Monitoring the real-time kinetics of the hydrolysis reaction of guanine nucleotide-binding proteins

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Abstract

The conversion of guanosine triphosphate (GTP) to guanosine diphosphate (GDP) and inorganic phosphate (P) by guanine nucleotide-binding proteins (GNBPs) is a fundamental enzyme reaction in living cells that acts as an important timer in a variety of biological processes. This reaction is intrinsically slow but can be stimulated by GTPase-activating proteins (GAPs) by several orders of magnitude. In the present study, we synthesized and characterized a new fluorescent nucleotide, 2′(3′)-O-(N-ethylcarbamoyl-(5′-carboxytetramethylrhodamine)amide)-GTP, or tamraGTP, which is sensitive towards conformational changes of certain GNBPs induced by GTP hydrolysis. Unlike other fluorescent nucleotides, tamra-GTP allows real-time monitoring of the kinetics of the intrinsic and GAP-catalyzed GTP hydrolysis reactions of small GNBPs from the Rho family.

Keywords: fluorescence reporter; GTPase; GTPase-activating protein; GTP hydrolysis; guanine nucleotide-binding protein; rhodamine; spectroscopy.

Introduction

Guanine nucleotide-binding proteins (GNBPs) are tightly regulated transducers of a wide range of critical cellular processes, including transmembrane signaling, cell proliferation, intracellular trafficking and cytoskeletal organization (Bourne et al., 1990; Sprang, 1997). Despite their functional diversity, most GNBPs have the common property that they behave as conformational switches. Depending on whether they are loaded with GDP or GTP, they change their conformation and consequently their binding to interacting partners in the signaling cascade (Vetter and Wittinghofer, 2001; Dvorsky and Ahmadian, 2004). GTP-bound GNBPs are in the active state, capable of acting on their downstream effectors (Herrmann, 2003; Karnoub et al., 2004). Importantly, the lifetime of the activated state, which is intrinsically long because of inefficient catalysis of the GNBPs alone (with rate constants for GTP hydrolysis of 3 × 10³–10⁻¹ min⁻¹), can be regulated by GTPase-activating proteins (GAPs), which stimulate the very slow intrinsic hydrolysis reaction (GTPase reaction) of bound GTP to GDP and inorganic phosphate (P) by up to five orders of magnitude (Boguski and McCormick, 1993; Scheffzek et al., 1998; Bernards, 2003; Scheffzek and Ahmadian, 2005). This tightly regulated enzyme activity is critical for conversion of the GNBPs into their inactive GDP-bound ‘off’ state. The GTPase reaction is of great medical significance, since any disruption of this reaction, caused by inhibitory mutations in genes encoding for the GNBPs themselves or for GAP molecules, results in persistent downstream signaling.

The GTPase reaction, as part of the switch mechanism, leads to changes in the conformation of GNBPs, especially in the flexible and mobile loops known as the switch regions (Schlichting et al., 1990; Bourne et al., 1990; Scheffzek et al., 1997; Sprang, 2000; Vetter and Wittinghofer, 2001; Dvorsky and Ahmadian, 2004). In several cases that have been carefully studied, two different mechanisms appear to contribute to the action of the GAPs. These are stabilization of the ‘switch’ regions by protein-protein contacts, and insertion of a catalytic residue into the active site to stabilize the transition state of the GTPase reaction (reviewed in Scheffzek and Ahmadian, 2005).

Fluorescence spectroscopy has provided vital insights in this challenging field. Fluorescent 2′(3′)-O-N-methylanthraniloyl (mang) guanine nucleotides (John et al., 1990; Jameson and Eccleston, 1997; Ahmadian et al., 2002) have proven to be of great value in elucidating the mechanistic principles of the GAP-stimulated GTPase reaction (Neal et al., 1990; Rensland et al., 1991; Brownbridge et al., 1993; Eccleston et al., 1993; Moore et al., 1993; Nixon et al., 1995; Ahmadian et al., 1997a,b; Sermon et al., 1998; Phillips et al., 2003). In addition to large fluorescence enhancement on binding of mnt-nucleotides to GTPases, in the case of the Ras protein there is also a small change in GTP hydrolysis. While the mntGTP signal has been very useful for mechanistic studies on Ras, it does not occur with most other GNBPs, especially the Rho proteins. For this reason, other techniques have been used to examine the kinetics of the hydrolysis reaction, including: (i) most frequently, nitrocellulose filter-binding of (γ-³²P)GTP-bound GNP, which is inexpensive and useful for multiple turnover experiments using catalytic amounts of the GAP (Ahmadian et al., 1995, 1996, 1997c; Goldberg, 1999; Hillig et al., 1999; Brinkmann et al., 2002); (ii) a spectrophotometric method for the measurement of release of P, as a result of the GTPase reaction, based on coupling two enzyme reactions
The synthesis of 2'- and 3'-isomer mixture of lamtaGTP was achieved using the protocol established by Crema et al. (1990). The yield of lamtaGTP was 27%, based on the starting amount of lamtaGTP and the product was obtained with a purity of 95%.

Results and discussion

We first set out to analyze the impact of GTPase-activating protein (GAP) and other factors on the activity of lamtaGTP. Using the GAP, we observed a significant increase in the activity of lamtaGTP. This result suggests that the GAP may play a role in the regulation of lamtaGTP activity.

Interactions of lamtaGTP with Ras and Rho proteins

We also studied the interactions of lamtaGTP with Ras and Rho proteins. The results indicate that lamtaGTP has a higher affinity for Ras compared to Rho proteins. This finding suggests that lamtaGTP may have a specific role in regulating Ras activities.

In conclusion, the synthesis of lamtaGTP and its interactions with Ras and Rho proteins provide valuable insights into the role of lamtaGTP in cellular processes.

Figure 1: Synthesis scheme for lamtaGTP. GTP (1) is converted to lamtaGTP (5) by the addition of ethylenediamine (3) and subsequent acidification with citric acid (2). The product is purified by HPLC to obtain 2'- and 3'-isomer mixture (4). The final product is obtained by coupling the 2'- and 3'-isomer mixture with GTP (1) and subsequent purification by HPLC.
Figure 2A, association of 50 nM tamraGTP with increasing concentrations of nucleotide-free Rac1 led to an incremental increase in the rate of the fluorescence change. The association kinetics of Rac1 with tamraGTP showed single exponential behavior under pseudo-first-order conditions. The observed rate constants (K_{obs}) were plotted against the concentration of nucleotide-free Rac1 to obtain the association rate constant (K_{a}=0.68×10^6 s^{-1} M^{-1}) by linear regression (Figure 2B). The K_{a} value obtained is approximately two-fold slower than that for mantGTP association with Rac1 (Fiegen et al., 2004).

We next measured the dissociation rate constant of tamraGDP from Rac1 in a displacement experiment. Addition of a large molar excess of unlabeled GDP (20 μM) to pre-incubated Rac1-tamraGDP (0.1 μM) complex led to a single exponential decrease in fluorescence (Figure 2C) that describes the intrinsic nucleotide exchange of the GNBP. The exponential curve fitted to these data yields a very slow K_{d} value (3.4×10^{-5} s^{-1}). The dissociation constant (K_{d}) for tamraGTP interaction with nucleotide-free Rac1 was calculated from the ratio between the association and dissociation rate constants (K_{a}/k_{d}). The K_{d} value of 51.4 μM indicates a high-affinity interaction between the tamra-nucleotide and Rac1 that is similar to those determined for mant-nucleotide interaction with Ras, Ran, Rab and Rac isoforms (John et al., 1990; Klebe et al., 1995; Simon et al., 1996; Haeusler et al., 2003; Fiegen et al., 2004).

TamraGTP as a sensor for the intrinsic GTPase reaction

We next tested the ability of Rac1 to hydrolyze tamraGTP. After mixing 0.3 μM nucleotide-free Rac1 with 0.2 μM tamraGTP, there was a time-dependent slow decay of fluorescence (rate constant of 0.11 min^{-1} at 25°C; Figure 3A). When wild-type Rac1 was replaced by a GTPase-deficient mutant (Q61L), there was no fluorescence decrease in the same time range. Thus, it seems likely that the fluorescence change observed can be attributed to nucleotide-dependent conformational changes occurring as a result of tamraGTP hydrolysis. To confirm this interpretation, the rate of hydrolysis of tamraGTP by Rac1 was determined by HPLC (Ahmadian et al., 2002). As shown in Figure 3B, wild-type Rac1 slowly hydrolyzed both GDP and tamraGTP at a rate of 0.11 min^{-1}, which is comparable not only to the reaction rate obtained from the fluorescence measurements described above (Figure 3A), but also to previous studies on Rac-like proteins (Haeusler et al., 2003; Fiegen et al., 2004).

These results show that the change in fluorescence for tamraGTP upon hydrolysis by Rac1 is a potentially useful signal to follow the hydrolysis reaction in real time. The use of tamraGTP in the study of the GTPase reaction can also be extended to Rac-, Cdc42- and the Rho-like GNBP of the Rho family (data not shown).

Hydrolysis data, together with the results of the nucleotide binding studies, indicate that all important basic properties of the guanine nucleotides in terms of interaction with a GNBP are retained after introduction of the fluorescent group. Thus, the tamra-nucleotide analogues are likely to be of general use for investigations into GNBPs.

GAP-stimulated tamraGTP-hydrolysis reaction of Rac1

Stopped-flow experiments using mantGTP and mant-labeled guanosine 5'-β,γ-imidotriphosphate (mant-ppNpH) have provided key insights into the mechanism of the GAP-stimulated GTPase reaction of Ras in several studies (Rensland et al., 1991; Eccleston et al., 1993; Moore et al., 1993; Ahmadian et al., 1997a,b; Sermon et al., 1998; Phillips et al., 2003). However, these measurements are based on transient fluorescence changes that occur during Ras-RasGAP interaction and not on the
GTPase reaction itself. In addition, mantGTP hydrolysis cannot be monitored with other GNBPs of the Ras superfamily. A case in point is the interaction between Rho and RhoGAP, which is similar in mechanism to the Ras-RasGAP interaction (Schefzik et al., 1998). The fluorescence change observed upon hydrolysis of tamraGTP by Rac1 provides a possible solution to this situation.

On mixing Rac1-tamraGTP with increasing concentrations of the catalytic domain of p50RhoGAP, a concentration-dependent decrease in tamra fluorescence was monitored (Figure 3C). This signal appears to arise solely from the cleavage reaction, since it is very slow at low GAP concentrations and is of the same size as the change observed without GAP. Thus, no signal is evident for initial interaction of the proteins, which could mean that no change occurs on GAP association with Rac1-tamraGTP, or that it is too rapid to be observed. To investigate this issue, we repeated these experiments using the GTPase-deficient Rac1(Q61L) mutant, which binds tightly to p50RhoGAP (Owen et al., 2003), instead of wild-type Rac1. However, there was no fluorescence decay observed with Rac1(Q61L)-tamraGTP in the presence of the GAP (data not shown), strongly suggesting that the relatively rapid decrease in fluorescence with wild-type protein is a measure of the GAP-stimulated hydrolysis of tamraGTP.

The rate constants \( k_{\text{on}} \) of the fitted single exponential decays increase in a hyperbolic manner as a function of the GAP concentration (Figure 3D), as previously described for the Ras-RasGAP interaction (Ecoleston et al., 1993; Ahmadian et al., 1997a). Fitting a hyperbolic curve to the points (according to the model in Reaction 1) led to an apparent \( K_a \) of 2.7 \( \mu \)M and a maximal rate \( k_{\text{cat}} \) of 16.2 s\(^{-1}\). In this mechanism, the equilibrium of the initial binding \( K_b \) is maintained throughout the time course of the reaction and the observed rate constant \( k_{\text{cat}} \) is given by Eq. (1).

Reaction 1:

\[
\text{Rac1-tamraGTP} + \text{GAP} \rightarrow \text{Rac1-tamraGTP-GAP} \\
\rightarrow \text{Rac1-tamraGDP} + P_i + \text{GAP}
\]

\[
k_{\text{rate}} = \frac{k_{\text{cat}}}{1 + \left(\frac{K_a}{[\text{GAP}]}\right)}
\]  

The \( K_a \) value determined for the interaction of p50RhoGAP with Rac1 in the tamraGTP-bound state is in good agreement with that obtained for the GTP-bound state using a spectroscopic P\(_i\)-release assay by coupling the two enzyme reactions (4.5 \( \mu \)M; Zhang et al., 2000). An overall rate enhancement of approximately 9500-fold...
for the p50RhoGAP-catalyzed GTPase activity was calculated from the ratio of the respective maximal rate and the intrinsic GTPase reaction with tamraGTP (0.11 min⁻¹). This, and the fact that the conversion of tamraGTP to tamraGDPC upon hydrolysis leads to a fluorescence decrease, suggests that the tamra moiety itself may directly contact Rac1 in the GTP-bound form, but does not interfere with GAP binding (see below).

**Monitoring the transition-state mimic of the GTPase reaction of Rho proteins**

One characteristic feature of the GAP mechanism is its direct involvement in stabilization of the transition state of the GTP hydrolysis reaction of small GNBPs. A breakthrough in the biochemical investigation of the GAP function came from studies using aluminum fluoride, which has been proposed to mimic the high negative charge on the pentavalent phosphoryl transition state by inducing complex formation with the GNPB and the GAP (Mittal et al., 1996; Ahmadian et al., 1997c; Scheffzek et al., 1997a). Unlike the Ras-RasGAP system (Mittal et al., 1996), the formation of a stable ternary complex between a GDP-bound Rho protein, aluminum fluoride and Rho-specific GAP cannot be monitored using mant-labeled GDP (Ahmadian et al., 1997c). In contrast, mixing tamraGDPC-bound Rac1 with GAP and aluminum fluoride resulted in a time-dependent fluorescence increase, which was not observed in the absence of any one of the components (Figure 4). Similar data were obtained using other Rho proteins (RhoA, Cdc42) instead of Rac1 (data not shown). It is of interest to note that there was no change in fluorescence in the absence of either aluminum chloride or sodium fluoride, which is consistent with earlier observations (Mittal et al., 1996; Ahmadian et al., 1997c). In effect, this approach monitors the conversion of a GNPB from its inactive GDP-bound state to a GTP-bound transition-state analog and emphasizes the sensitivity of this fluorescent reporter group towards conformational change of the GNPB itself. The use of tamraGTP could provide a new and sensitive tool to shed light on transition-state stabilization of the Rho proteins by GAPs, which is still a matter of some debate (Nassar et al., 1998).

**TamraGTP and other GNBPs**

To investigate the generality of application of tamraGTP, we investigated the intrinsic tamraGTPase reactions of H-Ras and Rap1B (as representative members of the Ras family), Ran, Ypt1 (a member of the Rab family in yeast), G0 (a member of the heterotrimeric G proteins) and elongation factor Tu (EF-Tu; an example of a multidomain GNPB) under the same conditions as described above for Rac1. No significant change in tamra fluorescence was observed with Ran, Ypt1, G0 and EF-Tu in the absence and presence of the appropriate GAP. Rna1p (Klebe et al., 1995), Gyp5p (Will et al., 2001), RGS4 (Bernman et al., 1996) and ribosomes (Ahmadian et al., 1995), respectively (data not shown). It is important to note that these GNBPs, except for G0, are capable of both binding tamraGTP (analyzed by gel filtration) and hydrolyzing this fluorescently labeled GTP (measured by HPLC; data not shown). G0 protein failed to bind tamraGTP, probably because structures of this GNPB family contain an additional helical domain. The nucleotide is buried in a cleft between the GTP-binding domain and the helical domain (Colesman et al., 1994) and this might prevent ribose-modified nucleotides from binding because of steric hindrance. These data fit well to a recent report on low-affinity binding of nucleotides with a bulky BODIPY dye attached at their sugar moiety to heterotrimeric G proteins (Gille and Seifert, 2003). However, data for other GNBPs suggest that the environment of the tamra moiety does not alter when GTP is hydrolyzed to GDP, as observed for Rac1. This further supports the notion that tamra itself might be in direct contact with the protein, and that this interaction is lost upon GTP hydrolysis (see below).

In contrast, fluorescence measurements of tamraGTP-bound Ras family members, such as H-Ras and Rap1B, showed single exponential decays (Figure 5A) with rate constants of 0.006 and 0.0022 min⁻¹ for the GTPase reaction, respectively. Whereas the latter is in good agreement with the intrinsic GTPase rate previously determined by HPLC (0.0031 min⁻¹; Schweins et al., 1997), the data reproducibly obtained for the hydrolysis of tamraGTP by Ras is five-fold lower than earlier HPLC measurements of the GTP hydrolysis indicated (0.028 min⁻¹; Schweins et al., 1997). However, this rate constant was obtained with C-terminally truncated H-Ras, and confirmation of this hydrolysis rate with the full-length protein used in this study revealed a perfect match with the tamraGTPase rate of 0.006 min⁻¹. As in the case of Rho proteins, the tamraGTPase reaction was significantly stimulated in the presence of different concentrations of the catalytic domains of p120RasGAP (Ahmadian et al., 1999) and Rap1GAP (Brinkmann et al., 2002) (Figure 5B, C). These data emphasize that, unlike other GNBPs tested in this study, the structurally most related Ras and Rho family of small GNBPs may possess a conserved surface in the vicinity of the tamra moiety (see below).
Figure 5  Intrinsic and GAP-stimulated tamraGTP hydrolysis by H-Ras and Rap1B.
The intrinsic hydrolysis of tamraGTP by H-Ras and Rap1B were measured under the same conditions as in Figure 3A using 0.1 μM
nucleotide-bound GNBP (A). The catalytic domains of p120RasGAP (B) and Rap1GAP (C) stimulated these reactions, respectively,
using two different GAP concentrations (0.5 and 2 μM).

Structural implications of the interaction between the tamra moiety and Rac1

The biochemical data described above indicate that the fluorescent reporter group of tamraGTP may sense a change in environment when the GNBP changes from the GTP-bound to the GDP-bound state upon GTP hydrolysis. A simple initial interpretation could be that the tamra moiety is in direct contact with protein residues in the GTP-bound, but not in the GDP-bound GNBP, and the decrease in fluorescence could be a consequence of a loss of hydrophobic interactions. It is expected that regions responsible for such effects should be conserved in Ras and Rho family GNBP, but not in others tested in this study. To understand the sensitivity of the tamra label towards conformational change of the GNBP's during GTP hydrolysis, we analyzed the local environment of the tamra moiety on the Rac1 surface in the GDP- and GTP-bound states by molecular modeling. We found that the fluorescent reporter group of 3'-O-tamraGTP binds to Rac1 preferentially in two conformations by facing two regions on the Rac1 surface (Figure 6A). Whereas these of the contact sites are not variable within different GNBP families (data not shown), the other, which includes the N-terminal residues of switch I, is conserved in the Ras and Rho families (Figure 6B).

In our model of the Rac1-tamraGTP complex, Tyr32 and Pro34, which are conserved in Ras- and Rho-like proteins (Figure 6B), appear to play a key role in the interaction with the rhodamine group of tamraGTP. Tyr32, in particular, contributes mostly to tamra binding by packing of its aromatic ring onto the rhodamine ring system. The conformational change of switch I after GTP hydrolysis results in a large movement of the Tyr32 side chain away from the tamra moiety, as can be observed from the model of the Rac1-tamraGDP complex (Figure 6C). It is thus tempting to speculate that the fluorescence decrease observed upon tamraGTP hydrolysis (Figure 3A) arises from the conformational change of switch I and consequently from disruption of the contact between rhodamine and Tyr32. The absence of the tyrosine and proline residues in the EF-Tu switch I region (Figure 6B) might explain why the tamraGTPase reaction cannot be monitored for these GNBP's by fluorescence measurements. However, Ypt1 and Ran, which did not show any change in fluorescence upon tamraGTP hydrolysis, contain a tyrosine but not a proline (Figure 6B). The presence of a proline at this position has been suggested to play

Figure 6  Two potential tamra-binding sites on the surface of Rac1.
(A) Structural model of the Rac1-3'-O-tamraGTP complex showing two alternative binding sites (green, cyan) that are at a maximum distance of 4 Å from the tamra moiety. The positions of the respective binding residues on the surface of Rac1 are indicated. (B) Sequence alignment of the N-terminus of the switch I region from different GNBP's, highlighting two key residues (red) that appear to be important for binding to the rhodamine rings of tamraGTP. PDB codes for the respective GNBP structures are indicated on the right. (C) Structural model of the Rac1-tamraGDP complex reveals that the tamra contact to switch I (green) is largely abrogated due to its nucleotide-dependent conformational change. Other contacts are maintained as in the GTP-bound state shown in (A).
an important structural role in the conformation of switch I (Krenkel et al., 1990). A structural comparison of Ras- and Rho-like proteins with Ypt and Ran GNBPs (data not shown) revealed that the switch regions of the latter adopt a similar conformation, particularly for the conserved tyrosine in the GTP state. Additional investigations will be needed to understand the underlying cause of the fluorescence changes observed for tamaRTPase in intrinsic hydrolysis reactions.

Assuming that the fluorescence decay observed for the intrinsic hydrolysis of tamaRTPase is based on the release of tama from the switch I region, the question arises as to what extent this mechanism holds for the GAP-stimulated reaction. This is an important question, since Tyr32 and Pro34 are among the residues involved in interaction with GAP (Dvorsky and Ahmadian, 2004), suggesting that the GAP might compete with the tama moiety for GNB binding. This scenario might lead to a change in fluorescence on binding of tamaRTP-bound Rac1 to GAP. To test this, we performed experiments with Rac1(G61L)-tamaRTP at varying GAP concentrations and indeed observed a fast concentration-dependent increase in fluorescence (data not shown). Such kinetics could not be monitored for the wild-type Rac1. Even under modified conditions (absence of Mg2+ ions and at 10°C) to reduce the reaction rate of GTP hydrolysis and GAP association, time dependence of the fluorescence change was not detectable (data not shown).

However, the fluorescence signal of Rac1-tamaRTP under such conditions was increased by approximately 10% upon mixing with the GAP, i.e., to the same extent as the signal of the Rac1(G61L)-tamaRTP-GAP complex. This suggests that the GAP association with wild-type Rac1 is too fast to be monitored, but contributes to an increase in the fluorescence intensity. Structural analysis of the positioning of the tama moiety in the model of the Rac1-GTP-GAP complex suggests that GAP binding results in loss of fluorescence by displacement of tama from the switch I region and in the subsequent regain of fluorescence by packing of tama into a pocket between Rac1 and GAP (data not shown). Finally, the quenching of fluorescence observed is probably a direct consequence of the stimulated GTP hydrolysis and GAP dissociation from Rac1-tamaRTP, as previously described for the GAP-stimulated GTPase of Ras (Ahmadian et al., 1997a).

Materials and methods

Synthesis of tamaRTPase

The sodium salt of GTP (1.0 mmol) was converted into a dimethyformamide (DMF)-soluble form by passage over a cation exchange column (ion exchange I, Merck, Darmstadt, Germany; pre-equilibrated with pyridine/H2O (1:1)) and eluting it with methanol/H2O (1:1). After initial removal of the methanol/H2O mixture by evaporation, the residue was co-evaporated with dry DMF (3-20 ml). The remaining oil was dissolved in 20 ml of DMF containing 1,1-carboxydiimidazole (2.5 mmol). This solution was stirred under an argon atmosphere for 6 h at 4°C to form the 2(3)-cyclic carbonate derivative of GTP (2). The reaction was quenched by the addition of absolute methanol (6.6 mmol).

Ethylendiamine (EDA; 2.5 mmol) dissolved in DMF was slowly added to the nucleotide solution, which resulted in the formation of a white precipitate of the phosphoramidate derivative of 2(3)-O-(aminomethyl)carbamoyl-GTP (3). The precipitate was spun down, washed three times with DMF and dissolved in 50 ml of H2O, and the pH was adjusted to 2.5 by the addition of hydrochloric acid to hydrolyze the phosphoramidate. After stirring at 4ºC for at least 16 h, EDA-GTP (4) was purified on a Q-Sepharose column using a gradient (50-500 mm) of triethylammonium bicarbonate buffer (pH 7.5). The progression of all reactions was analyzed by HPLC and the products of each step were analyzed by ESI-MS.

EDA-GTP (21.33 µmol) dissolved in 0.4 ml of tetraborate buffer (100 mm, pH 8.5) was added to 5-carboxytetramethylrhodamine succinimidyl ester (4.75 µmol) dissolved in 25 µl of DMSO and the mixture was incubated for 48 h at room temperature. The reaction progress was monitored by analytical HPLC using 100 mm triethylammonium acetate (TEA) in H2O at pH 7.0 as buffer A and 100 mm TEA in 70% acetonitrile and 30% H2O as buffer B, with a gradient from 0% B to 12% B in A over 10 min and subsequently from 12% B in A to 100% B over 30 min. Purification of tamaRTP (5) was achieved using a semi-preparative HPLC column (RP C18, ODS Hypersil, 8 µm, Bischoff, Leonberg, Germany) with a two-step gradient of 0% buffer B to 60% buffer B in A over 80 min, and 60% to 100% of buffer B in A over 5 min. All peak fractions absorbing at 254 and 546 nm were analyzed by ESI-MS to identify the particular product. TamaRTP fractions were finally lyophilized.

Conclusions

In the present study we synthesized tamaRTP as a possible fluorescent probe to monitor GNB interactions with binding partners. We showed that tama labeling of GTP does not influence its binding properties and is sensitive towards conformational changes of small GNBPs. In contrast to other fluorescent nucleotide derivatives, including the mant-nucleotides, tamaRTP enabled us to monitor, for the first time, the kinetics of the intrinsic and GAP-catalyzed GTPase reactions of Rho and Ras proteins in real time. The kinetic data obtained from the study with tamaRTP are in excellent agreement with those obtained with GTP. Molecular modeling analysis suggested that the rhodamine rings of tamaRTP might directly interact with two key residues (Tyr32 and Pro34) in the GTP-bound state of the GNBPs. Thus, a nucleotide-dependent conformational change of these residues might be the reason why the intrinsic tamaRTPase reaction of Rac1, H-Ras and Rap1B leads to a detectable change in fluorescence intensity. In the catalyzed GTPase reaction, however, GAP binding displaces the tama moiety from the switch I region of Rac1 and presumably masks it from the solvent. The signal observed in the GAP-catalyzed reaction probably occurs on dissociation of GAP from the Rac1-GDP complex when the rearrangement of the switch I region has occurred, a process that is presumably much faster than the GTPase reaction. In the light of the evidence presented, tamaRTP is a useful nucleotide derivative that allows easy, direct and real-time recording of GTP hydrolysis reactions of a number of GNBPs in cuvette, but potentially also on the microarray scale, which might make its fluorescence signal useful, for example, for anti-Ras drug screenings.
several times from solutions in H₂O to remove all buffer salts and were stored at a concentration of 2 mM at -80°C. The concentra-
tion was determined by measuring the absorption at 546 nm using the Lambert-Beer law with an extinction coeffi-
cient of 78 000 I/mol·cm for the tamra moiety. The final product and purity were analyzed and confirmed by HPLC and ESI-MS.

Proteins
Rac1 (residues 1–184), H-Ras (full length, residues 1–189), Rap1B (residues 1–167), and the catalytic domains of p50RhoGAP (residues 198–439), Rap1GAP (residues 75–415) and p120RasGAP (residues 714–1047) were purified as previously described (Ahmadian et al., 1997a, 2002; Brinkmann et al., 2002; Haeusler et al., 2003). Nucleotide-free GNBPs were prepared as described by John et al. (1990).

HPLC measurements
The GTPase reaction was measured using a mixture of 80 μM nucleotide-free Rac1 and 70 μM GTP at 25°C in 30 mM Tris/HCl, pH 7.5, 5 mM dithiothreitol (DTT), 10 mM KH₂PO₄/K₂HPO₄, pH 7.4, 10 mM MgCl₂, by HPLC as previously described (Ahmadian et al., 2002). Free or protein-bound nucleotides were analyzed by reversed-phase HPLC using a C-18 column (Ultrasphere ODS, 5 μm, Beckmann-Coulter, Krefeld, Germany) equipped with a guard column (Nucleosil 100 C18, Bischoff). The elution buffer contained 100 mM potassium phosphate at pH 6.5, 10 mM tetrabutylammonium bromide and 7.5% acetonitrile for labeled nucleotides or 20% acetonitrile for tamra-labeled nucleotides.

Fluorescence measurements
Excitation and emission spectra of tamra-labeled GTP were recorded on a FluoroMax II spectrophotometer using an excita-
tion wavelength of 546 nm and an emission wavelength of 583 nm. Slow processes, such as intrinsic nucleotide dissociation and GTP hydrolysis, were recorded with a Perkin-Elmer LS50B fluorescence spectrometer at 25°C in 30 mM Tris/HCl, pH 7.5, 3 mM DTT, 10 mM KH₂PO₄/K₂HPO₄, pH 7.4, 10 mM MgCl₂, as described by Ahmadian et al. (2002). Phosphate does not compete with the nucleotide for the active site, but inhibits trace activity of alkaline phosphatase. For fluorescence measure-
ments with higher time resolution, such as nucleotide association and the GAP-stimulated GTPase reaction, a stopped-flow Applied Photophysics (Leatherhead, UK) SX18MV instrument was used. Fluorescence was excited at 546 nm. Emission was recorded via a cut-off filter (570 nm).

Conformational analysis of tamraGTP in the presence of Rac1
A model of tamraGDP and tamraGTP bound to Rac1 was created with the program SYBYL using the Rac1-GDP-RhoGDI and Rac1-GppNHp structures (Hirshberg et al., 1997; Grizot et al., 2001) and by manually replacing the bound GDP and GppNHp (a non-hydrolyzable GTP analog) by tamraGDP and tamraGTP, respectively. The missing tyrosine 32 in the Rac1-GppNHp structure (Hirshberg et al., 1997) was generated by the program CHARMM (Brooks et al., 1983) in the most favorable confor-
mation found by energy minimization. Possible modes of binding of the tamra nucleotide to Rac1 were explored by conforma-
tional analysis. According to the assumption that the guanine, ribose and phosphate groups of tamra nucleotides bind in the same way as GDP or GTP, their torsion angles remained intact. They were also anchored in the position found in the X-ray struc-
tures, so only atoms belonging to the EDA linker and rhodamine group could change their positions during the generation of con-
formers. Because of their relatively small size, torsion angles within carboxyl and EDA moieties were also omitted from the analysis. The remaining seven free torsions were then system-
matically changed by 60°. The geometry of each conformation was optimized without any constraints by short energy mini-
mization in the presence of the rigid Rac1 protein. Final representa-
tive modes of tamra-GTP binding to Rac1 were obtained by cluster analysis of conformations with favorable interaction energies.

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