Review Article

Probing the GTPase cycle with real-time NMR: GAP and GEF activities in cell extracts

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ABSTRACT

The Ras superfamily of small GTPases is a large family of switch-like proteins that control diverse cellular functions, and their deregulation is associated with multiple disease processes. When bound to GTP they adopt a conformation that interacts with effector proteins, whereas the GDP-bound state is generally biologically inactive. GTPase activating proteins (GAPs) promote hydrolysis of GTP, thus impeding the biological activity of GTPases, whereas guanine nucleotide exchange factors (GEFs) promote exchange of GDP for GTP and activate GTPase proteins. A number of methods have been developed to assay GTPase nucleotide hydrolysis and exchange, as well as the activity of GAPs and GEFs. The kinetics of these reactions are often studied with purified proteins and fluorescent nucleotide analogs, which have been shown to non-specifically impact hydrolysis and exchange. Most GAPs and GEFs are large multidomain proteins subject to complex regulation that is challenging to reconstitute *in vitro*. In cells, the activities of full-length GAPs or GEFs are typically assayed indirectly on the basis of nucleotide loading of the cognate GTPase, or by exploiting their interaction with effector proteins. Here, we describe a recently developed real-time NMR method to assay kinetics of nucleotide exchange and hydrolysis reactions by direct monitoring of nucleotide-dependent structural changes in an isotopically labeled GTPase. The unambiguous readout of this method makes it possible to precisely measure GAP and GEF activities from extracts of mammalian cells, enabling studies of their catalytic and regulatory mechanisms. We present examples of NMR-based assays of full-length GAPs and GEFs overexpressed in mammalian cells.

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1. Introduction

1.1. Small GTPase protein family

The human genome encodes 167 Ras superfamily small GTPase proteins [1,2] that behave in a similar switch-like fashion, but control diverse cellular functions. These proteins have been grouped in five subfamilies on the basis of sequence homology and cellular function: Ras (39 proteins), Rho (22 proteins), Rab (65 proteins), Arf (30 proteins), and Ran (the sole member of its family), and an additional 10 sequences remain 'unclassified' [1,2]. When bound to GTP, these small GTPase proteins adopt a biologically active conformation capable of interacting with and activating effector proteins by regulating their subcellular localization or assembly into signaling complexes, with possible allosteric contributions. In general, GDP-bound small GTPases are inactive and fail to bind effector proteins. Ras family members display intrinsic catalytic activity to hydrolyze GTP, which limits the duration of the signaling event. GTPase activating proteins (GAPs) interact with GTPases and promote GTP hydrolysis by providing catalytic residues and/or stabilizing a catalytically competent conformation of a GTPase. Inactive GTPase can be reactivated through the release of GDP and binding of a new GTP molecule. Such nucleotide exchange occurs slowly due to the high affinity of both nucleotides, but is markedly accelerated by guanine nucleotide exchange factors (GEFs), which interact with their cognate GTPase and displace bound nucleotide. Collectively, these processes comprise the GTPase cycle (Fig. 1a).

1.2. GTPase assays

The regulation of GTPase signaling is of critical importance, as mutation or unregulated expression of small GTPase proteins or their respective GAPs and GEFs is frequently associated with diseases such as cancer [3]. Thus, a number of methods have been devised to measure the activity of these regulatory proteins, both
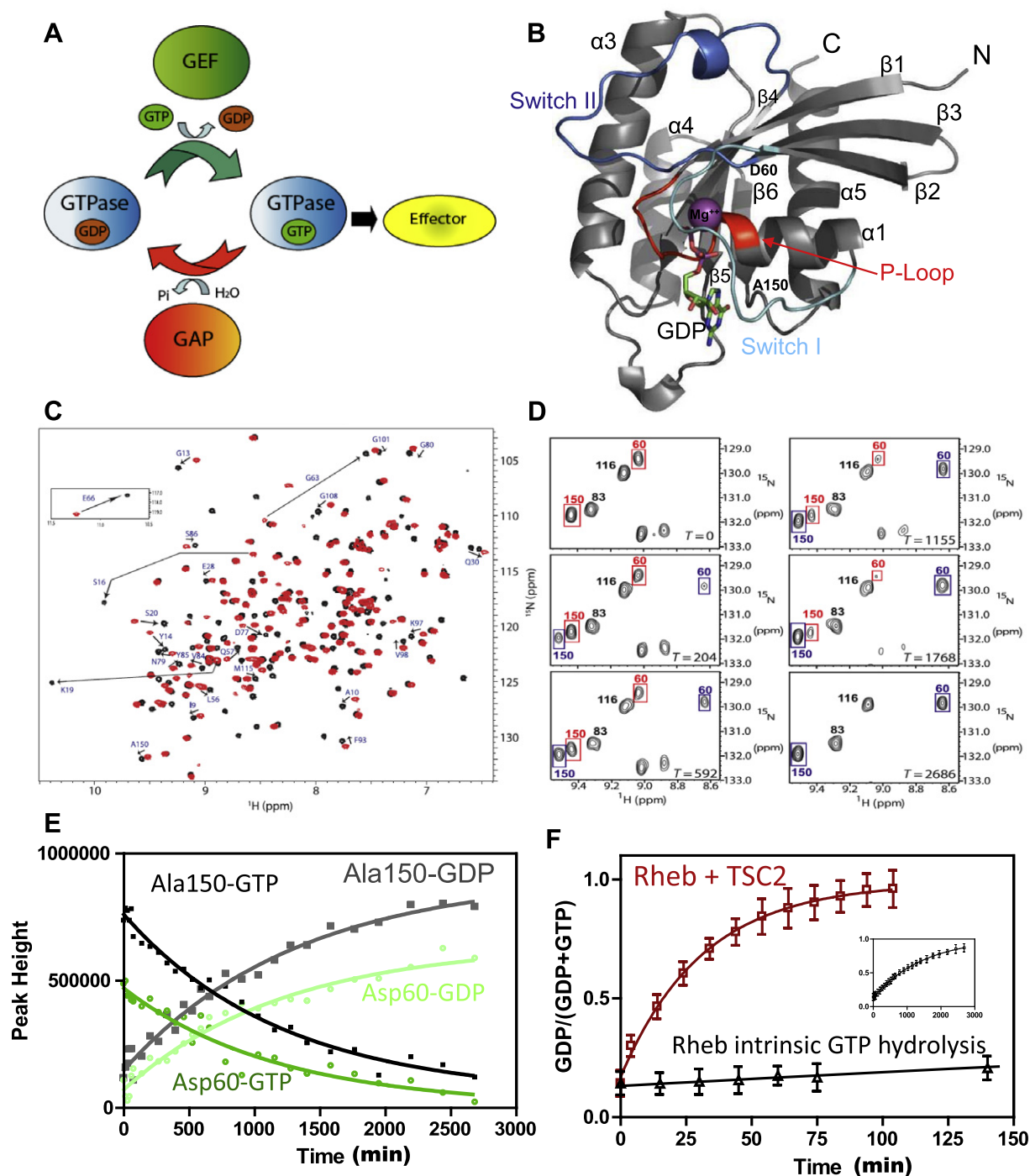
Fig. 1. Principle of real-time NMR-based GTPase assay. The method is illustrated using GTP hydrolysis by Rheb as an example. (A) Schematic of the GTPase cycle. GTP-bound small GTPase proteins adopt an activated conformation capable of interacting with effector proteins, whereas the GDP-bound conformation is inactive. GTPases possess an intrinsic catalytic activity to hydrolyze GTP, which is accelerated by the activity of GTPase activating proteins (GAPs). Inactive GTPase can be reactivated by binding a new molecule of GTP, which is markedly accelerated by guanine nucleotide exchange factors (GEFs) that promote the release of GDP. (B) Small GTPase structure. Small GTPases share the same basic topology, illustrated here by the example of Rheb-GDP (PDB ID: 1XTQ). The switch I and switch II regions, which undergo conformational change upon nucleotide cycling, are colored cyan and blue, respectively, the phosphate-binding loop (P-loop) is red, the nucleotide is colored by atom type, and a Mg^{2+} ion required for high-affinity nucleotide binding is magenta. The position of residues D60 and A150 are indicated, as their resonances are illustrated in panels D and E. (C) ^1H–^{15}N-HSQC spectra of Rheb in the GDP- and GMPPNP-bound states (black and red, respectively). Assignments of key residues are indicated with arrows showing chemical shift changes. The assay uses cross-peak intensities of ^{15}N-labeled amides of the GTPase protein that are specific to the activated or inactive form. (D) Snapshots of ^1H–^{15}N-HSQC spectra of GTP-loaded Rheb at various times (indicated in minutes) as GTP is hydrolyzed. Rheb-GTP and Rheb-GDP cross-peaks are indicated in red and blue boxes, respectively, and peaks that are insensitive to GTP hydrolysis are labeled in black. (E) Peak heights (arbitrary units) for Rheb residues Ala 150 (black) and Asp 60 (green) in the GTP- (dark shades) and GDP- (light shades) bound forms versus time. Well-resolved peaks from 22 Rheb residues were analyzed in this manner. (F) Hydrolysis of GTP by wild-type Rheb alone (triangles and inset) or in the presence of the GAP domain of TSC2 (squares) versus time. Data are presented as fraction GDP derived from peak intensities [I_{GDP}/($I_{GDP} + I_{GTP}$)] at each time point for 22 residues. This figure is adapted from Marshall et al. Science Signaling 2 (2009) ra3.

in vitro and *in vivo*. Using purified proteins *in vitro*, nucleotide hydrolysis can be monitored by loading the GTPase with GTP and subjecting aliquots harvested over a time course to HPLC analysis of bound nucleotides [4]. Alternatively, release of ^{32}Pi from a ^{32}Pi-GTP-loaded GTPase sample can be used as a readout for nucleotide hydrolysis [5]. With purified proteins, nucleotide exchange can also be monitored in real time using fluorescent nucleotide analogs such as 2'(3')-*O*-(*N*-methylanthraniloyl)-(mant)-GTP [6] on the basis of differences in the fluorescence of GTPase-bound nucleotide versus free nucleotide in solution. The assay can be performed by monitoring either the binding [7–9] or release [10,11] of the tagged nucleotide. In some cases nucleotide hydrolysis reactions can also be monitored by fluorescence, if hydrolysis of the tagged nucleotide and/or the structural change in the protein is associated with a sufficient change in fluorescence properties of a tagged nucleotide [12]. Although mant-tagged nucleotides have been extensively used for fluorescence-based GAP and GEF assays with a number of GTPase proteins, we have shown that the mant tag can substantially alter the kinetics of many of these reactions in an unpredictable manner [13]. Reactions with native nucleotides have been monitored in real time using biosensors developed to detect the release of Pi or GDP in nucleotide hydrolysis or exchange assays, respectively [14,15].

Most GAPs and GEFs are large multidomain proteins regulated by complex inputs involving autoinhibition, phosphorylation, interactions with other proteins or membranes, or changes in subcellular localization [16]. This makes reconstitution of these regulatory mechanisms *in vitro* a challenge. The activities of full-length GAPs or GEFs in cells are commonly assayed indirectly by analyzing the effect of their overexpression on the activation state of the cognate GTPase. This has been achieved by analysis of the nucleotides bound to the GTPase of interest, following immunoprecipitation from cell lysates. If the cellular pool of nucleotides is labeled by incorporation of ^{32}Pi, the GDP:GTP ratio can be analyzed by thin layer chromatography [17,18]. This method is highly sensitive, but requires the use of radioactivity and the availability of suitable antibodies, and is sensitive to GTP hydrolysis during processing of cell lysates. It has been used extensively for characterizing the intrinsic GTPase activity of Rheb and its sensitivity to the GAP activity of TSC2 [19–25].

More recently, affinity-based assays of cellular GTPase activation have been developed that exploit the GTP-dependent interaction between a GTPase and its effector protein. The fraction of a GTPase that is GTP-loaded can be pulled down by immobilized GTPase-binding domain, and compared to the total amount of the GTPase in the lysate. For example, the Ras-binding domain (RBD) of Raf-1 fused to glutathione-*S*-transferase (GST) can selectively pull down Ras-GTP from cell lysates [26,27]. The Cdc42/Rac interactive binding (CRIB) domain from PAK1 fused to GST has been used to isolate activated Rac and Cdc42, which can then be specifically detected by Western blotting [28]. Similarly, the Rho-binding domain of Rhotekin has been used to assay activation of Rho GTPases (RhoA, B, and C) [29,30]. These methods suffer from poor reproducibility, and can only be applied to those GTPases for which a well characterized effector domain is available.

An alternate approach is to immobilize mutant forms of GTPases shown to be constitutively nucleotide-free or GTP-bound to selectively pull down activated GEFs or GAPs, respectively [31]. This assay is based on the principle that a GTPase bearing mutations that disrupt the nucleotide binding site will form stable interactions with GEFs, if the active site of the GEF is accessible and functional. Likewise, GTPases with catalytic residue mutations will often form stable, non-productive interactions with activated GAPs. These assays need to be validated for each system as they may not be universally applicable. Another recently reported assay uses a split luciferase system in which the GTPase and an effector domain are each fused to luciferase fragments such that functional luciferase enzyme activity is assembled in a GTP-dependent manner [32]. These fusions are produced in *Escherichia coli*, and their chemiluminescence serves as a read out of GTPase activation that can be used as an indication of GAP or GEF activities in recombinant proteins, cell lysates or immunoprecipitates. Finally, numerous biosensors have been developed to visualize the temporal and spatial patterns of activation of several GTPase proteins in live cells. 'Unimolecular' biosensors are genetically encoded fusion proteins comprised of fluorescence resonance energy transfer (FRET) donor and acceptor fluorescent proteins, a small GTPase protein and an effector domain linked such that the GTP-dependent interaction between the GTPase and its effector generates FRET by bringing the donor and acceptor fluorescent proteins together [33–36]. Thus FRET-based biosensors provide powerful tools that report on the spatio-temporal activation of a GTPase in a living cell, but they do not dissect the contributions of GAP versus GEF activities, nor do they report on reaction kinetics.

We have developed a real-time NMR-based assay to monitor hydrolysis or exchange of native nucleotides by recording nucleotide-dependent structural changes in a ^{15}N- (or ^{13}C-) labeled GTPase protein over time [37]. This can be used to assay intrinsic nucleotide exchange or hydrolysis, as well as the activities of purified GEFs or GAPs. Further, the unambiguous readout allows real-time assay of the kinetics of nucleotide exchange or hydrolysis to be performed in a complex mixture of proteins, including mammalian cell extracts. Here we present examples of NMR-based GAP and GEF assays for several GTPases performed using purified recombinant GAP or GEF domains and full-length proteins in crude mammalian cell lysates.

1.3. Ras, Rheb, and RhoA signaling

Following is a brief overview of the proteins discussed in this manuscript, which are reviewed more thoroughly in references [38–40]. Ras is the founding member of the small GTPase family, and among the best studied due to the prevalence of mutations that impair Ras GTP hydrolysis in many types of cancer [41]. There are three main Ras isoforms (H-Ras, K-Ras and N-Ras), which have highly conserved GTPase domains (residues 1–171), but bear substantially different C-terminal hypervariable regions that regulate subcellular localization and biological function. Ras interacts with and activates a large number of effector proteins bearing Ras-binding domains (RBDs) or Ras association (RA) domains, including PI3 kinase, the Raf kinases, RalGEFs, and the RacGEF Tiam1 [41]. Ras is regulated by only a handful of GAPs (including neurofibromin and p120) and GEFs (including Cdc25-domain proteins Sos, RasGRP and RasGRF). Rheb is a conserved GTPase that is 34% identical to H-Ras [19,42,43] and activates the protein kinase mTOR, considered the master regulator of cell growth and protein synthesis [44]. Rheb is regulated by the GAP activity of TSC2, which heterodimerizes with TSC1 to form a complex that integrates signaling from growth factors, nutrients, oxygen and cellular energy status [22,25,44–52]. Rheb exhibits low intrinsic GTPase activity relative to Ras, and there is disagreement regarding the identity of the GEF [53–55]. Rho family GTPases are important regulators of the cytoskeleton and cell motility, and the RhoA protein plays a crucial role in cellular processes including proliferation, movement, cell shape, and interactions with other cells and the matrix [56]. The human genome encodes ~85 GEFs for Rho subfamily GTPases, most of which contain a catalytic Dbl-homology (DH) pleckstrin-homology (PH) domain [57,58] as well as a number of various scaffolding and regulatory domains [16,59]. A subset of RhoGEFs including PDZ-RhoGEF and GEF-H1 (also known as Lfc in the mouse) are activated by G-protein coupled receptors (GPCRs) through Gα$_{12/13}$. Unlike other RhoGEFs, Lfc is sequestered in an inactive state on

microtubules (MTs) [60,61], and can be activated by MT-depolymerizing compounds (e.g., nocodozole), or stimulation by lysophosphatidic acid (LPA) or thrombin [61–63].

Using our NMR assay with recombinant purified proteins we examined GAP activity from the GAP domains of TSC2 and p120GAP, and GEF activity of the Rem/Cdc25 domain of Sos (SOS-cat) and the DH-PH domain of PDZ-RhoGEF. We then extended this method to characterize GAP and GEF activities in cell extracts, studying full-length TSC1/2 and characterizing the complex regulation of Lfc, which involves numerous cellular proteins and could not be reconstituted in vitro.

2. GTPase assays of purified proteins by real-time NMR

NMR has proven to be a powerful tool for the study of enzymatic catalysis over the decades, with pioneering work by Mildred Cohn and Albert Mildevan on bacterial enzymes including pyruvate carboxylase and kinase, and alkaline phosphatase [64–66]. NMR studies on small GTPases started in the mid-1980s [67–69], but were focused on static structures and backbone dynamics of these proteins, alone or in complex with GAPs, GEFs or effectors. Real-time NMR describes a process of collecting sequential NMR spectra as a reaction or process occurs in a sample. By contrast, time-resolved NMR collects a single spectrum at each of multiple time points after a 'trigger' event in a series of experiments. For example, recovery of a light-sensitive LOV domain was monitored by time-resolved NMR following pulses of illumination [70]. Real-time NMR has been extensively used to study protein folding, generally by monitoring peaks in one-dimensional proton spectra known to derive from the unfolded or folded states, although slow folding events have been monitored using two-dimensional spectra [71,72]. Other examples of real-time NMR include monitoring the sequential deamination of cytidines in HIV DNA by a human anti-HIV protein [73], and the sequential phosphorylation of a labeled peptide microinjected into Xenopus oocytes [74]. In fact, real-time NMR has previously been applied to Ras, as it was demonstrated that the intensities of unassigned downfield resonances in the proton NMR spectrum of Ras change with a time course that correlates with GTP hydrolysis [75]. Here we describe the application of two-dimensional real-time NMR to small GTPase cycling.

2.1. The assay principle

Small GTPases undergo structural changes upon nucleotide cycling, adopting distinct conformations dependent on binding to GTP or GDP. The most substantial changes occur in two 'switch' regions (Fig. 1b), which are flexible and can be stabilized in specific orientations by interaction with the nucleotide. These structural changes perturb the chemical shifts of residues in the switch regions and nucleotide binding site, and often propagate extensively throughout the protein (Fig. 1b,c). Even in the absence of a substantial conformational change, nucleotide cycling would be expected to cause chemical shift perturbations because (i) the chemical environment of residues near the γ-phosphate is altered, and (ii) there are often profound nucleotide-dependent changes in the dynamics of GTPase proteins that promote effector binding of the activated form. This assay uses cross-peak intensity of ^{15}N-labeled amides that are specific to the GTP- or GDP-bound form of a GTPase as a readout of the fraction of GTPase bound to each nucleotide (Fig. 1d,e). GTPases bind guanine nucleotides with high affinity (low- to sub-nM K_d) and slow k_{off} rates, thus for all GTPases we have studied, each pair of cross-peaks corresponding to a residue in the GDP- and GTP-bound conformations exhibits slow exchange on the NMR chemical shift timescale. By collecting successive NMR spectra, it is possible to monitor intrinsic

hydrolysis (or exchange) of nucleotides in real time (Fig. 1f). Upon assigning these resonances, it is possible to monitor the protein on a residue-by-residue basis in real time as the reaction proceeds. The activity of GAPs (or GEFs) can be assayed by adding the respective unlabeled proteins to the reaction (Fig. 1f).

2.2. Experimental protocols

Labeled small GTPase proteins (Rheb, Ras, and RhoA) were expressed in E. coli in M9 media supplemented with ^{15}N ammonium chloride. Typically the GTPase domain alone (Rheb 1–169, Ras 1–171, RhoA 1–181) is used for these assays, however, the C-terminal hypervariable region can be included provided it does not substantially reduce protein solubility or quality of the NMR spectrum. Rheb, H-Ras and RhoA were expressed using pGEX2T, pET15b and pET28 vectors, respectively. As described previously, tagged GTPases were purified by affinity chromatography (glutathione Sepharose for GST-Rheb and Ni–NTA resin for His-H-Ras and His-RhoA), tags were removed by thrombin cleavage and monomeric GTPases were further purified by gel filtration chromatography (Superdex 75) [7,37,76]. Samples were concentrated, and if necessary exchanged into NMR buffer (e.g., 25 mM sodium phosphate or HEPES pH 7.0, 100 mM NaCl, 5 mM MgCl$_2$, 10% D$_2$O and 1 mM DTT).

The recent development of the NMR microcryoprobe (e.g., Bruker 1.7 mm), which offers significantly higher mass sensitivity (signal/noise ratio per unit mass) than conventional 5 mm probes, has substantially reduced the amount of labeled protein required to perform real-time NMR assays. The 1.7 mm probe is also more tolerant of salts in the sample. The sample concentration must be sufficiently high for a good quality HSQC spectrum to be collected in a short time relative to the half life of the reaction. A conventional 5-mm cryoprobe requires a 500 μl sample of protein concentration 100–300 μM (i.e., 1–3 mg for a typical ~20 kDa GTPase protein) to collect a high-quality HSQC in 5 min (2 scans). Using a microcryoprobe, the volume of the sample can be reduced to as little as 35 μL, and a concentration of 200–500 μM is usually sufficient, thus only ~200 μg protein is required per sample. Expression of the GTPases described above typically yield 5–15 mg of pure protein per liter of E. coli culture. Because of their high affinity for nucleotides, GTPases co-purify with guanine nucleotide from E. coli in the presence of Mg^{2+}, which is important to stabilize the protein. Once purification is complete, spectra of Ras and RhoA usually indicate that they are completely GDP loaded, whereas Rheb is typically bound to a mixture of GDP and GTP, due to its weak intrinsic GTPase activity. A uniform GDP-bound sample can be achieved by allowing GTP hydrolysis to proceed overnight at room temperature, or the sample can be loaded with GTP as described below.

Intrinsic nucleotide exchange is measured by adding excess GTP or a non-hydrolyzable analog to the sample before commencing data collection. GEF-catalyzed nucleotide exchange can be assayed by adding an appropriate quantity of GEF (full length protein or the catalytic domain). To assay intrinsic GTP hydrolysis, a GTP-loaded sample must be prepared, which can be achieved by incubating the protein (~10 min at 37 °C or longer at room temperature) in the presence of 10-fold molar excess GTP and 10 mM EDTA. EDTA sequesters the Mg^{2+} ion that is required for high affinity nucleotide binding, thus accelerating nucleotide exchange [77]. Nucleotide exchange with RhoA required the addition of 0.5 M urea to achieve full GTP loading [13]. An alternative protocol we have used to load RhoA with GTP involves the addition of a small amount of immobilized GEF (e.g., glutathione Sepharose-bound GST-fused DH-PH domain of PDZ-RhoGEF) with excess GTP. Following the exchange, MgCl$_2$ is added to a final concentration of 20 mM to stabilize the newly bound nucleotide, and the sample passed through a gel

filtration or desalting column (PD MidiTrap[TM] G-25 (GE healthcare)) equilibrated with NMR buffer to remove excess nucleotide. The eluted sample is then quickly concentrated before collection of successive HSQC spectra to monitor GTP hydrolysis. For the GAP assay, purified full-length GAP or a fragment comprising the GAP domain is added to the reaction.

The GAP domain of TSC2 (residues 1525–1742) was expressed as a GST fusion from the pGEX2T vector, and the GAP domain of p120 RasGAP (residues 715–1047, also called GAP-334) [78] was prepared as a His-tagged protein from the pET15b vector. The DH-PH catalytic fragment of PDZ-RhoGEF (residues 713–1081: hereafter termed DH-PH[PRG]) was prepared using pGEX4T1 vector, and the catalytic Rem/Cdc25 domain of the Son of Sevenless (SOS-[cat], residues 566–1049) was prepared as a His-tagged protein from the pET15b vector. These protein fragments were purified by affinity chromatography, and cleaved from their tags with thrombin, followed by final purification by gel filtration chromatography.

2.3. Results

2.3.1. Rheb and TSC2GAP

Although Rheb is closely related to Ras, substitutions at key residues suggested a different mechanism of hydrolysis. For example, Rheb has an arginine (Arg15) in the highly conserved position homologous to Ras Gly12, mutations of which (including G12R) impair the intrinsic GTPase activity of Ras [79–83]. While Rheb exhibits much lower intrinsic GTPase activity than Ras, mutagenesis suggested that this is not a direct function of the Arg substitution, as the Arg15Gly mutation did not restore GTPase activity [19,20,24]. Using the NMR method, we were able to determine that the Arg15Gly mutation actually decreased the intrinsic Rheb GTPase activity slightly [37], which was not previously detected by less sensitive methods. We found that the Q64L mutation only slightly decreased catalytic activity [37], highlighting mechanistic differences between Rheb and Ras. These results agree with previous in vivo and in vitro studies, but provided more detailed kinetic information.

To study the GAP activity of TSC2, we designed a GAP domain construct comprised of residues 1525–1742, which when added to Rheb-GTP at a 1:2 molar ratio increased the rate of GTP hydrolysis by 50-fold (Fig. 1d). At a 1:50 ratio to Rheb, no GAP activity could be detected for TSC2$_{1525–1742}$, thus the activity of TSC2$_{1525–1742}$ is less potent compared to RasGAPs (see Section 2.3.2), which may be related to low affinity for Rheb. It remains to be determined whether there are additional binding sites for Rheb in the TSC1/2 complex outside the GAP domain, but it should be noted that Rheb and TSC2 coimmunoprecipitation has not been reported, suggesting interaction between the native proteins is transient. TSC2-GAP utilizes an 'asparagine thumb' mechanism [37,84,85], that is distinct from the 'arginine finger' of the GAPs for Ras and Rho GTPases [80,86], and mutations of the putative catalytic residue of TSC2 (N1643) have been found in tuberous sclerosis patients [87–90]. We used our NMR-based method to assess the role of Asn1643 in TSC2-GAP activity, and found that the GAP activity of TSC2$_{1525–1742}$ was completely abolished by mutation of Asn 1643 to Ala, as well as by conservative mutations to Asp or Gln [37], demonstrating a strict requirement for precise positioning of a carboxamide for catalysis.

Hundreds of mutations in TSC1 and TSC2 have been found in tuberous sclerosis patients, several of which map to the TSC2 GAP domain [88]. We applied our assay to examine how some of these disease-associated mutations affect the GAP activity of TSC2$_{1525–1742}$ [37]. A disease-associated mutation of the catalytic residue, N1643I [90,91], rendered TSC2$_{1525–1742}$ inactive, as expected, as did two other proximal mutations (H1640Y [92] and K1638N [91]). Among disease-associated mutations predicted to

be located on the same face of the GAP domain as the catalytic residue, we found that the charge perturbing mutation E1558K [89,93] eliminated GAP activity and the conservative L1594M substitution [88] reduced GAP activity by approximately 80%, whereas the D1690Y mutation [94] did not impair GAP activity of TSC2$_{1525–1742}$, despite its association with tuberous sclerosis disease. We were also able to show that a TSC2 polymorphic variant (D1636N) found in a TSC patient carrying an additional mutation [95] had no direct effect on GAP activity.

2.3.2. RasGAPs and RasGEFs

Compared to TSC2, the GAP domain of p120 (GAP-334) had more potent GAP activity towards Ras: a molar ratio of only 1/5000 RasGAP to GTP-loaded H-Ras was required to achieve a 6-fold increase in the nucleotide hydrolysis rate (Fig. 2a). The GTPase reaction was accelerated in a linear manner with the amount of GAP-334 added over the range examined. The catalytic Cdc25 domain of Sos (Sos[cat]) accelerated nucleotide exchange 8-fold when added to Ras at a molar ratio of 1:5000, and exchange was similarly accelerated in a linear relationship with the addition of Sos[cat] (Fig. 2b).

2.3.3. PDZ-RhoGEF

The DH-PH domain of PDZ-RhoGEF exhibited very potent exchange activity, which could be detected even at sub-nM concentrations, representing a 1:500,000 ratio relative to RhoA [96]. Using this assay, we were able to compare nucleotide exchange of RhoA-GDP in the presence of free GTP versus the 'reverse' reaction (i.e., RhoA-GTP to GDP), without the interference of fluorescent tags (see Section 2.3.4). The general mechanism of GEF function involves interactions with the cognate GTPase that destabilize the bound nucleotide and promote its release [16]. Higher concentrations of GTP than GDP in mammalian cells favor activation of the small GTPase, because it is more likely that the displaced nucleotide will be replaced with GTP than GDP. It has often been assumed that GEFs simply accelerate equilibration, i.e., that they would displace GDP and GTP from the GTPase equally well, thus catalyzing nucleotide exchange in both directions. In contrast, we found that DH-PH[PRG] catalyzes exchange of RhoA-GDP to GTP much more strongly than the reverse reaction, suggesting that DH-PH[PRG] is selective for the GDP-bound conformation of RhoA [96].

2.3.4. Effects of fluorescently tagged nucleotides

Using this assay, we examined how modification of nucleotides with the commonly used mant fluorophore affects hydrolysis and exchange reactions. Mant did not substantially affect intrinsic Ras nucleotide hydrolysis, however mant-GTP was hydrolyzed by Rheb >10 times faster than was native GTP, whereas RhoA hydrolyzed mant-GTP 3.5-fold more slowly than GTP [13]. Further, mant-GTP was hydrolyzed by Ras 5-fold faster than GTP in the presence of p120GAP, whereas the GAP activity of TSC2 for Rheb was severely impaired by the presence of the mant tag [13]. Finally the ability of DH-PH[PRG] to catalyze nucleotide dissociation from RhoA was 6-fold lower for mant-GDP than GDP, and the Cdc25-catalyzed association of mant-GTP with Ras was 3-fold slower than GTP [13]. These results indicate the unpredictable effects of mant-tagged nucleotides and highlight the advantage of the NMR method, which does not require any chemical modifications.

2.4. General considerations

The assay requires that the GTPases be soluble and stable for at least 1–2 days. In the majority of structural studies, only the GTPase domains are used, with the hypervariable regions and lipidation sites truncated. The assay readout is based on
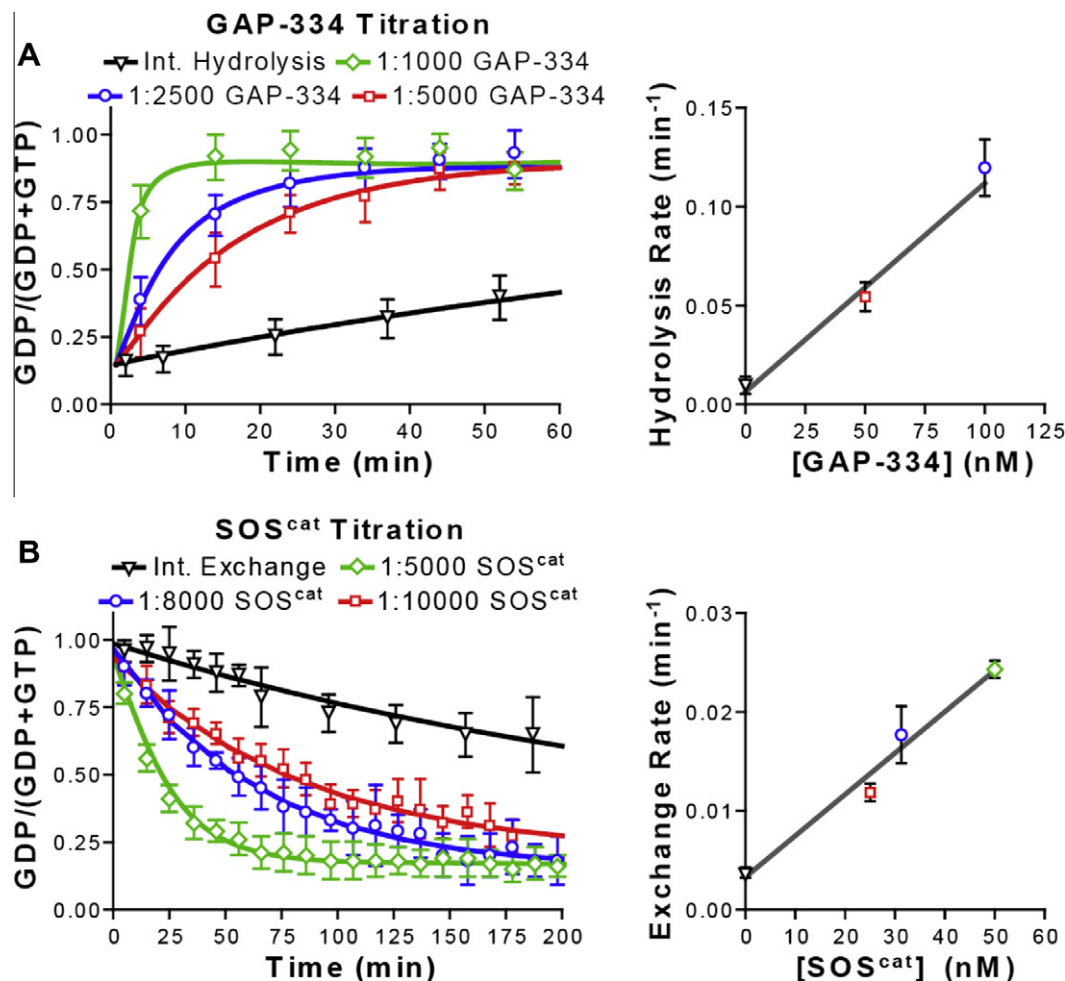
Fig. 2. Catalytic activity and concentration dependence of Ras GAP and GEF domains monitored by real-time NMR. (A) The catalytic activity of GAP-334 (catalytic domain of p120 Ras GAP). Hydrolysis of GTP by Ras alone (triangles) or in the presence of increasing ratios of GAP-334:Ras, as indicated. Right panel, dependence of reaction rate on GAP concentration. (B) The nucleotide exchange activity of Sos^{cat} (catalytic domain of Sos). Intrinsic Ras nucleotide exchange (GDP to GTP) (triangles), and exchange catalyzed by increasing ratios of Sos^{cat}:Ras, as indicated. Right panel, dependence of exchange rate on Sos^{cat} concentration.

conformation-specific NMR amide resonances, thus any misfolded protein that may be present does not contribute to these peaks. Furthermore, aggregated protein is generally invisible to NMR due to the large particle size, thus the assay contains a 'built-in' control for folding of the GTPase. Aliquots of GTPase can be snap frozen in NMR buffer, stored at −80 °C and thawed for assays as required, with no appreciable effect on spectral quality. For GTP hydrolysis assays, it is particularly useful to freeze several aliquots of GTP-loaded protein, thawing them immediately prior to performing an assay.

Acquisition of data of sufficient quality to perform kinetic analyses may require range-finding experiments to match the timing of data collection to the rate of the reaction. For intrinsic hydrolysis or exchange assays, each spectrum can usually be collected using 2–4 scans (requiring 5–10 min). In assays of GAP or GEF activities, it is necessary to determine a suitable GAP or GEF concentration that accelerates the reaction rate significantly, but not beyond the limit of the rate of sampling. For example, DH-PH^{PRG} showed robust GEF activity at a ratio of 1:100,000 GEF:RhoA [96]. If this ratio was increased to 1:5000 the half life of the exchange reaction decreased to ~5 min, and could not be monitored using a standard HSQC pulse sequence. However, fast reactions can be monitored using specific pulse sequences, such as the SOFAST HMQC [72], which allowed us to collect spectra in 30 s using a 5 mm cyroprobe [37]. In this case, the limiting factor becomes the time required to add

nucleotide and GEF to the NMR tube, insert it into the magnet, and tune, match and shim the instrument and calibrate the ^{1}H pulse. However, it should be possible to initiate the reaction in the magnet using the rapid mixing devices designed for real-time NMR protein folding studies [97]. In contrast to the potent exchange activity of DH-PH^{PRG}, Sos^{cat} should be added at ~10-fold higher concentrations for optimal rates in exchange assays. Whereas our analysis of the GAP activity of TSC2 on Rheb required that the GAP domain be added at a ratio of 1:2 to achieve a substantial increase in hydrolysis, GAP-334 can be used at ~100-fold lower concentrations. These examples illustrate the need to optimize the quantity of GAP or GEF used.

In nucleotide exchange assays the choice of nucleotide analog is important, and should be validated for the GTPase used. We performed a RhoA exchange assay using native GTP, and by subtracting the known rate of GTP hydrolysis, we calculated a theoretical GTP exchange curve [96]. While guanosine 5′-[γ-thio]triphosphate (GTPγS) exchanged at a rate similar to this theoretical curve, guanosine 5′-[βγ-imido]triphosphate (GMPPNP) exchanged at half this rate, presumably due to lower affinity. Further the spectrum of RhoA-GTPγS was more similar to RhoA-GTP than was RhoA-GMPPNP [96]. For a GTPase such as Rheb, where the exchange rate is much faster than the hydrolysis rate, native GTP can be used in exchange assays because the contribution of hydrolysis is negligible.

The reaction buffer used should be optimized for solubility of the GTPase protein and quality of NMR spectra. A typical buffer is 20 mM HEPES pH 7.0, 100 mM NaCl, 5 mM MgCl$_2$, 10% D$_2$O with DTT or TCEP as a reducing agent, if required. The assay can be carried out over a range of temperatures. We usually perform GTPase assays at 20–25 °C although we have used temperatures between 15 and 30 °C for analysis of thermodynamics of GTP hydrolysis. The low temperature limit will be defined by the NMR sensitivity, whereas the upper temperature limit will be set by the stability of the proteins.

While we usually label the GTPase with ^{15}N, we have also used ^{13}C labeling and ^{1}H^{13}C HSQCs for detection. ^{13}C labeling offers the advantage of higher sensitivity detection of methyl groups, which could be further enhanced by deuterating the protein and selectively ^{1}H^{13}C-labeling Ile, Leu and Val [98], which may enable cleaner and more accurate detection of GTPase signals at low concentrations (<100 μM).

3. GTPase assays of cell lysates by real-time NMR

In the examples discussed above, the real-time NMR assay was used to measure the activities of purified GAP or GEF domains. Because the assay observes a single labeled protein, it is possible to measure GAP or GEF activities from cell lysates. Although there are multiple GAPs and GEFs for many GTPases (e.g., ~80 RhoGEFs in mammalian cells), we have found that in many cases the activity of a single overexpressed protein can be readily detected above the background of the endogenous proteins.

3.1. Principle and experimental protocols

Isotopically labeled small GTPase proteins were prepared as described above. Mammalian cells (e.g., HEK293T) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and transfected with mammalian expression vectors encoding the GAP or GEF of interest. Transfection and expression conditions should be optimized for high protein expression (see Section 3.3.2), and some considerations are discussed below. After a period of 24–48 h following transfection, cells were harvested using one of two methods. Cells were either scraped from the plate in a minimal volume (150 μl for a 6 cm plate) of lysis buffer (e.g., 1% Triton-X, 20 mM HEPES pH 7.5, 100 mM NaCl with Complete Protease Inhibitor cocktail (Roche) and PMSF), or harvested in PBS by scraping, then pelleted by centrifugation and snap frozen for future lysis.

Cell lysates were cleared by brief centrifugation (16,000 g for 30 s) and the total cellular protein in the supernatant was analyzed using the Bradford assay (BioRad). A concentration of 10–20 μg/μl total protein in the lysate is desirable. If the catalytic activity of an overexpressed GEF or GAP is to be compared under different conditions it is important to standardize the amount of the exogenous protein used in each assay to adjust for variations in expression levels. This can be achieved by Western blots of the lysates, although the time required for this analysis necessitates freezing the sample until the result is available. While freezing had no impact on the activity of many of the proteins we have studied, we consistently observed a loss of Lfc GEF activity after cell lysates were frozen and thawed. Thus, to immediately assess the Lfc expression level, we expressed eGFP-tagged Lfc and measured the eGFP fluorescence of a 1/10 dilution of the lysate using a Shimadzu RF-5301PC spectrofluorophotometer with excitation and emission wavelengths of 488 nm and 509 nm, respectively. The amount of lysate added to each assay was normalized according to the eGFP fluorescence, and subsequently verified by Western

blots [99]. Using this approach we have achieved highly reproducible exchange activity between lysates.

We prefer to perform assays of cell extracts using a 1.7 mm cryoprobe to reduce the sample requirements. First, a reference spectrum is collected from a 35 μl sample of the labeled GTPase alone (0.2–0.5 mM). In a GAP assay, this spectrum also confirms that the GTPase is fully GTP-loaded, then 3.5 μl of standardized cell extract is added (Fig. 3). In a GEF assay, the nucleotide exchange reaction is initiated by the simultaneous addition of GTPγS or GMPPNP (to a 10-fold molar excess over the GTPase) and 3.5 μl of standardized cell lysate. Data collection is subsequently initiated as rapidly as possible (Fig. 3).

3.2. Results

3.2.1. GAP activity of TSC1/2

Rheb is an example of a GTPase for which no effector-binding assay of activation is available, as no soluble Rheb-binding domain of mTOR has been identified. Thus in vivo assays of Rheb activation have relied primarily on TLC analysis of the bound radiolabeled guanine nucleotides, or the detection of phosporylation of p70 S6K or 4EBP1 as indicators of the kinase activity of the Rheb effector mTOR [19–25,100]. The development of an NMR-based assay for Rheb GAP activity in cell extracts makes a valuable contribution to the analysis of mTOR signaling.

Using the NMR-based assay, we have shown that we can detect (i) the endogenous GAP activity for Rheb in crude extracts of mammalian cells, (ii) an increase in Rheb GAP activity associated with TSC2 overexpression, and (iii) a loss of this activity in TSC2 knockout cells (limitations of this approach are discussed in Section 3.3.3). HEK293T cells were grown on 10 cm plates in DME media with 10% FBS, serum was removed and cells were transfected with both TSC1 and TSC2 (12 μg of each plasmid, using calcium phosphate-mediated transfection) or left untransfected. Cells were harvested in 200 μl lysis buffer, and 10 μg of cellular protein was added to a 35 μl sample of 0.4 mM ^{15}N Rheb in each assay. The addition of cell lysate from untransfected cells reduced the half-life for Rheb GTP hydrolysis from 14 h in the presence of lysis buffer, to 7.5 h, whereas lysate from cells coexpressing TSC1/2 reduced this time to 3.5 h (Fig. 4a). To investigate whether we could detect a loss of Rheb GAP activity associated with knockout of the TSC2 gene, we assayed the GAP activity of 15 μg of cellular protein in lysates from TSC2$^{-/-}$ and littermate TSC2$^{+/+}$ MEFs (obtained from David Kwiatkowski [101]) grown in the absence of growth factors (serum-free DME media) to stimulate TSC2 activity. Relative to Rheb's intrinsic GTPase activity in the presence of lysis buffer, TSC2$^{+/+}$ lysate accelerated GTP hydrolysis fourfold whereas only a small increase in GTPase activity was associated with the addition of TSC2$^{-/-}$ extracts (Fig. 4b). These results demonstrate the power of the real-time NMR method to detect Rheb GAP activity in a whole cell extract, enabling new approaches to study the TSC–Rheb–mTOR signaling pathway.

3.2.2. GEF activity of PDZ-RhoGEF

In contrast to GAP activity for Rheb, whereby TSC2 is the only validated specific GAP, mammalian cells contain >80 GEFs for Rho family small GTPases. While the specificities of many of these GEFs remain unknown, several have been shown to activate RhoA. To investigate whether we could detect the exchange activity of a single overexpressed RhoGEF against this background, we expressed full-length PDZ-RhoGEF (15 μg DNA per 10 cm plate using calcium phosphate-mediated transfection) and examined the RhoA exchange activity in the lysate. Compared to untransfected cells, lysates of cells overexpressing PDZ-RhoGEF (40 μg total protein) exhibited a 16-fold increase in RhoA exchange activity (Fig. 5), demonstrating that the activity of this overexpressed GEF can be
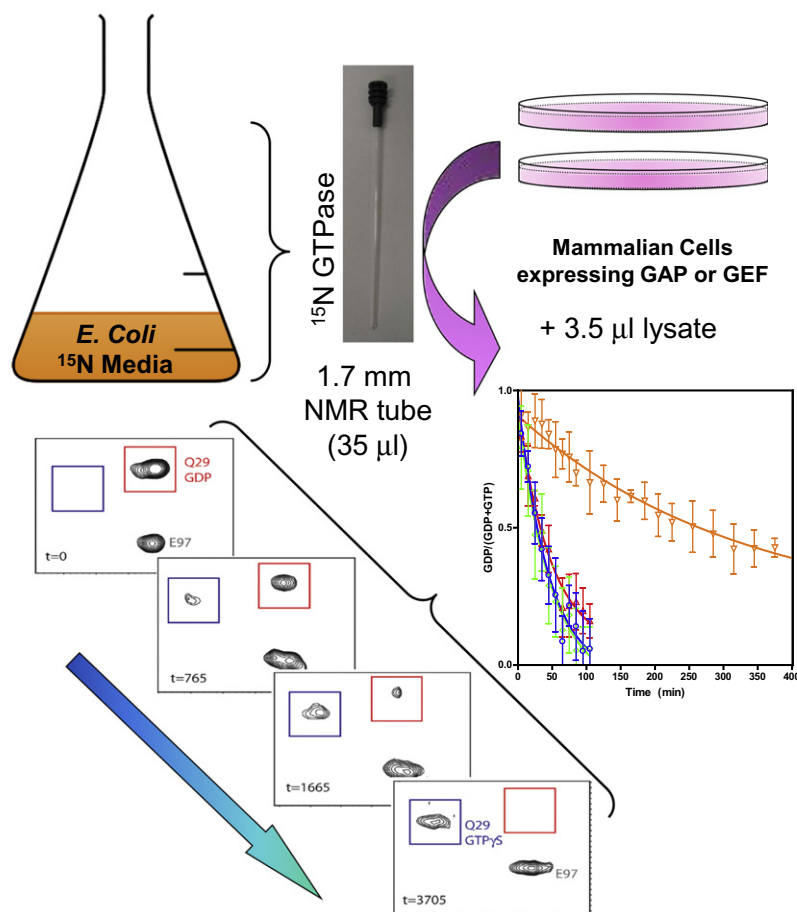
Fig. 3. Overview of real-time GTPase assay of GAP or GEF activities in cell extracts. ^{15}N-labeled small GTPases proteins are overexpressed in *Escherichia coli* and purified. For a GAP assay, the sample is loaded with GTP. With a 1.7 mm microcryoprobe, 3.5 µl of cell lysate (containing ~10–50 µg total cellular protein) is added to a 35 µl GTPase sample. In a GEF assay, GTP (or a non-hydrolyzable analog) is added to a 10-fold excess relative to the GTPase. This sample is inserted into the magnet and successive ^{15}N–^{1}H-HSQC spectra are collected (snapshots adapted from Meiri et al. Mol. Cell 45 (2012) 642–655). Nucleotide exchange or hydrolysis curves are plotted from cross-peak intensities of the GTPase protein that are specific to the activated or inactive form as illustrated in Fig. 1.

clearly detected above the endogenous GEFs. Lysate from untransfected cells increased the exchange rate ~2-fold relative to lysis buffer alone.

3.2.3. Correlation between DNA transfected and Lfc GEF activity

Because the cell lysate is a complex mixture of proteins, and the composition can be perturbed by overexpression of signaling factors, we sought to demonstrate that the readout is specific to the protein of interest. HEK293T cells were transfected with peGFP-C1 (Invitrogen) encoding a Lfc-GFP fusion protein, using a range of DNA concentrations to determine the relationship between the measured GEF activity and the level of over-expression of the trans gene. HEK293T cells cultured on 6 cm plates were transfected with 0–4 µg vector using PolyFect transfection reagent (Qiagen). After 48 h cells were harvested in 100 µl of lysis buffer containing 1% Triton X-100, which yielded samples containing 14–21 mg/ml total protein. After normalizing samples on the basis of total protein, GEF assays were performed using 36 µg of cellular protein from each lysate and samples were analyzed by Western blot using anti-Lfc for detection. The Western blot confirms that Lfc expression increased with the amount of DNA transfected (Fig 6, inset), and the RhoA nucleotide exchange rates in the presence of the cell lysates correlated well with the amount of DNA transfected (Fig. 6). As a demonstration of specificity of the assay, we have shown that overexpression of Lfc has no effect on the exchange activity of

Rac1, and that overexpression of Lfc T247F, a structure-guided mutation predicted to impair the catalytic activity of Lfc, has a severely reduced effect on RhoA exchange [99].

3.2.4. Inhibition of Lfc GEF activity by Tctex-1

We next sought to investigate whether this assay is sufficiently sensitive to detect the effect of a regulatory protein on the activity of a GEF. Lfc is a GEF that can be sequestered on microtubules (MTs) in an inactive state [102]. We identified Tctex-1, a dynein motor light chain, as a factor that interacts with Lfc and is required for Lfc recruitment to MTs [99]. To investigate whether Tctex-1 directly inhibits the RhoA nucleotide exchange activity of Lfc, we purified recombinant full-length Lfc and Tctex-1 proteins for NMR assays. Recombinant Lfc was catalytically active and interacted with recombinant rTctex-1, although its GEF activity was not inhibited by rTctex-1 [99]. However, the addition of rTctex-1 to lysates of cells overexpressing Lfc reduced the RhoA nucleotide exchange rate by 50%, and coexpression of Tctex-1 with Lfc inhibited its GEF activity more effectively, implicating another cellular factor in the mechanism of inhibition [99]. An Lfc variant lacking the Tctex-1 binding site (δ87–151Lfc) was slightly more active than wild-type Lfc and was not inhibited by co-expression of Tctex-1 [99]. To investigate whether polymerized microtubules are required for the inhibition of Lfc by Tctex-1, cells were treated with the microtubule depolymerizing agent, nocodazole. Upon
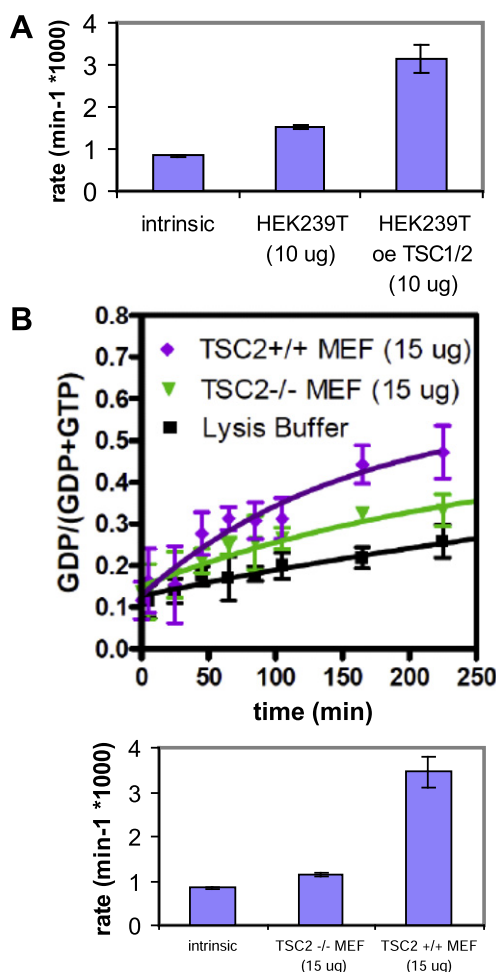
Fig. 4. GAP activity of overexpressed and endogenous TSC2 in cell lysates. (A) GTP hydrolysis rates for Rheb alone versus Rheb plus cell extract (10 μg total protein) from untransfected HEK293 cells or cells overexpressing TSC1 and TSC2. (B) Hydrolysis of GTP by Rheb in the presence of lysis buffer, or extracts (15 μg total protein) from TSC2$^{+/+}$ or TSC2$^{-/-}$ MEFs.

Fig. 5. GEF activity of overexpressed PDZ-RhoGEF in cell lysates. (A) RhoA nucleotide exchange curves in the presence of extracts from untransfected HEK293 cells versus those overexpressing PDZ-RhoGEF (adapted from Meiri et al. Mol. Cell 45 (2012) 642–655). (B) Rates of RhoA nucleotide exchange in the presence of lysis buffer (intrinsic), or 40 μg total protein from extract of untransfected HEK293 cells, or HEK293 cells overexpressing myc-tagged PDZ-RhoGEF, which was detected in a Western blot (anti-myc, inset).

Fig. 6. Correlation of amount of DNA transfected with Lfc expression level and GEF activity. HEK293T cells cultured on 6 cm plates were transfected with increasing amounts of peGFP-C1 DNA encoding Lfc-eGFP (0–4 μg). GEF assays were performed using 36 μg of cellular protein from each lysate. Inset, Lfc expression in each lysate was assessed in a Western blot (anti-Lfc) and GAPDH levels were analyzed to ensure equal loading.

nocodazole treatment, the RhoA nucleotide exchange activity in lysates of cells overexpressing Lfc increased 1.5-fold, and the co-expression of Tctex-1 failed to inhibit Lfc [99].

We had previously shown that Lfc is inhibited by PKA phosphorylation of S885 [103], thus we investigated whether PKA plays a role in Tctex-1-mediated inhibition of Lfc. The Lfc mutant S885A, which blocks phosphorylation, interacted with Tctex-1 normally and was recruited to microtubules; however this did not affect its exchange activity [99]. Furthermore the PKA inhibitor H89 impaired the ability of Tctex-1 to inhibit Lfc exchange activity. These results suggest that recruitment of Lfc to the microtubules by Tctex-1 promotes its phosphorylation and inhibition by PKA. In this example, even if the macromolecular microtubule and dynein motor structures are not preserved upon cell lysis, the inhibitory effects of the phosphorylation events are maintained. These results show how the cell extract-based NMR assay can be used to dissect a complex cellular regulatory mechanism (see Section 3.3.3).

3.2.5. Scaling down cell culture for increased throughput

Assays using the 1.7 mm micro-cryoprobe require only a small amount of cell lysate. Although we usually culture cells in 3.5, 6.0 or 10 cm plates, only 1–5% of the lysate from these plates is required for each assay. To investigate whether the assay could be adapted to the 96-well arrays utilized in various high throughput
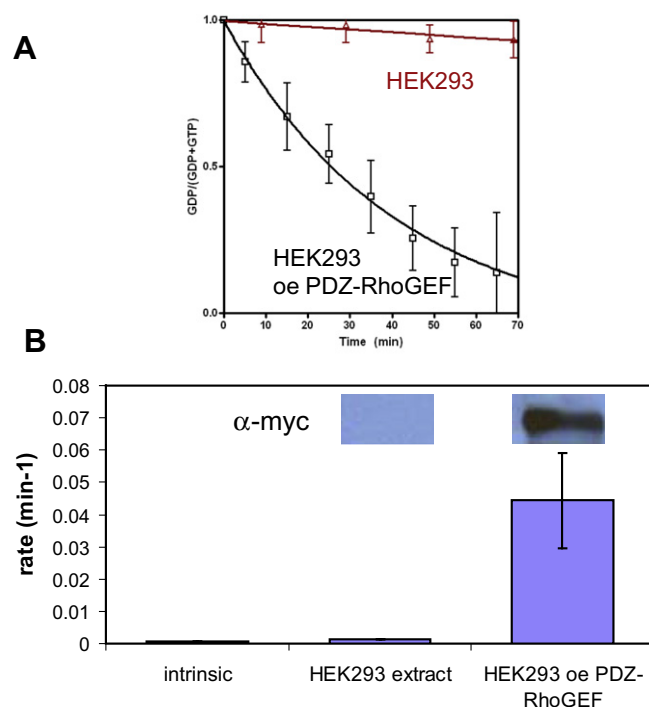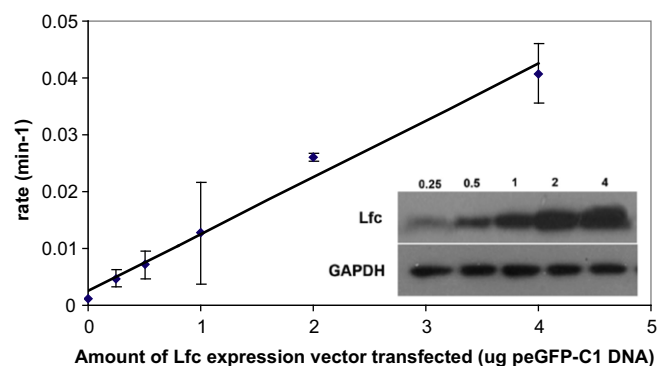
methods, HEK293T cells were cultured in 3.5 mm wells of a 96-well plate and transfected with Lfc-GFP or GFP. The cells were harvested in 10 μl 2× lysis buffer yielding total protein concentrations of 10–15 μg/μl, and exchange assays were performed with 35 μg of protein (Fig. 7). A robust 15-fold increase in RhoA exchange activity was seen in cells overexpressing Lfc-GFP versus GFP. Thus the cost media and transfection reagents can be substantially reduced by scaling down the cell culture.

Availability of NMR spectrometer time is the most limiting factor in performing these assays. Assays of rapid reactions (high GEF
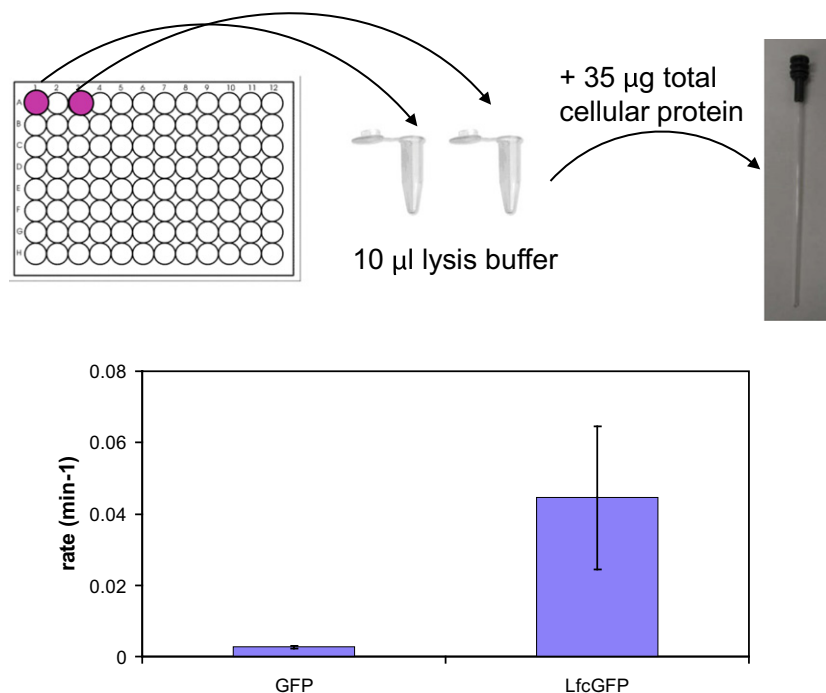
Fig. 7. Scaling down cell culture for real-time NMR assays. HEK293T cells cultured in 3.5 mm wells of a 96-well plate were transfected with Lfc-GFP or GFP. The cells were harvested in 10 µl lysis buffer and RhoA nucleotide exchange assays were performed with 35 µg total cellular protein. Resulting exchange rates are shown in the lower panel.

or GAP activity) take ∼ 1 h, whereas control or inhibited samples require several hours and are usually run overnight. With slow reactions, it is possible to assay several samples in parallel using an automated sample changer (e.g., Bruker SampleJet), although this necessitates reducing the frequency of spectra acquisition (temporal resolution) for each sample.

3.3. General considerations

3.3.1. Controls and standardization

It is important to run several controls to distinguish the activity of overexpressed proteins of interest from endogenous GEF/GAP activities and intrinsic exchange/hydrolysis reactions. Intrinsic exchange/hydrolysis rates of the GTPase should be determined with the addition of lysis buffer alone. Next, the basal activity of endogenous GAPs or GEFs should be established by the addition of lysate from untransfected cells, or cells transfected with empty vector or an unrelated gene (e.g., GFP). Depending on the experimental design, addition of lysates can be standardized on the basis of total cellular protein, Western blots for the protein of interest, or the fluorescence of a GFP-tagged overexpressed protein.

Overexpression of one GAP or GEF may affect the activity or expression level of endogenous proteins, and as with any cell-based assay, this has the potential to impact the NMR method. When the protein-of-interest is highly overexpressed, the contribution of endogenous proteins should be relatively minor; nevertheless this should be considered and controlled for. It should also be noted that a small amount of guanine nucleotide is carried into the reaction with the cell lysate. However the intracellular guanine nucleotide concentration (∼500 µM GTP, ∼150 µM GDP [104]) is diluted by at least 2–3 orders of magnitude in the NMR sample, thus the final concentration is very low relative to the GTPase protein concentration (200–500 µM). The cellular guanine nucleotide does not appreciably affect most assays, and is controlled for by assaying lysates of untransfected (or empty vector-transfected) cells to establish basal rates.

3.3.2. Cell type, transfection and lysis conditions

We have overexpressed proteins in a variety of cell types for this assay (e.g., MEFs, NIH3T3, Rat2, HEK293/293T). In general, HEK 293T cells are particularly well suited, because they are rich in protein, efficiently transfected, and express high yields of the trans-gene. We expect that any expression vector and transfection method that leads to high expression would be suitable. We have used various vectors including pcDNA3.1, pFlag-CMV2, and peG-FP-C1 with a variety of transfection methods including calcium phosphate, polyethylenimine, and Polyfect (Qiagen). We generally use 1% Triton X-100 in our lysis buffer, and have increased this to 2% to compensate for dilution when cells are harvested in a very small volume of lysis buffer in order to increase protein concentration. It is important that the detergent does not negatively impact the quality of the NMR spectra of the GTPase, and does not affect the rate of the nucleotide hydrolysis/exchange reactions being monitored. If necessary, detergent can be avoided by using hypotonic lysis, freeze/thaw cycles, or passage through a low-gauge syringe. Inclusion of protease inhibitors in the lysis buffer is recommended, but we have generally avoided using general phosphatase or kinase inhibitors in an attempt to preserve the overall balance of phosphorylation of the target protein. Lfc and TSC1/2 are heavily phosphorylated proteins and most of the sites are uncharacterized, thus there is not sufficient information regarding which kinases and phosphatases to inhibit. In a more completely characterized system, it may be desirable to employ inhibitors to 'lock' the phosphorylation state of the protein of interest.

3.3.3. Limitations

Most small GTPases are found tethered to cellular membranes through prenylated C-termini, and thus many regulatory events involve subcellular localization of signaling proteins (GAPs, GEFs and effector proteins) to the membranes where they encounter the small GTPases with which they interact. For example, the RasGEF Sos is recruited to the plasma membrane via interactions with Grb2 at activated growth factor receptors, resulting in co-localiza-

tion and activation of its target, Ras [105,106]. This type of regulatory mechanism may not be detected in the NMR-based assay, as cell membranes are disrupted upon lysis and the ^{15}N-labeled GTPase providing the readout is not attached to a membrane. In the case of Lfc, the inhibitory effect of MT-localization was preserved after disruption of the cell, due partly to inhibitory post-translational modifications. Another important consideration is that many GEFs are regulated by complex autoinhibitory mechanisms [105,107–112], thus it may be necessary to determine specific conditions to activate these proteins in order to measure GEF activity.

In the case of Rheb, we were able to observe a substantial reduction in GAP activity in TSC2-null versus wild-type MEFs. This is likely because TSC2 is probably the major, if not the only GAP with specific activity towards Rheb. It will be more challenging to detect the loss of activity associated with depletion of a GAP or GEF whose activity overlaps other proteins. For example, to date we have not successfully detected a loss of RhoA exchange activity in lysates of Lfc-null versus wild-type MEFs.

4. Data processing

Data processing involves picking the amide cross-peaks associated with one form of the protein (GDP-bound in an exchange assay and GTP-bound in a GTPase assay) in the first spectrum, and the alternate form in the final spectrum. Residues that exhibit distinct, well-resolved amide cross-peaks in each nucleotide-bound state were used as indicators of the reaction progress, and all report consistent rates. Often, some peaks derived from the switch regions are broadened in the GTP-bound form. Such peaks should be avoided if possible, but if not severely broadened, they can be included in the analysis by normalizing the peak heights. The heights (I) of the reporter peaks are extracted from the spectra collected at each time point, which can be performed by adapting any NMR software with the capacity to analyze NMR relaxation data. We processed spectra with NMRPipe [113] and analyzed peak heights using the NMRPipe script SeriesTab, Sparky3 (Goddard and Kneller, University of California, San Francisco) or NMRView [114]. In most cases, the progress of the reaction is monitored by quantifying the fraction of GTPase protein in the GDP-bound state $[I_{GDP}/(I_{GDP} + I_{GTP})]$ for each reporter residue at each time point. To determine the hydrolysis rate (k_{hy}), these data are then fitted to a one phase exponential association function using PRISM (GraphPad software):

$$[I^*_{GDP}/(I^*_{GDP} + I^*_{GTP})](t) = 1 - \exp(-k_{hy} \times t) \quad (1)$$

where I^*_{GDP} and I^*_{GTP} are the peak heights of the GTPase bound to GDP or GTP, respectively.

RhoA bound to native GTP exhibited broadened and split peaks that complicate peak height extraction [96], thus we used the increasing intensities of the resonances from the GDP-bound form to monitor hydrolysis:

$$[I^*_{GDP}/I^{*f}_{GDP}](t) = 1 - \exp(-k_{hy} \times t) \quad (2)$$

where I^{*f}_{GDP} is the GDP peak height upon completion of hydrolysis.

Nucleotide exchange assays performed with non-hydrolyzable GTP analogs were fitted to a single phase exponential decay equation to determine the exchange rate k_{ex}.

$$[I_{GDP}/(I_{GDP} + I_{GTP\_analog})](t) = \exp(-k_{ex} \times t) \quad (3)$$

Analyses of nucleotide exchange with native GTP must consider the hydrolysis of the newly bound GTP. Equations to estimate the true exchange rate based on the observed exchange rate and known hydrolysis rate are described in reference [13].

5. Discussion, conclusions and future directions

We have shown that real-time NMR can be applied to assay GEF and GAP activities of either purified recombinant proteins, or a complex mixture of proteins in cell lysates by virtue of detection of a single isotopically labeled GTPase protein. This method requires recombinant expression of a labeled, soluble, and stable GTPase domain. While we have demonstrated the method for a number of examples, the variety of small GTPases that have been studied successfully by NMR and crystallographic methods suggests this method will be widely applicable to numerous Ras superfamily members.

Unlike effector domain pull-down assays, this method can be used to study the many GTPases for which a well-characterized, specific effector domain is not available (e.g., Rheb). The method does not require chemical modification of the nucleotide or the GTPase, such as the fluorescent tags that significantly perturb some GTPase reactions. The specific isotopic labeling could also be advantageous by enabling the monitoring of one GTPase in a system that may be regulated by other GTPases, for example, activation of the RhoGAP activity of RLIP76 by Ral GTPases [115], or the interplay between the subunits of the heterodimeric Rag GTPases [116,117].

Using cell lysates, we have shown that we can detect the activity of endogenous GAPs and GEFs, and that the activity of an over-expressed protein can be clearly observed above this background. Our analysis of TSC2-null MEFs indicates that the assay can detect the loss of activity associated with the depletion of a GAP or GEF, at least in cases where there is no substantial overlapping enzymatic activity present in the cell. This suggests that the assay could also be used to characterize the effects of transient gene knockdowns. However, we have not demonstrated a loss of enzymatic GEF activity in Lfc-null MEFs due to the abundance of other RhoGEFs in the cells, indicating a limitation of this approach. Nevertheless, we were able to dissect a complex mechanism by which a RhoGEF is regulated by coexpressing Lfc with its inhibitor Tctex-1, and probing the effects of mutagenesis and pharmacological agents on GEF activity. Although the regulation of Lfc by Tctex-1 depends on subcellular localization, i.e., recruitment to microtubules, the inhibition was maintained after cell lysis, probably involving phosphorylation. Regulatory mechanisms involving biological membranes may not be accessible by this method, which uses GTPase domains that lack prenylation sites. We are actively pursuing development of NMR methods to study membrane-tethered GTPases, which are less sensitively detected by NMR. Ultimately, the challenge will be to monitor GTPases in living cells by NMR, which requires efficient delivery of labeled proteins across the plasma membrane and a highly sensitive detection strategy.

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