Targeting RGS Proteins using Engineered Galpha Proteins and the Transcreener® GDP Assay

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Abstract

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 contains “GTPase-accelerating protein” (GAP) activity, RGS proteins attenuate GPCR signaling by deactivating heterotrimeric G-protein alpha subunits. The steady-state GTPase activity of isolated Galpha proteins is limited by GDP dissociation, so GTP hydrolysis cannot be used to measure GAP activity in simple biochemical assays. To overcome this hurdle, we produced a series of mutated Galpha proteins predicted to exhibit decreased GTP hydrolysis rates and increased GDP dissociation rates and used the Transcreener® GDP Assay to identify variants that showed RGS protein-dependent increases in GTPase activity. The RGS proteins and RGS domains were coupled with the Transcreener® GAP assay to screen a small library of bioactives, and RGS4-specific inhibitors were identified. The combined use of a GDP detection assay with a rate-altered Galpha substrate represents a unique strategy to screen for compounds that selectively modulate GPCRs via their specific interactions with RGS proteins.

Figure 3. The R178M/A326S double mutation in Gαi3 allowed detection of a 6.5-fold GAP effect by RGS4. Dashed lines are in the absence and solid lines in the presence of 50 nM RGS4. Transcreener® GAP assay assays were conducted to enzyme reactions at zero time and plates were read at intervals starting at 15 s. All Gαi3 proteins were present at 0.1 μM except R178C, which was at 100 μM. Control reactions lacked Gαi3 protein. Reactions were run in 20 mM Tris-HCl, 1 mM EGTA, 10 mM MgCl2, 10 mM GTPγS (9 μM GTPm) and 2 μM AOX3/AX3/Tracer in a final 20 μl volume. 6.5 GAP effect is based on initial rates determined after converting polarization data to GDP formation.

Figure 4. The specific combination of R178M and A326S enhanced Koff eff-GDP-synergistically. Radioligand measurements of A) GTPγS binding (GDP dissociation) and B) single turnover γ-32P-GTP hydrolysis. Single turnover GTP hydrolysis assays, which are not stabilized by GDP dissociation, were used to measure the intermid, and GDP/GTP binding assays were used to measure GDP dissociation. The single turnover assay measured γ-32P-GTP; reactions are terminated before auto-inactivation of phosphatase is formed. Binding of the non-hydrolyzable GTP analog, GTPγS, to Gαi3, which had been preincubated with GDP was used as a measure of the rate of GDP dissociation; the assumption is that Koff eff for GTPγS is much more rapid than for GDP. Rates calculated from the linear portion of the curves are shown in the table at right.

Figure 5. R178M/A326S mutations did not disrupt binding interface or alter selectivity for RGS. A)Sensorgrams derived from 600 second injections of various concentrations (3 nM to 10 μM) of GDP over 5 nM of wildtype Gαi3 were recorded. B) Gαi3 GDP binding (left panel) or GDP (right panel) were used to derive dissociation constants (Kd) values. In the Transcreener® GDP Assay was used to measure the GAP effects of various RGS proteins with Gαi3 R178M/A326S. GAP 4 and 6 are known from previous studies to interact functionally with Gαi3 whereas RGS4 is known to be highly specific for Gαi3.

Table 1. Orthologous double mutations in related Gα proteins allowed detection of GAP activity with functionally interacting RGS proteins. Gα formation was measured for each Gα protein in the presence and absence of theophylline-activated RGS protein to determine GAP effects. RGS4-10, -14 and -17 are expected to functionally interact with Gα proteins, whereas RGS2 is highly specific for Gα12. The dendrogram shows percentage amino acid identity with Gα12.

Table 6. Screening in the presence (A) and absence (B) of RGS 4 allowed identification of compounds with specific effects on RGS catalytic activity. GDP assay compounds were added to wells containing 50 nM Gαi3 (R178M/A326S) with (A) or without (B) 25 nM RGS4 protein and GDP/GTP compound was added at 5 μM final concentration of GDP/GTP. 150 nM of active RGS inhibitor CGCG4986, 0.5 mM DMSO only, as indicated in the legend. The range of signal observed [3 standard deviations] about the mean ± 0 is denoted by the dashed lines for the 96 well compound library screen using RGS4 and Gαi3 (+/-) in wells a and b, b sedimentation nM GDP (γ-32P-GTP) IP production rate of Gαi3 (R178M/A326S) in the absence of RGS4 GTPase-accelerating protein (GAP) activity.

Figure 2. Measuring RGS protein GAP activity is prevented by the slow dissociation of GDP from Ga proteins. Goal: Increase Koff eff-GDP at 150 μM (from 0.03 to 5) by mutagenesis. A)RGS proteins act catalytically on GTP complexes to stimulate GTP hydrolysis rates as much as 100fold thereby attenuating GPCR agonist signals. B) Detection of GTP 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 assays. To overcome this kinetic limitation, we combined Gα mutations that increased GDP dissociation and decreased GAP activity.

Figure 6. Screening in the presence (A) and absence (B) of RGS 4 allowed identification of compounds with specific effects on RGS catalytic activity. GDP assay compounds were added to wells containing 50 nM Gαi3 (R178M/A326S) with (A) or without (B) 25 nM RGS4 protein and GDP/GTP compound was added at 5 μM final concentration of GDP/GTP. 150 nM of active RGS inhibitor CGCG4986, 0.5 mM DMSO only, as indicated in the legend. The range of signal observed [3 standard deviations] about the mean ± 0 is denoted by the dashed lines for the 96 well compound library screen using RGS4 and Gαi3 (+/-) in wells a and b, b sedimentation nM GDP (γ-32P-GTP) IP production rate of Gαi3 (R178M/A326S) in the absence of RGS4 GTPase-accelerating protein (GAP) activity.

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.
- Gαi3-related Gα proteins can be mutationally altered to allow the use of steady state GTPase assays as a direct measure of RGS GAP activity.
- Mutated Gαi3-related Gα proteins functionally interact with RGS isoforms with the same specificity as the wild type proteins.
- The mutated Gαi3 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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