[42] Quantitative Assays for GTPase-Activating Proteins

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G protein signaling is fundamentally kinetic. The rate of receptor-stimulated GTP binding determines the rate of signal initiation, and the rate of hydrolysis determines the rate of termination. The balance of these rates determines signal amplitude. GTPase-activating proteins (GAPs) accelerate GTP hydrolysis up to 2000-fold, and thereby confer the speed needed for reliable cellular signaling. A G protein GAP can perform one or more diverse regulatory functions depending on its kinetic behavior, regulation, and subcellular localization.\(^1\) First, a GAP will increase the speed with which signaling terminates on removal of agonist, and GAP activity is clearly necessary to reconcile the slow rate of hydrolysis of G\(\alpha\)-bound GTP in vitro with the rapid signal termination displayed in cells. GAPs can also inhibit signaling by decreasing the steady-state level of active G\(\alpha\)-GTP in the face of constant stimulation of GDP/GTP exchange by receptor. Last, complex effects of a GAP on the dynamics of the overall GTPase cycle can lead to two initially unsuspected effects: the damping of basal (background) G protein activation with little effect on the agonist-stimulated signal and/or the enhancement of selectivity of a G protein among multiple receptors. GAP activity may be detected in cells or tissues as increases in the rate of deactivation of a G protein-mediated signal on removal of agonist or as inhibition of the steady-state amplitude of an agonist-initiated signal. Diverse biochemical regulators can also produce these effects, of course, and in vitro determination of GAP activity is therefore desirable. The mechanism of G protein GAP activity has been reviewed.\(^2\)

Assays for G protein GAP activity are all based on the reaction pathway for GAP-accelerated GTP hydrolysis shown in Scheme I.

\[
\begin{align*}
G\alpha-GTP & \xrightarrow{k_h} \text{G}\alpha-GDP + P_i \\
\text{GAP-}G\alpha-GTP & \xrightarrow{k_{\text{gap}}} \text{GAP} + \text{G}\alpha-GDP + P_i
\end{align*}
\]

SCHEME I

A GAP binds reversibly to the GTP-ligated G\(\alpha\) subunit and promotes the rapid hydrolysis of GTP in the GAP–G\(\alpha\)–GTP complex (i.e., \(k_{\text{gap}} \gg k_h\)). \(P_i\) dissociates essentially immediately, and most GAP assays monitor the production of the \(P_i\) product with time. It is possible, but usually less convenient, to measure the conversion of G\(\alpha\)–GTP to G\(\alpha\)–GDP by chromatography of bound nucleotide,

as is routine in assays of GAPs for small monomeric G proteins. Dissociation of the GAP from the $\text{G}_\alpha$–GDP product is fast and is not shown separately, although the binding of phospholipase (PLC)-$\beta$ to $\text{G}_\alpha_q$ during steady-state GTP hydrolysis may be stable enough to persist over tens of seconds on the surface of a membrane bilayer.\(^3\)

Two general approaches are used to measure GAP activity. The simplest and most quantitative assay is to measure the rate of hydrolysis of $\text{G}_\alpha$-bound GTP in a single enzymatic turnover. This assay is usually performed by binding $[\gamma-^{32}\text{P}]$GTP to a $\text{G}_\alpha$ subunit under conditions where hydrolysis is minimal and then monitoring hydrolysis as release of $[^{32}\text{P}]\text{P}_1$ under optimal assay conditions.\(^4,5\) "Single turnover" refers to the hydrolysis of a single molecule of GTP by each molecule of $\text{G}_\alpha$; the GAP itself turns over multiple $\text{G}_\alpha$–GTP complexes during the assay and thus acts catalytically. Single-turnover assays are preferred for assaying GAP activity in crude preparations (during purification, for example), for routinely standardizing the concentration of active GAP under defined conditions, or for studying the mechanism of GTP hydrolysis and its acceleration. The second general sort of GAP assay is to measure the increase in a G protein’s steady-state GTPase activity.\(^6\) Steady-state GAP assays depend on receptors to catalyze GDP/GTP exchange, which would otherwise be rate limiting for the overall GTPase reaction cycle. If exchange is relatively fast, acceleration of the hydrolytic step by a GAP will appear as an increase in the steady-state GTPase rate. The steady-state assay provides information on the role of the GAP in modulation of the overall GTPase cycle, reflects the importance of the membrane as an organizing structure, and measures activities under conditions closer physically to those encountered in cells. In some cases, the steady-state assay can detect GAP activity that is below the limits of sensitivity of a single-turnover assay. Rates of the individual reactions of the GTPase cycle can also be measured during steady-state hydrolysis (elsewhere in this volume\(^6^a\)). As is true for any biochemical measurement, the choice of GAP assay and the analysis of the data depend on the investigator’s goals. GAP assays may be used to identify novel GAPs, to monitor GAP purification or standardize concentration, to study the regulation of GAP activity by allosteric ligands or covalent modification, or to evaluate the affinities and selectivities with which GAPs regulate different G proteins. The answer you get depends on how you ask the question.

\(^5\) It is also possible to measure hydrolysis of GTP bound to a $\text{G}_\alpha$ subunit fluorometrically because the GTP-activated state displays a higher intrinsic tryptophan fluorescence than does the GDP-bound state. [T. Higashijima and K. M. Ferguson, Methods Enzymol. 195, 321 (1991); T. Higashijima, K. M. Ferguson, P. C. Sternweis, M. D. Smigel, and A. G. Gilman, J. Biol. Chem. 262, 762 (1987)].
Single-Turnover GAP Assays

To perform a single-turnover GAP assay, $[\gamma-^{32}\text{P}]\text{GTP}$ is first bound to the $G\alpha$ target, residual nucleotide is usually removed, and hydrolysis of $G\alpha$-bound GTP is monitored either over time or at a single time point. GAP activity is thus measured as acceleration of the hydrolytic rate. This is the preferred assay for most applications.

Hydrolysis of $G\alpha$-bound GTP should follow a monoexponential time course (Scheme I, Fig. 1). The observed first-order rate constant, $k_{\text{app}}$, reflects a combination of $k_h$, the intrinsic hydrolytic rate of the $G\alpha$ subunit, and $k_{\text{gap}}$, the GAP-stimulated rate, weighted according to the concentration of the GAP and its affinity for the $G\alpha$–GTP complex ($k_{-1}/k_1$). GAPS act catalytically. Each GAP molecule sequentially binds multiple $G\alpha$–GTP molecules to promote their hydrolysis, and $k_{\text{app}}$ is proportional to the concentration of GAP for GAP concentrations well below that of $G\alpha$–GTP (Fig. 1B).

Reliable quantitation of GAP activity in single-turnover assays is based on the analogy of Scheme I to the Briggs–Haldane formalism for an “enzyme” that catalyzes conversion of a $G\alpha$–GTP “substrate” to $G\alpha$–GDP and $P_i$ products. GAP-independent hydrolysis during the assay is subtracted as background. This analogy allows application of the Michaelis–Menten equation, defining $K_m = (k_{-1} + k_{\text{gap}})/k_1$ and $V_{\text{max}} = [\text{GAP}] \cdot k_{\text{gap}}$ (Scheme I). $V_{\text{max}}$, in units of moles of $P_i$ produced per unit time, is a measure of total GAP activity. When $k_{\text{gap}}$ is less than $k_{-1}$, the value of $K_m$ approximates the affinity of the GAP for the particular $G\alpha$–GTP substrate because $K_m \approx K_s = k_{-1}/k_1$, and provides a measure of GAP–$G\alpha$ selectivity that is independent of absolute hydrolysis rates. The value $k_{\text{gap}}/K_m$ is an overall measurement of GAP activity, but reflects both relative activity of the GAP and the hydrolytic capacity of the $G\alpha$ substrate. Overall, this Michaelian analysis is the preferred method of data presentation for describing GAP activity.

A unit of GAP activity, like any enzyme unit, is defined as the amount of GAP that will elevate $k_{\text{app}} (V_{\text{max}})$ by a fixed amount, usually 1 min$^{-1}$. The number of GAP units displayed by a particular concentration of a GAP depends on the assay conditions, but such a unit is a reliable measure of activity in that it scales linearly

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7 For Scheme I in rapid binding equilibrium that defines $K_1 = k_1/k_{-1}$, the differential rate equations can be solved to yield a simple first-order equation for the concentration of phosphate released [$P$] as a function of time.

$$\frac{[P]_t}{[P]_{\infty}} = 1 - e^{-k_{\text{app}}t}$$

For $[\text{GAP}] << 1/K_1$, the apparent first-order rate constant $k_{\text{app}}$ is defined as

$$k_{\text{app}} = \frac{k_h + k_{\text{gap}}K_1[\text{GAP}]}{1 + K_1[\text{GAP}]}$$

and $k_{\text{app}}$ will increase linearly with [GAP].

Fig. 1. Quantitative detection of Gz GAP activity in a particulate fraction from steer cerebral cortex. (A) Gz-[γ-32P]GTP was incubated at 15° with a suspension of brain membranes, and release of [32P]Pi was monitored as described in the text. Assays contained 125 fmol of Gz-[γ-32P]GTP and either 5 μg (△), 10 μg (○), 15 μg (△), or 30 μg (▽) of untreated membranes or 15 μg of boiled membranes (□). Hydrolysis approached 100% and approximated a family of monophasic exponential curves. Parallel binding experiments showed that loss of bound [γ-32P]GTP equaled production of [32P]Pi, indicating that dissociation of [γ-32P]GTP was negligible. The boiled membranes had no effect compared with a buffer control. (B) The data shown in panel A were well fit by a single exponential rate equation to determine \( k_{\text{app}} \), which is shown plotted vs the amount of membrane added. Activity in 30 μg of membranes is about equal to that of 10 fmol of purified RGSZ1. Hence, each molecule of GAP turned over about 10 molecules of Gz-[γ-32P]GTP. Reproduced with permission from J. Wang, Y. Tu, J. Woodson, X. Song, and E. M. Ross, J. Biol. Chem. 272, 5732 (1997).
with GAP concentration over a wide range of conditions. It is therefore useful for quantitating a GAP, particularly during purification. Note that a GAP unit is defined with reference to a single Gaα-GTP substrate and set of assay conditions. Most importantly, a GAP unit reflects both the intrinsic GTPase activity of the particular Ga subunit and the relative stimulation of hydrolysis (“-fold increase”). For example, 1.5 units of a Gz GAP would increase k_{app} for Gaα from 0.015 min^{-1} to 1.515 min^{-1}, about 100-fold. An equal increment in the k_{app} of Gaα, from 3 min^{-1} to 4.5 min^{-1}, would be only a 50% increase.4

Single-turnover assays depend absolutely on obtaining either reliable initial rates—while the reaction is linear with time—or a complete enough description of the entire reaction time course to allow calculation of the rate constant k_{app}. GAP activity must also be linear with increasing amounts of the GAP protein. Many published assays have grossly underestimated G protein GAP activity because the investigators ignored these points. This section presents methods for single-turnover GAP assays. The method for Gz GAP activity is presented first and in greatest detail as an example. The following section on GAPs for Gi, Go, and Gz is a variation, and sections on Gq and Gt describe their unique problems. Procedures for purifying G protein subunits9 and other ancillary techniques are described in this and previous volumes (see also Wang et al.10).

**Prototypical Single-Turnover Assay for Gaα GAP Activity**

Gaα hydrolyzes bound GTP more slowly than does any other Ga subunit, with k_{hyd} ~ 0.014 min^{-1}. This facilitates the preparation of Gaα-[γ-32P]GTP and allows its relatively stable storage during extended experiments.

*Preparation of Gaα-Bound [γ-32P]GTP.* Before beginning the binding reaction, prepare a 0.5 x 10 cm (2 ml) gravity-feed column of Sephadex G-25 in buffer B [25 mM Na–HEPES (pH 7.5), 1 mM EDTA, 0.1% Triton X-100, 3 mM dithiothreitol, 2.5 μM GTP]. To bind [γ-32P]GTP, incubate purified Gaα, 1–60 pmol Gaα per column assay, in a total of 200 μl of buffer B plus ~3 x 10^7 cpm of [γ-32P]GTP11 for 20 min at 30°. Chill the reaction and determine total radioactivity.


11 [γ-32P]GTP can be purchased or easily synthesized by the method of R. A. Johnson and T. F. Walseth, *Adv. Cyclic Nucleotide Res.* 10, 135 (1979). Purity is 98–99% in either case. This is adequate for single-turnover assays in which Ga-bound [γ-32P]GTP is separated from free [γ-32P]GTP and [32P]P. For steady-state assays, however, contamination with [32P]P must be below 0.5%, and is below 0.1% with freshly purified [γ-32P]GTP. We purify [γ-32P]GTP by anion-exchange HPLC with a 10–700 mM gradient of KP, pH 7.0. KP concentrations depend on the anion exchanger.3 Adjust elution rates and gradient using unlabeled nucleotide and a UV absorbance monitor so that the [γ-32P]GTP (up to 5 mCi per preparation) is eluted in less than 0.5 ml with greater than 99.9% purity. Storage at −80° with inclusion of 10 mM Tricine maintains purity for 3 weeks.
in a 2 μl volume to calculate the specific activity of the \([γ^{−32}\text{P}]GTP\). The amounts specified here should give about 60 cpm/fmol. Before continuing, check \(Gα_x\)-
bound nucleotide in a 2 μl sample of incubation mix by the standard nitrocellulose filter assay.\(^{12}\) About 25% of the \(Gα_x\) should have \([γ^{−32}\text{P}]GTP\) bound. The remainder is bound to GDP.\(^{13}\) \(Gα_x−[γ^{−32}\text{P}]GTP\) may be stored at −80°C at this point.

Load the binding mixture onto the gel filtration column at 0–4°C and wash the column with 350 μl of cold buffer B. Elute \(Gα_x−[γ^{−32}\text{P}]GTP\) with an additional 750 μl of buffer B. (Adjust elution volumes for the volume and geometry of the column.) Dilute the \(Gα_x−[γ^{−32}\text{P}]GTP\) to a predicted concentration of \(\sim 0.2\) pmol \(Gα_x−[γ^{−32}\text{P}]GTP\)/70 μl. This concentration is designed to give about 3 nM \(Gα_x−[γ^{−32}\text{P}]GTP\) in the assay, just above the \(K_m\) for brain \(G_x\), GAP,\(^4\) and may be altered according to the experiment. Add 0.05 volume of 0.1 M GTP (5 mM final), 0.05 volume of 0.2 mg/ml albumin (10 μg/ml final), and 0.01 volume of 0.68 M MgCl₂ (calculated free Mg\(^{2+}\) concentration of 1 mM; see below).\(^{14}\) These volumes can be modified to allow for addition of other agents, to vary the concentration of \(Gα−[γ^{−32}\text{P}]GTP\), or to allow for addition of GAP in larger volumes.

Before starting the assay, check bound \([γ^{−32}\text{P}]GTP\) again by nitrocellulose binding assay of a 70 μl aliquot, and check residual \([32\text{P}]P_i\) by the charcoal precipitation assay described below. Background \([32\text{P}]P_i\) should be less than 5% of total \(Gα_x\)-bound radioactivity. \(Gα_x−[γ^{−32}\text{P}]GTP\) is relatively stable at 0°C and in the absence of Mg\(^{2+}\) (\(t_{1/2} \sim 4.5\) hr). Regardless, prepare and isolate \(Gα_x−[γ^{−32}\text{P}]GTP\) just before use to maximize yield and minimize free \([32\text{P}]P_i\). In our experience, dissociation of \([γ^{−32}\text{P}]GTP\) is much slower than its hydrolysis under all common conditions.

**GAP-Stimulated Hydrolysis of \(Gα_x−[γ^{−32}\text{P}]GTP\)**. \(G_x\) GAP activity is measured at 15°C, where the unstimulated rate of hydrolysis of bound GTP \(k_h = 0.014\) min⁻¹ (\(t_{1/2} = 50\) min) (Fig. 1). The assay can potentially be carried out over any time span, but 10 min is usually convenient and produces a low and reproducible level of unstimulated hydrolysis (\(\sim 10%\) of total). Assays are initiated by adding the \(Gα_x−[γ^{−32}\text{P}]GTP\) in 70 μl to prewarmed polypropylene tubes that contain the GAP (or buffer as a blank), Mg\(^{2+}\), and any other additives in a total volume of 10 μl (80 μl total assay volume). Individual volumes of the components can be


\(^{13}\) In this and other single-turnover GAP assays, the \(Gα−[γ^{−32}\text{P}]GTP\) substrate is substantially contaminated with \(Gα−GDP\) that was either not bound to \([γ^{−32}\text{P}]GTP\) originally or was formed by hydrolysis after binding. Although competition for GAP binding by \(Gα−GDP\) is not a problem because of its low affinity, the presence of \(Gα−GDP\) should not be forgotten. For example, residual \(Gα\) can chelate stoichiometric amounts of the \(Gβγ\).

\(^{14}\) Many algorithms and computer programs are available to calculate the concentrations of free divalent cations in the presence of one or more chelators.\(^4,10\) Under these assay conditions, values of \(K_q\) for Mg\(^{2+}\) of EDTA and GTP of 1 μM and 100 μM have yielded calculated values of [Mg\(^{2+}\)]\(_{\text{free}}\) within 10% of the concentrations determined experimentally.\(^4\)
varied as needed to accommodate other additions. Controls in each experiment should include a sample with no added GAP to provide a parallel measure of basal hydrolysis ($k_b$) (although this rate is quite reproducible) and a reagent blank (Gαζ-[γ-32P]GTP plus charcoal, no incubation, no GAP) to correct for contaminating [32P]P, counter background, inefficient precipitation of GTP, etc.

Each assay is terminated by addition of 920 µl of a 5% slurry of activated charcoal in H3PO4, pH 3 (Norit A or equivalent) that is continually stirred at 4°C. Assay tubes are vortexed and kept on ice until the experiment is completed, and all are centrifuged at 1500g for 10 min. [32P]P, in a convenient volume of supernatant, usually 600 µl, is then determined by either Cerenkov or liquid scintillation counting.

The assay medium described here, buffer B plus 1 mM Mg2+, is optimal for members of the RGSZ subfamily of RGS proteins and for RGS4. The GTP in the buffer is used to protect any free [γ-32P]GTP (carried over from the binding reaction or dissociated from Gαζ) from hydrolysis by contaminating nucleoside triphosphatases. This caution is particularly important for crude preparations of GAPs. In our experience, most GAPs are not sensitive to the concentration of Mg2+ over the range of 1–1000 μM, although activity declines above 1 mM. RGSZ1 (Ret-RGS1) displays a distinct activity optimum at 1 mM free Mg2+, however, and we recommend maintaining this concentration routinely. Because the assay contains 1 mM EDTA and 5 mM GTP, it is important to calculate the final concentration of free Mg2+ carefully, taking into consideration any Mg2+ or chelator that may be added as a component of the GAP dilution buffer. BSA is added both to increase recovery of Gαζ-[γ-32P]GTP during gel filtration and to stabilize protein during the assay. A detergent is required to maintain the solubility of Gα subunits and many GAPs; Triton X-100 was found to be the best detergent for Gζ GAP. Components of the assay cocktail may be changed as necessary for other GAPs, as suggested by variations described in other assay protocols.

Calculation of Data. GAP activity is most simply calculated as the initial (linear) hydrolytic rate (moles [γ-32P]GTP hydrolyzed per minute) after subtraction of the “no GAP” control value.15 This format is simple and direct and should be used when GAP activity is analyzed according to the Michaelis–Menten equation ($V_{max}$, $K_m$). Data can also be presented as $k_{app}$ or in GAP

15 To calculate the specific activity of Gαζ-[γ-32P]GTP, divide the radioactivity in the sample of the [γ-32P]GTP binding cocktail by the amount of GTP in the same volume. This value is valid for the Gαζ-[γ-32P]GTP complex because Gαζ bound to GDP will not interfere in the assay and is therefore irrelevant. After subtracting radioactivity in the reagent blank (no GAP, no incubation) from that in the experimental samples, use the specific activity to convert radioactivity in each sample to moles of Gαζ-[γ-32P]GTP hydrolyzed. Remember to correct for the fraction of the charcoal supernatant sampled, usually 600 µl of a total of 1000 µl. Gαζ-[γ-32P]GTP is measured in the binding assay performed at the beginning of the experiment.
units (see above). To calculate the net hydrolysis rate constant $k_{\text{app}}$ from the amount of [γ-32P]GTP hydrolyzed, the equation in footnote 7 is rearranged to give

$$k_{\text{app}} = (-1/t) \ln(1 - [P]/[P]_\infty),$$

where $[P]/[P]_\infty$ is the fraction of [γ-32P]GTP hydrolyzed during the assay time $t$. Alternatively, a complete hydrolysis time course can be fit to yield $k_{\text{app}}$ (Fig. 1). $k_{\text{app}}$ increases linearly with the concentration of GAP (for low concentrations) and is therefore a good measure of GAP activity (Fig. 1B).

Note that data are usable only when total hydrolysis is both (1) significantly above the “no GAP” background level, such that incremental GAP-stimulated hydrolysis can be determined accurately, and (2) significantly below complete hydrolysis of all the $G\alpha$–GTP substrate. Do not use data where $[P]/[P]_\infty$ exceeds 80%, because small errors in determining this fraction cause large errors in the calculated GAP activity. We have observed that efficient $G_z$ GAPs increase the hydrolytic rate for $G\alpha_z$–GTP about 600-fold, with $K_m \sim 2 \text{nM}$.16

**Modification of Single-Turnover Assays for $G\alpha_i$, $G\alpha_o$, and $G\alpha_s$**

GAP assays for $G\alpha_i$, $G\alpha_o$, and $G\alpha_s$ are designed and executed essentially as described for $G\alpha_z$, but the intrinsically faster hydrolytic rates of these $G\alpha$ subunits require that $G\alpha$–[γ-32P]GTP be purified and used promptly after preparation and that the assay itself be adjusted to account for the higher $k_{\text{gap}}$ values that are achieved. Modifications suggested below have been applied routinely to $G\alpha_i$ and $G\alpha_o$, and should also be applicable to $G\alpha_s$.

To prepare $G\alpha$–[γ-32P]GTP, incubate $G\alpha$ subunit (usually 50 pmol) for 15–20 min at 30° with [γ-32P]GTP (1 μM or a 0.5 μM stoichiometric excess over $G\alpha$, whichever is greater, usually 20 cpm/fmol) in 100 μl of Buffer C (50 mM Na–HEPES (pH 7.5), 0.05% dodecyl polyoxyethylene (n=9; Lubrol PX, Genapol 24-L-75, etc.), 1 mM dithiothreitol (DTT), 5 μg/ml albumin) plus 10 mM EDTA. Because $G_i$ is usually stored in the presence of MgCl2, 10 mM EDTA is needed to reduce free Mg$^{2+}$ to below 10 nM and thereby inhibit hydrolysis during loading. Chill the reaction mixture on ice and immediately purify $G\alpha$–[γ-32P]GTP by centrifugal gel filtration on a 3 ml column of Sephadex G-25 in buffer C plus 10 mM EDTA at 0–4°. Incubation time for binding [γ-32P]GTP is not critical, but the $G\alpha$–[γ-32P]GTP complex must be chilled, purified, and used as quickly as possible! About 5–10% of bound [γ-32P]GTP is hydrolyzed per 10 min at 0°, which decreases the substrate concentration and increases the assay background. Design experiments so that a reasonably small number of assays is performed with one batch of $G\alpha$–[γ-32P]GTP to keep its concentration acceptably constant for all the assays. Measure the specific activity of the [γ-32P]GTP and the concentration of $G\alpha$-bound [γ-32P]GTP as described for $G\alpha_z$.

Hydrolysis of Gα-bound GTP is measured as described for Gαz-[γ-32P]GTP. Because other Gα subunits hydrolyze bound GTP 50- to 100-fold faster than does Gαz, the assay is executed over a shorter period of time and, usually, at a lower temperature, 0–10°. Note that hydrolysis rates of Gα subunits may vary idiosyncratically with temperature; extrapolating rates from one temperature to another is dangerous. Regardless, it is important to choose assay time and temperature and the concentration of GAP so that an acceptable amount of Gα-[γ-32P]GTP is hydrolyzed (more than basal, less than 75%). Our routine assay medium is Buffer C plus 1 mM GTP and 1 mM free Mg2+. The concentration of free Mg2+ is achieved by controlling for chelation by EDTA and GTP (see above).14 Optimal assay conditions must be established by the investigator. Assays are terminated, usually after 30 sec, by addition of 950 μl of a 5% (w/v) charcoal slurry in 50 mM H3PO4, pH 3, and radioactivity is determined in a 600 μl sample of the supernatant. GAP activity for Gαo, Gαi, or Gαs is calculated exactly as described above for Gαz.

Modification of Single-Turnover Assays for Gαq

Because members of the Gq family bind GTP slowly, it is not possible to load wild-type Gαq with [γ-32P]GTP fast enough to avoid complete hydrolysis, but Gq GAP activity can be measured in a single-turnover assay using the hydrolysis-defective R183C mutant.17 Gαq R183C hydrolyzes bound GTP about 0.7% as fast as wild-type, which allows loading with [γ-32P]GTP, and retains sensitivity to the GAP activity of both RGS proteins and PLC-β. The Gαq R183C GAP assay provides a convenient way to quantitate Gq GAP activity during purification or to standardize the activity of a Gαq GAP; it has been described in detail elsewhere.17 This GAP assay is not nearly so straightforward as those for other Gα subunits. First, meaningful conclusions clearly depend on the assumption that the GAP responses of Gαq R183C are unaltered from those of wild-type Gαq. Further, both spontaneous hydrolysis and dissociation of [γ-32P]GTP are significant relative to GAP-stimulated hydrolysis, and appropriate corrections must be applied. In addition, hydrolysis time courses are not monophasic and frequently do not go to completion. The magnitude of the initial GAP-stimulated phase is crucially dependent on the identity and concentration of detergent. Investigators should evaluate the complete time course of GAP-stimulated hydrolysis and restrict assays to the period over which hydrolysis can be fit to a single exponential, where the first-order rate constant kapp increases linearly with the concentration of GAP, and where total hydrolysis is less than 50% complete.

Single-Turnover Assay for G<sub>i</sub>

A variation of the single-turnover assay has been used by the groups of Arshavsky and Wensel\textsuperscript{18,19} to study the GAP activity of RGS proteins on G<sub>i</sub>, which is essentially impossible to load with [\(\gamma\)-\textsuperscript{32}P]GTP in the absence of rhodopsin or a related receptor. In this assay, photoreceptor membranes (disks) are washed to remove G<sub>i</sub> and as many other proteins as possible. Rhodopsin is then activated by light and substoichiometric G<sub>i</sub> is added. The G<sub>i</sub> releases bound GDP when it binds the bleached rhodopsin. The reaction is initiated by addition of an amount of [\(\gamma\)-\textsuperscript{32}P]GTP substoichiometric to G<sub>i</sub> and the time course of its hydrolysis is monitored using charcoal precipitation of substrate as described above. This method is useful to demonstrate the presence of endogenous GAP activity in the disk membranes (RGS9/G\(\beta\)5) or in preparations of exogenous proteins, and to measure the potentiation of GAP activity by the \(\gamma\) subunit of cyclic GMP phosphodiesterase. It probably underestimates GAP activity substantially, however. The assay strategy assumes that initial binding of [\(\gamma\)-\textsuperscript{32}P]GTP is fast and that chemical hydrolysis is uniquely rate-limiting, both of which are uncertain. Skiba \textit{et al.} have measured GAP-stimulated hydrolysis rates for G<sub>i</sub> GTP that are much higher than those measured with the older assay and which are commensurate with those measured for G<sub>q</sub> and G<sub>i</sub>\textsubscript{q}.\textsuperscript{20} Interested readers are referred to this work.

\textit{GAPs with High K<sub>m</sub>}

Values of \(K_m\) for different GAPs and their Go-GTP substrates vary from \(\sim 2 \text{ nM}\) to \(\sim 1 \mu M\).\textsuperscript{20-22} High values of \(K_m\) reflect in part the high rates of GAP-promoted GTP hydrolysis (\(K_m = (k_1 + k_{gap})/k_1\), Scheme I), but low affinity for the Go-GTP substrate (high \(K_a\)) may also contribute. It is therefore often impossible to perform single-turnover GAP assays at concentrations of Go-[\(\gamma\)-\textsuperscript{32}P]GTP well above the \(K_m\), either because background hydrolysis is high or because substrate with appropriate specific activity cannot practically be prepared at adequate concentrations. Similarly, low values of \(V_{max}\) may necessitate using high concentrations of the GAP. In such situations, \(K_m\) and \(V_{max}\) can still be determined by measuring GAP activity as a function of GAP concentration at several low concentrations of the Go-GTP substrate. Assay protocols are otherwise identical to those described above, and data are analyzed according to an alternative derivation of the Briggs–Haldane equation for steady-state enzyme activity.\textsuperscript{8} When the concentration of Go-GTP is

low, the initial rate of GTP hydrolysis (after subtraction of background) is defined as:

$$v = [G\alpha - GTP]k_{gap} \left( \frac{[GAP]}{K_m + [GAP]} \right)$$

Thus, initial rate is proportional to the concentration of G\(\alpha\)-GTP and "saturates" at \(V_{max} = k_{gap} [G\alpha - GTP]\) as the GAP concentration increases above \(K_m\). Practically, it is also possible to assay GAPs reproducibly even when \(K_m\) is unmeasurably high. If activity is reproducible over a reasonable range of G\(\alpha\)-GTP concentrations, plots of rate vs concentration will have the slope \(k_{gap}/K_m\), which is the virtual second-order reaction rate constant for the productive interaction between GAP and substrate.\(^8\)

**Monitoring Dissociation of G\(\alpha\)-[\(\gamma\text{-}^{32}\text{P}\)GTP**

When first establishing a single-turnover GAP assay, it is important to verify both that the G\(\alpha\)-[\(\gamma\text{-}^{32}\text{P}\)GTP complex is stable throughout the assay period and that no [\(^{32}\text{P}\)P]i is formed from hydrolysis of free [\(\gamma\text{-}^{32}\text{P}\)GTP (dissociated from G\(\alpha\) or carried over from the loading reaction). The most convenient control is to confirm that loss of G\(\alpha\)-bound [\(\gamma\text{-}^{32}\text{P}\)GTP measured in a nitrocellulose filter binding assay\(^23\) exactly equals the formation of [\(^{32}\text{P}\)P]i during the assay interval (Fig. 1A). This control should be performed periodically when using impure GAP preparations or when adding any reagent that might alter nucleotide dissociation. Definitive demonstration that [\(^{32}\text{P}\)P]i is derived quantitatively from G\(\alpha\)-bound [\(\gamma\text{-}^{32}\text{P}\)GTP is to measure production of G\(\alpha\)-bound [\(\alpha\text{-}^{32}\text{P}\)GTP from G\(\alpha\)-bound [\(\alpha\text{-}^{32}\text{P}\)GTP directly. For this assay, G\(\alpha\)-[\(\alpha\text{-}^{32}\text{P}\)GTP is prepared exactly as described above for [\(\gamma\text{-}^{32}\text{P}\)GTP.\(^24\) The GAP assay is also performed similarly, but the reaction is quenched with cold buffer and G\(\alpha\) is trapped by binding to a nitrocellulose filter.\(^4\,\,23\) The filter is dissolved in 1 ml of cold acetone, and bound [\(\alpha\text{-}^{32}\text{P}\)GTP and [\(\alpha\text{-}^{32}\text{P}\)GTP are resolved by thin-layer chromatography on polyethyleneimine cellulose in a solvent composed of 0.75 \(M\) Tris base plus 0.45 \(M\) HCl.\(^23,\,25\) Radioactivity in each spot is quantitated by Cerenkov counting.

**Competitive Inhibition by G\(\alpha\) Subunits**

Depending on its affinity for the GAP, a second G\(\alpha\) subunit bound to GTP or a GTP analog will competitively inhibit binding of the G\(\alpha\)-[\(\gamma\text{-}^{32}\text{P}\)GTP substrate in a single-turnover GAP assay. Such competition allows the interaction of a GAP with diverse G\(\alpha\) subunits to be analyzed according to standard enzymologic

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\(^{24}\) Commercial [\(\alpha\text{-}^{32}\text{P}\)GTP (>98% pure) is acceptable.

strategies for measuring competitive inhibition).\textsuperscript{10} If the concentration of the \(G\alpha-[\gamma^{32}\text{P}]\text{GTP}\) substrate and its \(K_m\) are known, then the concentration dependence of inhibition will directly yield the affinity of binding of the competing \(G\alpha\) (\(K_d = K_i = IC_{50}/(0.1 + [S]/K_m)\)). This approach is particularly useful when comparing \(G\alpha\) subunits with different intrinsic values of \(k_h\) or \(k_{\text{gap}}\), for studying the effects of mutation or covalent modification of a \(G\alpha\) on GAP binding, where the \(K_m\) for a \(G\alpha\)-GTP complex is too high to allow convenient preparation of high concentrations of \(G\alpha-[\gamma^{32}\text{P}]\text{GTP}\) substrate, or where the \(G\alpha\) is bound to a nucleotide other than GTP.

Once assay conditions have been established for a particular GAP and \(G\alpha-[\gamma^{32}\text{P}]\text{GTP}\) substrate, \(K_i\) is determined by evaluating the apparent \(K_m\) at increasing concentrations of competitive inhibitor.\textsuperscript{8} Plotting the apparent \(K_m\) against the concentration of inhibitory \(G\alpha\) should yield a straight line whose slope is equal to \(K_m/K_i\). More simply, GAP assays are performed at a fixed concentration of \(G\alpha-[\gamma^{32}\text{P}]\text{GTP}\) substrate (near or just above \(K_m\)) in the presence of a range of inhibitor concentrations adequate to yield fractional inhibition of 10–90%. Fractional inhibition should follow a simple saturation function, with half-maximal inhibition occurring at IC\textsubscript{50}. This method is also valuable in that relative values of IC\textsubscript{50} for different inhibitors can be compared even without knowledge of \(K_m\).

For inhibition assays, the concentration of native inhibitor should be determined directly according to nitrocellulose filter binding assays.\textsuperscript{23} Note that the binding of \(\text{AlF}_4^-\) to \(G\alpha\)-GDP is relatively unstable. If the \(G\alpha\)-GDP-\(\text{AlF}_4^-\) is isolated before the assay, it must be used quickly to avoid dissociation.\textsuperscript{26,27} Alternatively, fixed concentrations of \(G\alpha\)-GDP plus 20 \(\mu\text{M}\) \(\text{AlCl}_3\) and 10 \(\text{mM}\) \(\text{NaF}\) can be added directly to the assay medium.\textsuperscript{4} However, because single-turnover GAP assays always contain some \(G\alpha\)-GDP that is formed by hydrolysis of the \(G\alpha-[\gamma^{32}\text{P}]\text{GTP}\) substrate during loading, the excess \(\text{Al}^{3+}\text{F}^-\) will bind this \(G\alpha\)-GDP and thus form another potent inhibitor. Thus, if \(\text{Al/F}\) is not removed before assay, its additional inhibitory activity must be subtracted before inhibition by the alternative \(G\alpha\)-GDP-\(\text{AlF}_4^-\) is evaluated.\textsuperscript{4}

**Steady-State GAP Assays**

Because steady-state GTPase activity depends on both GTP hydrolysis and GDP/GTP exchange, GAP activity can be observed and measured as stimulation of a G protein's steady-state GTPase activity. Stimulation is significant only if the nucleotide exchange rate is sufficiently fast, however. In most cases, GTPase


activity is limited by the rate of GDP release and GTP binding, such that increasing the rate of hydrolysis itself has little effect on steady-state rates (Table I). To observe GAP activity in a steady-state assay, GDP/GTP exchange must therefore be accelerated by a receptor or receptor-mimetic peptide.

Steady-state GAP assays are most useful for studying the coordinated activities of receptor, G protein, and effector during signal transduction, their mechanisms and regulation. In cells, GAPs stimulate the steady-state GTPase activity of a coupled receptor–G protein system during concurrent stimulation of GDP/GTP exchange by agonist-bound receptor and multiple modulating inputs. These interactions are complex and depend both on a network of protein–protein interactions and on the balance of the rates of many partial reactions. Acceleration of hydrolysis can alter the nucleotide exchange activity of receptors, increased availability of Gα-GTP can alter GAP activity, and location of all the proteins at a membrane surface will have an impact on steady-state GTPase activity. GAPs interact with Gβγ, receptors, and other regulatory proteins in addition to Gα, and steady-state assays can be used to evaluate these interactions. Measurement of GAP activity at steady state thus gives the most applicable information about how well a GAP can interact with other signaling components and what its effects on signal output will be: enhanced turn-off rate, attenuated signal, enhanced receptor selectivity, etc. Steady-state GAP assays are also useful when it is difficult to prepare the Gα-GTP substrate for a single turnover assay but where a coupled receptor-G

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**Table 1**

<table>
<thead>
<tr>
<th>Compound</th>
<th>-PLC</th>
<th>+PLC</th>
</tr>
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<tbody>
<tr>
<td>Atropine</td>
<td>0.14</td>
<td>0.25</td>
</tr>
<tr>
<td>Carbachol</td>
<td>0.41</td>
<td>14.4</td>
</tr>
</tbody>
</table>

GTPase depends on both GDP/GTP exchange and GTP hydrolysis steady-state GTPase activity was measured in phospholipid vesicles that contain m1 muscarinic cholinergic receptor and Gβγ, with or without 5 nM phospholipase C-β (PLC) and in the presence of either atropine (antagonist) or carbachol (agonist). Stimulating nucleotide exchange alone (agonist) increases activity only about 3-fold to where hydrolysis becomes rate-limiting. Stimulating hydrolysis alone (with PLC) has little effect because exchange is already rate-limiting. Both agents combine to accelerate steady-state hydrolysis about 100-fold. Data, expressed as moles of P$_i$ released per mole of Gβγ per min, are recalculated from Biddlecome.$^{34}$
protein preparation is available. Last, steady-state assays are much more sensitive for detection of GAP activities than are single turnover assays.\textsuperscript{22,28}

Steady-state assays are less useful for determining a GAP's absolute activity or affinity for substrate because GDP/GTP exchange can become rate-limiting as the hydrolysis reaction is accelerated, and maximal stimulation of hydrolysis cannot be observed. Relative stimulation of steady-state GTPase by a GAP cannot be interpreted as proportional acceleration of the hydrolysis step. In addition, accelerated hydrolysis can itself increase the rate of receptor-catalyzed GDP/GTP exchange and alter its mechanism,\textsuperscript{3} further complicating kinetic analysis of GAP effects. The overwhelming reason to study GAP activity during steady-state GTP hydrolysis is that this is the situation in which GAPs act in cells: on the surface of a membrane, as part of a rapidly cycling multiprotein complex that includes receptor and (probably trimeric) G protein, and in kinetic "competition" with the receptor to determine the fractional activation of the Go subunit.

Receptor–G Protein Vesicles

Steady-state GTPase measurements must be made when both receptor and G protein are incorporated in either natural membranes or reconstituted phospholipid vesicles. Because natural membranes contain enormous nucleoside triphosphatase activity, membrane-based GTPase assays produce low signals over high background; purified and reconstituted systems are the practical alternative. The methods described below are applicable to the G\textsubscript{q}, G\textsubscript{i}, and G\textsubscript{s} families and to appropriate adrenergic and muscarinic cholinergic receptors. These methods should be generally applicable, but each investigator must validate conditions to the receptor and G protein of interest.

Methods for purifying receptors, working with phospholipids, and reconstitution of heterotrimeric G proteins and receptors into unilamellar phospholipid vesicles are outside the scope of this chapter. We have reviewed methods for reconstituting components of G protein signaling pathways\textsuperscript{29,30} and described in detail methods for preparing large unilamellar phospholipid vesicles containing several different receptors and heterotrimeric G proteins.\textsuperscript{3,13,31,32} For most signaling studies, vesicles are prepared with receptor and heterotrimeric G protein in an approximately 1:10 molar ratio, similar to that in cell membranes. Changing


this ratio will have predictable effects on signaling reactions. Equimolar receptor and G protein are more appropriate in a GAP assay, where the intent is to accelerate GDP/GTP exchange and thus make hydrolysis as close as possible to rate-limiting. We also routinely use a Gα:Gβγ ratio of less than 1, usually 0.5, to increase the fractional recovery of Gα during reconstitution and to enhance receptor–G protein coupling. Note that all protein components must be quantitated after reconstitution because recoveries are not uniform. It is advisable to prepare large batches of vesicles so that a single batch can be assayed for its content of receptor and G protein, stored under argon in small aliquots at −80°, and used for multiple experiments. We thaw vesicles only once for use in signaling assays.

**Steady-State GTPase Assays**

GTPase assays are carried out at 30° in a total volume of 30–50 μl in polypropylene tubes for 15 min or less, according to the total GTPase activity and the needs of the experiment. It is generally convenient to prepare a cocktail of assay reagents and to add to it the vesicles, GAP, and any other effectors. GTPase reactions frequently display a pronounced lag before reaching steady state, probably a consequence of the time needed for receptor and G protein to associate in the membrane. Therefore, preincubate all components, including nonradioactive GTP and agonist/antagonist, for 2–4 min at 30° before initiating the assay by addition of [γ-32P]GTP. Preincubation of the GAP with the vesicles should also be evaluated. Soluble GAPs may be added directly to the assay medium, but GAPs that require detergents for solubility must be coreconstituted with receptor and G protein during formation of the vesicles.

The assay buffer contains 20 mM Na HEPES (pH 8.0), 0.1 M NaCl, 1 mM EDTA, 2 mM MgCl₂ (~1 mM free Mg²⁺), 1 mM dithiothreitol, 0.1 mg/ml albumin, 1–10 μM [γ-32P]GTP (concentration depends on observed Kₘ; specific activity ~10 cpm/fmol), 0.1 mM adenyl-5ʹ-yl imidodiphosphate [App(NH)p], and receptor agonist or antagonist. Under these conditions, it is usually convenient to add vesicles that contain 50–200 fmol of G protein. The concentration of GAP to be assayed is determined by its EC₅₀, the minimum detectable signal, and the total amount of GTP hydrolyzed during the assay period. App(NH)p is used to inhibit any contaminating nucleoside triphosphatases and may be omitted if background activity is absent. EGTA may replace EDTA to buffer free Ca²⁺ as desired, with appropriate adjustment to maintain the concentration of free Mg²⁺. We have not seen effects of micromolar Ca²⁺ on GTPase or GAP activities. HEPES buffer is preferred because it decreases background.

GTPase assays are terminated by addition of 950 μl of a cold 5% slurry of charcoal in H₃PO₄, pH 3, and [³²P]P₁ is determined in the supernatant. Background can be high in steady-state GTPase assays, particularly if [γ-32P]GTP is not fresh.
Both zero-time and zero-protein controls should be run routinely. They should give identical results, and the background value should be subtracted from experimental values before data are analyzed.

**Evaluation of Steady-State GTPase Data**

Calculate \[^{32}\text{P}]\text{P}_1\) formed in the total reaction volume, after subtraction of background \[^{32}\text{P}]\text{P}_1\), according to the specific activity of the \[^{\gamma-32}\text{P}]\text{GTP}\) substrate. GTPase rates are expressed in terms of moles of GTP hydrolyzed per unit time. Further normalization to the amount of G\(\alpha\) (mol GTP/min/mol G\(\alpha\)) is useful if other parameters are constant—concentration of receptor, lateral density of the proteins in the bilayer, etc.\(^{33}\) In general, a complete data set consists of four values: GTPase activities measured the presence of agonist, GAP, both, and neither. Data should ideally be expressed as a molar turnover number: moles of GTP hydrolyzed per minute per mole of G\(\alpha\) (determined in a parallel \[^{35}\text{S}]\text{GTP}\gamma\text{S}\) binding assay). Because even unliganded receptors can often promote GDP/GTP exchange at a low level, a better estimate of activity at the basal GDP/GTP exchange rate can sometimes be obtained by adding receptor ligands that inhibit exchange catalyst activity ("inverse agonists").

GAP activity during steady-state GTP hydrolysis can be evaluated at various mechanistic or descriptive levels. Routinely, relative (-fold) stimulation of agonist-stimulated GTPase activity at saturating GAP concentration is a reliable measure of a GAP’s net activity.\(^{22}\) However, as the GAP accelerates steady-state GTPase activity by increasing the hydrolytic rate, GTPase activity will reach a maximum when GDP/GTP exchange becomes rate-limiting. A low exchange rate will therefore put a somewhat artifactual upper limit on the observable GAP activity.

This change in the predominantly rate-limiting step can be inferred from both the small effect of GAPs observed in the absence of agonists and from the change in agonist potency in the presence and absence of a GAP. Without agonist, a GAP has little effect; the same is true for an agonist without and with a GAP (Table I). In the absence of a GAP, the EC\(_{50}\) for agonist will frequently be well below the \(K_d\) (apparent "spare receptors") because stimulation of GDP/GTP exchange by only a small number of agonist-bound receptors increases GTPase activity to the point where hydrolysis of G\(\alpha\)-bound GTP becomes rate-limiting. In the presence of a GAP, the EC\(_{50}\) for agonist approximates the agonist–receptor \(K_d\) because more agonist-bound receptors will be required to keep up with hydrolysis.\(^{6}\) Similarly, the potency of a GAP in a steady-state assay, its EC\(_{50}\) at saturating agonist, may

\(^{33}\) Not all of the G protein in reconstituted vesicles is accessible to receptors, presumably for geometric reasons.\(^{29,30}\) The pool of "coupled" G protein, usually 30–50% of the total, is defined as that which undergoes rapid GDP/GTP exchange in response to agonist. For most mechanistic studies, the concentration of coupled G protein should be used to calculate reliable turnover numbers and specific activities.
or may not approximate its $K_d$ for binding to the Gα–GTP complex. GAPs and receptors can influence each other's activities in complex ways. However, when rate constants for the individual reactions of the GTPase cycle were used to predict steady-state $K_m$ and $V_{max}$ according to the Briggs–Haldane equation, the predicted values were consistent with those measured directly at steady state.

The interactions of these kinetic parameters, determined by careful parallel monitoring of hydrolytic and GDP/GTP exchange rates, can give considerable mechanistic information about the coordinated action of receptor and GAP on the activation of the G protein. Such studies form the basis for analyzing secondary regulatory effects: allosteric modulators, covalent modification of the proteins, and modulation of protein concentration.

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