Interactions of the ras-like protein p25^{rab3A} with Mg²⁺ and guanine nucleotides

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The *rab3A* gene product is a 25 kDa guanine-nucleotide-binding protein which is expressed at high levels in neural tissue and has about 30 % sequence similarity to *ras*. Purified p25^{rab3A} has been used as a substrate to examine its kinetics of nucleotide binding and hydrolysis, and to study the effects of Mg²⁺ on these processes. p25^{rab3A} binds GDP and GTP similarly well, with nanomolar affinity. Mg²⁺ increases the affinity between p25^{rab3A} and guanine nucleotides by 3- and 7-fold for GTP and GDP respectively, primarily by drastically decreasing the nucleotide off-rates. The Mg²⁺ binding affinity to p25^{rab3A}·[α -³²P]GDP was determined to be about 4 μ M using entrapment of [α -³²P]GDP as a measure of Mg²⁺ binding. At a Mg²⁺ concentration of 11 mM, GTPase activity was rate-limited by the GDP off-rate. Surprisingly, at a Mg²⁺ concentration of 80 nM, GTPase activity was comparable with that in the presence of excess Mg²⁺. In this case, k_{cat} was rate-limiting. At Mg²⁺ concentrations below 10 nM there was no detectable GTPase activity, indicating that Mg²⁺ is required for the GTPase activity of p25^{rab3A}.

INTRODUCTION

Guanine-nucleotide-binding proteins function as molecular switches to control a wide variety of cellular processes [1–4]. Three major classes of GTP-binding proteins have been described: the large heterotrimers which transduce hormonal signals, the initiation and elongation factors which control vectorial processes in protein synthesis, and the ras protein-like monomeric GTP-binding proteins, the functions of which are largely unknown [2,3,5,6]. All of these GTP-binding proteins have a high specificity for GTP and GDP and a low intrinsic GTPase activity. Hydrolysis of bound GTP switches these proteins between their active (GTP-bound) and inactive (GDP-bound) conformations. The hydrolysis rate acts as a timing device which determines how long a GTP-binding protein is 'on'. Alterations of normal kinetics of GTP binding and hydrolysis can have drastic consequences; the *ras* proteins and G_s are rendered oncogenic by mutations which cause either a decrease in intrinsic GTPase activity or an increase in the rate of nucleotide exchange [5,7–10].

GTP-binding proteins also bind Mg²⁺. The interaction of Mg²⁺ with the α -subunit of the heterotrimeric G-proteins (G α) has been studied in detail and is quite complex. Mg²⁺ ions have been shown to promote association of guanosine 5'-[γ -thio]triphosphate (GTP[S]) with G α . Mg²⁺ induces a conformational change in the protein which stabilizes the GTP[S]·G α complex, but has no effect upon the GDP·G α complex [11,12]. Mg²⁺ is also absolutely required for the GTPase activity of G α , but at a much lower concentration than is necessary for stabilization of GTP[S]·G α , which suggests there are at least two binding sites for Mg²⁺ to GTP[S]·G α [11–14]. X-ray crystallographic analysis of p21^{ras} and EF-tu shows that Mg²⁺ is associated with the β - and γ -phosphates of GTP [15,16].

The kinetics of the nucleotide and Mg²⁺ interactions with members of the family of the ras-like GTP-binding proteins have not been investigated in detail. However, it is clear that they differ substantially from those of both the heterotrimeric G-proteins and the initiation and elongation factors. It is becoming apparent that substantial differences also exist between members

of the *ras* superfamily, although except for *ras* [17,18], few detailed studies have been reported [19,20].

p25^{rab3A} is a ras-like GTP-binding protein of unknown function, with approx. 30 % sequence similarity with p21^{ras}. We and others have shown that p25^{rab3A} is expressed at detectable levels only in exocrine tissues and is distributed approximately equally between cytosolic and particulate fractions [21–25]. In the present paper, the kinetics of nucleotide binding and hydrolysis by p25^{rab3A} are described. We also examine the Mg²⁺ binding kinetics and its effects upon the GTPase activity of p25^{rab3A}.

MATERIALS AND METHODS

Materials

[α -³²P]GDP (approx. 3000 Ci/mmol) and [γ -³²P]GTP (5000 Ci/mmol) were purchased from Dupont–New England Nuclear. Protease inhibitors and CHAPS detergent were from Boehringer Mannheim. Nitrocellulose filters were from Schleicher and Schuell and Millipore. The remainder of the reagents were from standard vendors. Conversion of [α -³²P]GTP to [α -³²P]GDP was performed as described previously [26].

Purification of rab3A

Rab3A protein was purified from bovine brain membranes to apparent homogeneity as described previously [21,27,28]. All of the GTP-binding activity of the preparation could be immunoprecipitated by antisera raised against recombinant rab3A protein [28], indicating that there were no other contaminating GTP-binding proteins in the preparation.

Nucleotide binding assays

Binding of α - or γ -³²P-labelled guanine nucleotides to purified p25^{rab3A} was performed in 50 mM-Hepes, pH 7.4, 100 mM-KCl, 0.6 % CHAPS, 1 mM-dithiothreitol (DTT) and 10–20 μ Ci of labelled nucleotide (buffer A). Insulin (400 μ g/ml) was added as a carrier protein. In order to efficiently load p25^{rab3A} with labelled nucleotide, it was necessary to ensure that the protein was predominantly in the apo form. This was accomplished by first diluting p25^{rab3A} in buffer A containing 10 mM-EDTA, in a

Abbreviations used: G α , α -subunit of G-protein; DTT, dithiothreitol; GTP[S], guanosine 5'-[γ -thio]triphosphate.

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volume such that the final concentration of endogenous nucleotide released from p25^{rab 34} would be < 20% of the dissociation constant. Labelled nucleotide was then added for 1–5 min at a specific radioactivity of 0.5–3.0 $\mu\text{Ci}/\text{pmol}$. A 2–5 μl portion of stock solution of nucleotide–protein complex was then added to reaction buffers (50–100 μl). Binding experiments were performed at 30 °C unless noted. All solutions were prewarmed prior to use. Aliquots (5–25 μl) of the reaction mix were removed at the indicated time intervals, added to 5 ml of ice-cold quenching solution (20 mM-KH₂PO₄, pH 6.5, 10 mM-MgCl₂, 1 mM-ATP and 1 mM-DTT) and washed through 0.45 μm nitrocellulose filters [29]. Radioactivity remaining on the filters was a measure of bound nucleotide. Background counts were no more than 2% of total bound ³²P.

Determination of kinetic constants

Guanine nucleotide off-rates were determined as follows. p25^{rab 34} was prebound to [α -³²P]GDP or [α -³²P]GTP as described above. The labelled complex (2–5 μl) was added to buffer A (100 μl) containing either 10 mM-MgCl₂ or 10 mM-EDTA plus a 1000-fold excess of unlabelled nucleotide, and aliquots were withdrawn at specified time intervals and assayed by filter binding. Because of the intrinsic GTPase activity of p25^{rab 34}, the off-rate of GTP in the presence of 10 mM-MgCl₂ was determined indirectly. p25^{rab 34} prebound to [γ -³²P]GTP was incubated with buffer A under the conditions described above, and assayed by filter binding. Loss of radioactivity as a function of time under these conditions is due both to cleavage of the γ -phosphate and to exchange of [γ -³²P]GTP with unlabelled GTP; thus it is a measure of $k_{\text{cat.}} + k_{\text{off}}$ for GTP·Mg²⁺ (method 3). By subtracting the value for $k_{\text{cat.}}$ determined independently (see below, methods 1 and 2), we were able to obtain an estimate of k_{off} for GTP·Mg²⁺.

Dissociation constants were obtained for GDP and GTP by incubating apo-p25^{rab 34} (0.5 pmol) for 5 min (3 min for GTP) at 30 °C in buffer A containing 10 mM-EDTA and known concentrations of the appropriate labelled nucleotide. The dissociation constant for GDP·Mg²⁺ was determined in a similar manner, except that the incubation was for 120 min with 10 mM-MgCl₂ in the buffer. On-rates were calculated from k_{off}/K_d .

Mg²⁺ kinetic determinations

We made use of the stabilization of the p25^{rab 34}·GDP complex by Mg²⁺ to estimate the apparent cation dissociation constant. p25^{rab 34} was loaded with [α -³²P]GDP as described above. Known amounts of Mg²⁺ were added and allowed to equilibrate for 10 min at 30 °C. Unlabelled GDP (2 mM) was added, and filter binding was performed after 5 min as described above. Under these conditions, any GDP bound without Mg²⁺ to p25^{rab 34} will not be bound to the protein, while GDP·Mg²⁺·p25^{rab 34} will still be > 95% bound (see Fig. 1). Therefore, under these conditions, the amount of bound [α -³²P]GDP is a measure of bound Mg²⁺. Free [Mg²⁺] was determined from the known total Mg²⁺, total GDP and EDTA concentrations and the appropriate dissociation constants for Mg²⁺·EDTA [11] and Mg²⁺·GDP [30] by computer program, using an iterative procedure to solve the multiple simultaneous equations (T. Gunter, University of Rochester, Rochester, NY, U.S.A.).

An upper value for the Mg²⁺ off-rate from p25^{rab 34}·GDP was estimated in a similar fashion. p25^{rab 34} was prebound to [α -³²P]GDP in buffer A as described above, Mg²⁺ (500 μM final concentration) and unlabelled GDP (1 mM final concentration) were added, and the mixture was incubated for 10 min at 30 °C to allow equilibration. Excess EDTA was added (10 mM final concentration) and aliquots were withdrawn at time intervals

and assayed by filter binding. Loss of bound radioactivity is a measure of Mg²⁺ dissociation. Under these conditions, a Mg²⁺ off-rate slower than the off-rate for GDP in the absence of Mg²⁺ would be observable as a lag time between the addition of EDTA and the loss of GDP.

GTPase activity: effects of Mg²⁺

To determine the requirement for Mg²⁺ of GTPase activity (method 1), p25^{rab 34} (200 ng, 8 pmol) was added to buffer A (30 μl) containing 1.5 μM -[γ -³²P]GTP (0.25 $\mu\text{Ci}/\text{pmol}$) and final free concentrations of 11 mM-Mg²⁺, 80 nM-Mg²⁺ (10 mM-EDTA) or less than 10 nM-Mg²⁺ (the reaction mixture was first dialysed against buffer plus 10 mM-EDTA). [³²P]P_i released was measured as described previously [14,31]. Protein-independent formation of [³²P]P_i was subtracted. Under these conditions, the release of P_i as a function of time is described by eqn. (1) [14,32]:

$$[P_i](t) = \frac{G \cdot k_{\text{cat.}} \cdot k_{\text{off}}}{C} \left(t - \frac{1}{C} + \frac{e^{-Ct}}{C} \right) \quad (1)$$

where $k_{\text{cat.}}$ is the intrinsic GTPase catalysis rate constant, k_{off} is the rate constant for GDP dissociation from the GTP-binding protein, G is the total concentration of GTP-binding protein, and $C = k_{\text{cat.}} + k_{\text{off}}$. Once steady state is attained, eqn. (1) reduces to:

$$[P_i](t) = G \cdot k_{\text{cat.}} \cdot k_{\text{off}} / C \quad (2)$$

Thus, by measuring P_i released as a function of time, we can fit the data to eqn. (1) by non-linear least-squares analysis and derive values for $k_{\text{cat.}}$ and k_{off} (T. Gunter, University of Rochester).

Determination of GTPase catalysis rate constant

To measure $k_{\text{cat.}}$ directly, we assayed the extent of conversion of [α -³²P]GTP into [α -³²P]GDP as a function of time under conditions of single turnover kinetics (method 2). p25^{rab 34} (0.1–2 pmol) prebound to [α -³²P]GTP was added to buffer A containing 10 mM-MgCl₂ and 2 mM each of unlabelled GTP and GDP. Conversion from GTP into GDP was assessed using t.l.c. [33]. Prebinding p25^{rab 34} to [α -³²P]GTP eliminates the association rate constant as a factor affecting the hydrolysis rate. The large excess of unlabelled nucleotides ensures that we are measuring single turnover of the enzyme. Thus we can obtain a good estimate of the intrinsic GTPase rate constant, $k_{\text{cat.}}$, for p25^{rab 34}. As a control, an equivalent amount of [α -³²P]GTP was added without p25^{rab 34} to buffer A under identical conditions and assayed by filter binding and t.l.c. [33]. Background binding was less than 2% of that determined with p25^{rab 34} and GTP hydrolysis was negligible under control conditions.

RESULTS

Guanine-nucleotide-binding kinetics: effects of Mg²⁺

To provide a basis for the investigation of the regulation of p25^{rab 34} function, and for comparison with p21^{ras}, we characterized the kinetics of guanine nucleotide binding to p25^{rab 34}. Off-rates from p25^{rab 34} were measured in the presence of a 1000-fold excess of unlabelled guanine nucleotide and either 10 mM-MgCl₂ or 10 mM-EDTA, at 30 °C. As shown in Fig. 1, Mg²⁺ decreased the off-rate for GDP by approx. 56-fold. Because of the intrinsic GTPase activity of p25^{rab 34}, the k_{off} for GTP·Mg²⁺ could only be determined indirectly. The loss of bound radioactivity from p25^{rab 34}·[γ -³²P]GTP, as detected by filter binding (see the Materials and methods section), is a function of both $k_{\text{cat.}}$ and k_{off} for GTP·Mg²⁺. Thus, by determining $k_{\text{cat.}}$ independently (see the Materials and methods section), we could calculate the

Fig. 1. Effect of Mg^{2+} on nucleotide dissociation from $\text{p25}^{\text{rab3A}}$

$\text{p25}^{\text{rab3A}} \cdot [\alpha\text{-}^{32}\text{P}]\text{GDP}$ was incubated in the presence of 2 mM-GDP and either 10 mM- MgCl_2 ($\blacklozenge$) or 10 mM-EDTA ($\circ$). $\text{p25}^{\text{rab3A}} \cdot [\alpha\text{-}^{32}\text{P}]\text{GTP}$ was incubated in the presence of 2 mM-GTP plus 10 mM-EDTA ($\triangle$). Aliquots of the reaction mixture were removed at the indicated time intervals, washed through nitrocellulose filters, and the radioactivity remaining on the filters was counted. The natural log of radioactivity bound at time t (B)/radioactivity bound at time zero (B_0) was plotted versus time and the slopes determined by least-squares analysis to derive the first-order dissociation rate constants. The results shown represent three independent experiments in each case.

Table 1. Constants for nucleotide and Mg^{2+} binding to $\text{p25}^{\text{rab3A}}$

The K_d is the equilibrium dissociation constant between $\text{p25}^{\text{rab3A}}$ and the indicated species (except for Mg^{2+} , which was equilibrated with $\text{p25}^{\text{rab3A}} \cdot \text{GDP}$). k_{off} is the first-order dissociation rate constant; k_{on} is the second-order association rate constant derived from k_{off}/K_d . Values represent means of three separate experiments.

	GDP	GDP· Mg^{2+}	GTP	GTP· Mg^{2+}	Mg^{2+}
K_d (nM)	420	63	130	(46)*	4000
k_{off} (min^{-1})	0.63	0.011	0.22	(0.024)†	> 0.63
k_{on} ($\mu\text{M}^{-1} \cdot \text{min}^{-1}$)	1.5	0.18	1.7	0.52	> 0.16

* K_d for $\text{GTP}[\text{S}] \cdot \text{Mg}^{2+}$ (data taken from [27]).

† Value determined by subtracting k_{cat} , obtained directly using method 2 (see the Materials and methods section) from $k_{\text{off}} + k_{\text{cat}}$, obtained using method 3.

value for k_{off} for $\text{GTP} \cdot \text{Mg}^{2+}$ (Table 1). The k_{off} for GTP in the absence of Mg^{2+} could be determined directly because it is sufficiently large that hydrolysis over the time course examined was negligible. Dissociation constants for GDP, $\text{GDP} \cdot \text{Mg}^{2+}$ and GTP were determined from double-reciprocal plots of equilibrium binding data shown in Fig. 2, and were used to estimate values for k_{on} from the ratio of K_d/k_{off} . These values are presented in Table 1. They demonstrate that nucleotides can be rapidly loaded on to the protein in the presence of EDTA, and then effectively trapped by the addition of Mg^{2+} . We used this approach to study the kinetics of Mg^{2+} binding to $\text{p25}^{\text{rab3A}}$.

Mg^{2+} binding kinetics

Binding of Mg^{2+} to $\text{p25}^{\text{rab3A}}$ was measured in an indirect manner, using the fact that Mg^{2+} dramatically slows the off-rate

for GDP from $\text{p25}^{\text{rab3A}}$. A similar approach was used to measure the affinity of Mg^{2+} for $\text{G}\alpha \cdot \text{GTP}[\text{S}]$ [11]. By measuring the amount of $[\alpha\text{-}^{32}\text{P}]\text{GDP}$ that remained bound to $\text{p25}^{\text{rab3A}}$ 5 min after chasing with a large excess of unlabelled GDP, we could be sure that > 95 % of the $\text{p25}^{\text{rab3A}} \cdot \text{GDP}$ without bound Mg^{2+} had dissociated, whereas only < 5 % of the complex bound to Mg^{2+} had dissociated. Thus, under these conditions, bound GDP is a measure of bound Mg^{2+} . By this method we estimated an apparent dissociation constant for Mg^{2+} from $\text{p25}^{\text{rab3A}} \cdot \text{GDP}$ of about 4 μM (Table 1, Fig. 3a). This value is about 95-fold lower than the dissociation constant for the free $\text{GDP} \cdot \text{Mg}^{2+}$ complex in solution under similar conditions of ionic strength, pH and temperature [30].

We were also able to estimate a minimum value for the k_{off} for Mg^{2+} by adding excess EDTA to a solution containing a $\text{p25}^{\text{rab3A}} \cdot \text{Mg}^{2+} \cdot [\alpha\text{-}^{32}\text{P}]\text{GDP}$ complex and measuring the loss of bound radioactivity as a function of time. If the off-rate for Mg^{2+} were slower than the off-rate for GDP in the presence of EDTA, then one would predict a lag time between the addition of EDTA and the loss of bound radioactivity. The results are shown in Fig. 3(b) and in Table 1. Since no lag time was observed, we conclude that the k_{off} for Mg^{2+} was > 0.63/min (the k_{off} for GDP in the presence of EDTA).

Mg^{2+} requirement for GTPase activity

One of the fundamental properties of all known GTP-binding proteins, except for the ADP-ribosylation factor [34], is that they possess a low, but detectable, intrinsic GTPase activity. It has been reported that Mg^{2+} is essential for the GTPase activity of the heterotrimeric G-proteins [11,12]. The role of Mg^{2+} in the GTPase activity of the ras superfamily of GTP-binding proteins has not been studied in detail. Therefore we investigated the effects of Mg^{2+} upon GTPase activity of $\text{p25}^{\text{rab3A}}$. We measured the GTPase activity of $\text{p25}^{\text{rab3A}}$ as described previously [31] in the presence of millimolar or nanomolar concentrations of free Mg^{2+} . As shown in Fig. 4, in the presence of 11 mM- Mg^{2+} , $\text{p25}^{\text{rab3A}}$ exhibited a gradual increase in the rate of P_i released, which can be modelled according to eqn. (1). Using as initial estimates the values of k_{cat} and k_{off} determined independently, and allowing both parameters to vary, we obtained values of 0.010/min for k_{off} and 0.028/min for k_{cat} (Table 2). The predicted function is shown as a solid curve (without symbols) in Fig. 4.

In the presence of 10 mM-EDTA, free $[\text{Mg}^{2+}]$ was calculated to be 80 nM (assuming the K_d for $\text{Mg}^{2+} \cdot \text{EDTA}$ is 1 μM , at pH 7.4, and that $\text{GTP} \cdot \text{Mg}^{2+}$ binding is negligible). Surprisingly, GTPase activity was substantial, surpassing GTPase activity in the presence of excess Mg^{2+} at all early time points (Fig. 4). At later times (> 40 min), eqn. (1) predicts that the steady-state hydrolysis rate in the presence of Mg^{2+} will be about 2-fold greater than that measured without Mg^{2+} . However, partial inactivation of $\text{p25}^{\text{rab3A}}$ during prolonged incubations at 30 °C prevented the extension of these studies to longer time intervals. In the presence of excess EDTA, a steady state was rapidly attained and eqn. (2) was applicable. Furthermore, since k_{off} is much greater than k_{cat} , in the presence of excess EDTA, eqn. (2) reduces to $\text{P}_i(t) = G \cdot k_{\text{cat}}$. The measured rate of P_i release was 0.032 pmol/min (Fig. 4), and thus k_{cat} was 0.004/min in the presence of excess EDTA (Table 2).

In order to determine if Mg^{2+} was required for the GTPase activity of $\text{p25}^{\text{rab3A}}$, we first dialysed the reaction mixture against buffer containing 10 mM-EDTA, and then supplemented the reaction mixture with 10 mM-EDTA. We could thus be sure that the free Mg^{2+} concentration was less than 10 nM. There was no detectable GTPase activity under these conditions, indicating that some Mg^{2+} is required (results not shown). Replenishing the

Fig. 2. Equilibrium binding of GDP, GDP·Mg²⁺ and GTP to p25^{rab3A}.

(a) p25^{rab3A} (0.5 pmol) was incubated for 3 min in the presence of 10 mM-EDTA and known concentrations of [α-³²P]GTP (●), or for 5 min in the presence of 10 mM-EDTA and known concentrations of [α-³²P]GDP (○). (b) p25^{rab3A} (0.5 pmol) was incubated for 120 min in the presence of 10 mM-MgCl₂ and known concentrations of [α-³²P]GDP. Samples were washed through nitrocellulose filters, and the radioactivity remaining on the filters was counted. The results shown represent two independent experiments in each case.

Fig. 3. Kinetics of Mg²⁺ binding to p25^{rab3A}·[α-³²P]GDP.

(a) Equilibrium dissociation constant for Mg²⁺ from p25^{rab3A}·[α-³²P]GDP; (b) estimation of Mg²⁺ off-rate; ↓ denotes time at which excess EDTA was added. Since there was no lag time observed between the addition of EDTA and the release of [α-³²P]GDP, we conclude that the Mg²⁺ off-rate is at least as fast as the GDP off-rate. Mg²⁺ affinity was determined from the ratio of [α-³²P]GDP·Mg²⁺·p25^{rab3A} to GDP·p25^{rab3A} at different concentrations of free Mg²⁺ (see the Materials and methods section). The results represent two independent experiments in each case.

Mg²⁺ in the reaction mixture restored GTPase activity (results not shown).

DISCUSSION

We have investigated the kinetics of guanine nucleotide binding to and hydrolysis by p25^{rab3A}, as well as the influence of Mg²⁺ upon these processes. We have found that Mg²⁺ dramatically decreases the off-rate for GDP and GTP from p25^{rab3A}, in agreement with observations made for the ras [29] and ralA [20] proteins, but not for the SEC4 gene product of *Saccharomyces cerevisiae* [19]. The dissociation constants measured for GTP and GDP were comparable, unlike SEC4 which exhibits a 20-fold higher affinity for GTP over GDP [19], and eukaryotic initiation factor 2 which has a 400-fold higher affinity for GDP [35]. Using a modification of the technique used previously [11], we have

calculated an apparent dissociation constant and estimated a minimum k_{off} for Mg²⁺ from p25^{rab3A}·GDP. We calculate an apparent K_d for Mg²⁺·p25^{rab3A}·GDP of 4 μM. The reported K_d for Mg²⁺·GDP under similar conditions of pH and ionic strength is 380 μM [30]; therefore Mg²⁺ and GDP/GTP either bind to separate sites on p25^{rab3A}, or Mg²⁺ is co-ordinated to both. X-ray crystallographic data for p21^{ras} support the second hypothesis [16]. The Mg²⁺ off-rate is not rate-limiting for the dissociation of GDP in the presence of excess EDTA, and cannot account for the dramatically decreased guanine nucleotide off-rates in the presence of Mg²⁺. A model depicting these interactions is shown in Fig. 5.

The effects of Mg²⁺ on GTPase activity were examined. At a Mg²⁺ concentration of 11 mM, GDP release was the rate-limiting step for GTPase activity. Using non-linear least-squares analysis, we were able to fit the data for P_i(t) to eqn. (1), and derive values

Fig. 4. GTPase activity: requirement for Mg^{2+}

p25^{rab3A}, as purified (200 ng), was incubated in buffer A (30 μ l) containing 1.5 μ M- $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ (0.25 μ Ci/pmol) and either 10 mM-EDTA (▼) or 11 mM- $MgCl_2$ (△). Samples were assayed for release of P_i as described in the Materials and methods section. Free $[Mg^{2+}]$ in the presence of EDTA was calculated to be 80 nM (assuming the K_d for Mg^{2+} -EDTA is 1 μ M at pH 7.4; ionic strength 0.15). The results shown represent three independent experiments in each case. The solid curve represents data for GTPase activity in the presence of excess Mg^{2+} , fitted to a mathematical model for $P_i(t)$ using non-linear least-squares analysis (see the Materials and methods section).

Table 2. Estimates of k_{cat} : effects of Mg^{2+}

In the presence of EDTA, the free $[Mg^{2+}]$ was calculated to be 80 nM. In method 1, k_{cat} was calculated from measurements of the rates of P_i released under saturating conditions. In method 2, k_{cat} was measured directly under conditions of single turnover. In method 3, a value for $k_{cat} + k_{off}$ was obtained by measuring the rate of loss of bound radioactivity from p25^{rab3A} prebound to $[\gamma\text{-}^{32}\text{P}]\text{GTP}$. See the Materials and methods section for further details. Results are the means $\pm$ S.E.M. of at least three to six experiments. Standard errors are in parentheses.

	Constant (min^{-1})	
	+ EDTA	+ Mg^{2+}
Method 1 (k_{cat})	0.004 ± 0.0002	0.028 ± 0.002
Method 2 (k_{cat})	—	0.024 ± 0.004
Method 3 ($k_{cat} + k_{off}$)	—	0.048 ± 0.017

for k_{off} and k_{cat} . The derived values were in excellent agreement with the values for k_{off} and k_{cat} determined independently. Surprisingly, at a Mg^{2+} concentration of 80 nM, GTPase activity was comparable with GTPase activity in the presence of excess Mg^{2+} . In this case, k_{cat} was rate-limiting. In fact, the catalytic capacity of p25^{rab3A} was decreased; the k_{cat} was 0.004 min^{-1} compared with 0.024 min^{-1} in the presence of excess Mg^{2+} . However, since the off-rate for GDP is so much greater in the presence of excess EDTA, the overall GTPase activity was not impaired. At sufficiently low magnesium concentrations, k_{cat} was decreased to zero; thus it is concluded that Mg^{2+} is required for the GTPase activity of p25^{rab3A}.

The abundance of p25^{rab3A} in neurons of the hippocampus and

Fig. 5. Model for the interaction of p25^{rab3A}, guanine nucleotides and Mg^{2+}

Solid lines indicate processes measured in the present paper; dashed lines represent processes presumed to occur. R3 designates the apo form of p25^{rab3A}.

hypothalamus, in synaptic vesicles and in other exocrine tissues suggest that this small GTP-binding protein may be involved in stimulus-coupled secretion [21–25]. Related members of the *rab* gene family have been shown to play roles in vesicular trafficking [36]. Of particular interest is the *SEC4* gene product, which has about 45% sequence similarity with *rab3A*, and is involved in a late stage of constitutive secretion in yeast. Despite these similarities, the two proteins exhibit very different biochemical properties. For example, the GDP off-rate from SEC4 is not decreased in the presence of Mg^{2+} [19], and the GTPase activity of *rab3A* is 25-fold greater than that of SEC4. It is interesting to note that both proteins have a serine instead of a glycine residue at position 12, and *rab3A* also has a serine at position 13. Mutation of either of these two residues in *ras* (both glycines) severely impairs GTPase activity and renders the protein oncogenic. The extremely low GTPase activity of SEC4 is understandable in this context, but the relatively high GTPase activity of *rab3A* demonstrates that other factors are involved. The striking differences in kinetic properties between *rab3A* and SEC4 may reflect fine tuning of the respective systems.

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