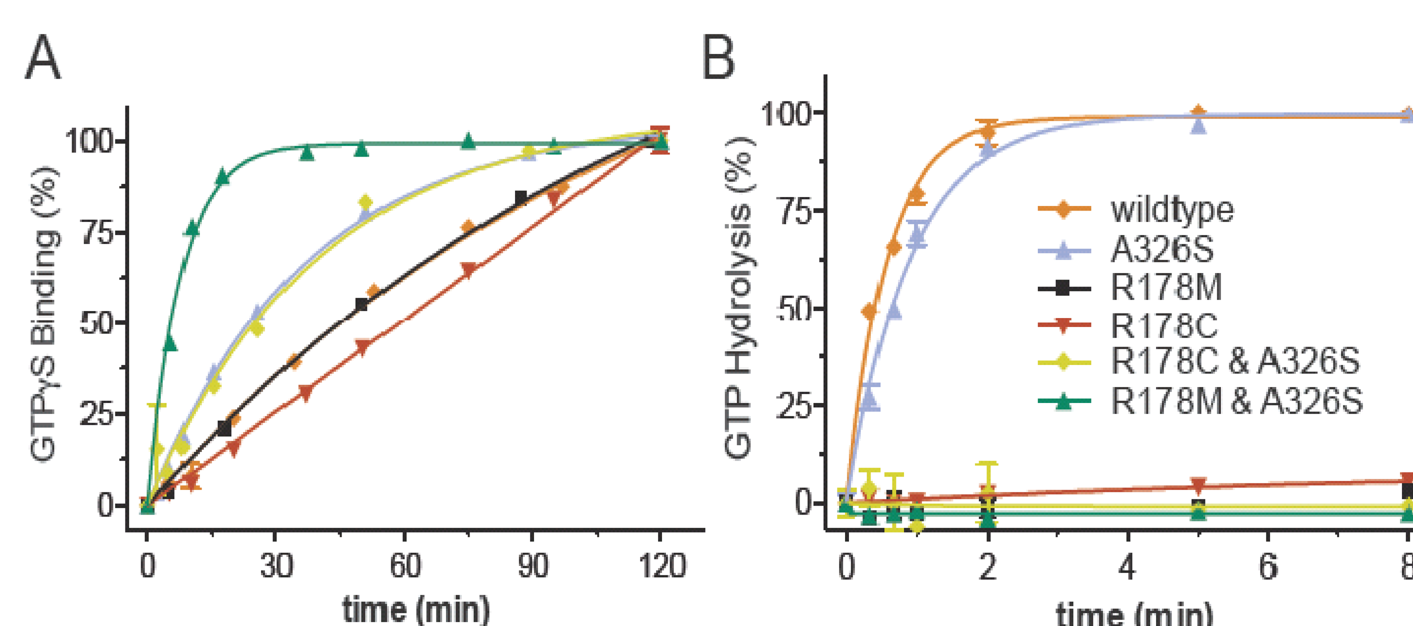


Figure 4. GAP effects of RGS isoforms measured using Transcreener GDP Assay with mutated $G\alpha_{i1}$ (R178M/A326S). Reactions conditions were as described for Figure 3. RGS4 and RGS16 have been previously shown to interact with $G\alpha_{i1}$ whereas RGS2 is known to be specific for $G\alpha_q$. These enzymatic results were confirmed by direct binding measurements using surface plasmon resonance (data not shown).



$G\alpha_{i1}$	GTP binding (min^{-1})	GTP hydrolysis (min^{-1})
WT	0.009	1.718
R178M	0.008	0.001
R178C	0.009	0.008
A326S	0.027	1.097
R178M/ A326S	0.130	0.000
R178C/ A236S	0.025	0.000

Figure 5. Radioassay measurements of A) GTPγ-^{35}S binding (GDP dissociation) and B) single turnover γ-^{32}P-GTP hydrolysis confirmed the properties of the wild type and mutated $G\alpha_{i1}$ proteins. Single turnover GTP hydrolysis assays, which are not rate-limited by GDP dissociation, were used to measure the intrinsic k_{cat}, and GTPγS binding assays were used to measure GDP dissociation. The single turnover assay measures ^{32}Pi released from enzyme-bound γ-^{32}P-GTP; reactions are terminated before a stoichiometric amount of phosphate is formed. Binding of the non-hydrolyzable GTP analog, GTPγ-^{35}S, to $G\alpha_{i1}$ which had been preloaded with GDP was used as a measure of the rate of GDP dissociation; the assumption is that k_{on} for GTPγ-^{35}S is much more rapid than k_{off} for GDP. Rates calculated from the linear portions of the curves are shown in the table at right.

Conclusions:

- The Transcreener GDP Assay, a competitive fluorescence polarization immunoassay for GDP, provides a robust, flexible HTS assay platform for the detection of GTPase activity.
- $G\alpha_{i1}$ can be mutationally altered to allow the use of steady state GTPase assays as a direct measure of RGS GAP activity.
- The specific combination of the R178M and A326S mutations results in an unexpected enhancement of the GDP dissociation rate for $G\alpha_{i1}$ that is more than additive.
- Mutated $G\alpha_{i1}$ functionally interacted with RGS isoforms with the same specificity as the wild type protein.
- The mutated $G\alpha_{i1}$ proteins coupled with the Transcreener® GDP Assay provide an HTS-compatible assay system to identify inhibitors of RGS protein GAP activity as a novel approach for selective modulation of GPCRs.

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