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RhoGDI facilitates geranylgeranyltransferase-I-mediated RhoA prenylation

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ABSTRACT

Protein prenylation is a post-translational modification where farnesyl or geranylgeranyl groups are enzymatically attached to a C-terminal cysteine residue. This modification is essential for the activity of small cellular GTPases, as it allows them to associate with intracellular membranes. Dissociated from membranes, prenylated proteins need to be transported through the aqueous cytoplasm by protein carriers that shield the hydrophobic anchor from the solvent. One such carrier is Rho GDP dissociation inhibitor (RhoGDI). Recently, it was shown that prenylated Rho proteins that are not associated with RhoGDI are subjected to proteolysis in the cell. We hypothesized that the role of RhoGDI might be not only to associate with prenylated proteins but also to regulate the prenylation process in the cell. This idea is supported by the fact that RhoGDI binds both unprenylated and prenylated Rho proteins with high affinity *in vitro*, and hence, these interactions may affect the kinetics of prenylation. We addressed this question experimentally and found that RhoGDI increased the catalytic efficiency of geranylgeranyl transferase-I in RhoA prenylation. Nevertheless, we did not observe formation of a ternary RhoGDI*RhoA*GGTase-I complex, indicating sequential operation of geranylgeranyltransferase-I and RhoGDI. Our results suggest that RhoGDI accelerates Rho prenylation by kinetically trapping the reaction product, thereby increasing the rate of product release.

1. Introduction

Rho GTPases are post-translationally prenylated on their C-termini with either 20-carbon geranylgeranyl or, less commonly, 15-carbon farnesyl groups. This protein modification is catalyzed by the geranylgeranyltransferase type I (GGTase-I) and farnesyltransferase (FTase) enzymes, respectively [1]. Prenylation is critical for the functioning of Rho proteins, as it enables them to reversibly associate with intracellular membranes [2] and promote binding to their key regulators, such as GDP exchange factors (GEFs) [3] and Rho GTPase-activating proteins (GAPs) [3,4]. An additional level of Rho regulation is brought about by the Rho GDP dissociation inhibitors (RhoGDIs) [5]. Three human RhoGDIs have been identified with the first isoform, RhoGDI-1 (here, RhoGDI), being most ubiquitously expressed and associating with most of the Rho proteins. RhoGDI forms tight complexes with prenylated Rho GTPases, thereby withdrawing them from the membrane and association with GEFs [6,7], GAPs [8], and downstream effectors.

Recently, it has become evident that the function of RhoGDI goes beyond its originally postulated role. It has been shown that the absence of RhoGDI leads to depletion of the cytosolic fraction of prenylated Rho proteins, where the highly hydrophobic isoprenoid moiety functions as a signal for degradation by intracellular proteases [9]. As a result, competition between prenylated Rho proteins for limiting amounts of RhoGDI [10] results in RhoGDI-mediated regulation of their intracellular levels [9,11]. Thus, it may be a physiological requirement for the prenylated Rho proteins to be in complex with RhoGDI after dissociation from the membranes or following their prenylation. The latter assumption is supported by the fact that impairment of the Rho:RhoGDI interaction leads to concentrations of RhoA, Cdc42, and Rac1 in the putative protein prenylation sites: the endoplasmic reticulum [9] and Golgi apparatus [12].

Further, RhoGDI's ability to bind both unprenylated and prenylated forms of Rho with physiologically relevant affinities [5,13] suggests that it may be involved in Rho pre- and postprenylation processing. This may be similar to the function of Rab escort protein (REP), which binds newly synthesized Rab proteins and presents them to the RabGGTase [14,15]. This function, however, cannot be attributed to RhoGDI, as GGTase-I does not require an accessory protein for its activity. Yet, RhoGDI may accelerate

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prenylation by kinetically trapping the product of the reaction and delivering it to the membrane compartment, similar to REP.

In this study, we assessed a possible role of RhoGDI in the prenylation reaction. We found that RhoGDI accelerates GGTase-I-mediated RhoA prenylation *in vitro* with a fluorescent analog of farnesyl pyrophosphate or with geranylgeranyl pyrophosphate by increasing the enzyme's processivity. This was not accompanied by formation of a RhoGDI∗RhoA∗GGTase-I ternary complex, indicating sequential operation of the proteins. Together with previous findings, these results strongly suggest that RhoGDI may be an essential component of the Rho prenylation machinery *in vivo*.

2. Materials and methods

2.1. DNA cloning

The C-terminal eGFP-RhoGDI and N-terminal mCherry fusions of RhoB and Cdc42 were cloned using the in-fusion protocol into the pOPINE vector [16] at the Queensland University Protein Expression Facility. The constructs for expression of RhoA, RhoGDI, and GGTase-I are reported elsewhere [5].

2.2. Protein expression and purification

GGTase-I, RhoA, and RhoGDI were expressed and purified as described previously [5].

2.3. Preparative enzymatic prenylation of RhoA

Purified RhoA was prenylated *in vitro* by GGTase-I using geranylgeranyl (Sigma) or NBD-geranyl pyrophosphate as described earlier [5].

2.4. Preparation of RhoA-GG∗RhoGDI-TMR complex

RhoA was prenylated in the presence of RhoGDI labeled with TMR as reported earlier [5].

2.5. Labeling of RhoGDI with maleimide dye

Bovine wild-type RhoGDI was labeled with tetramethylrhodamine maleimide (TMR) (Life Technologies, USA) according to the manufacturer's protocol.

2.6. Fluorescence anisotropy measurements

The time course of RhoA prenylation reaction by GGTase-I was performed at 37 °C in 1-ml quartz cuvettes (Hellma, Germany) on a Spex Fluoromax-4 spectrofluorometer fitted with polarizers of L-geometry (Jobin Yvon Inc., USA). The experiments were carried out in FA buffer containing 25 mM Hepes–NaOH, pH 7.2, 40 mM NaCl, 2 mM $MgCl_2$, and 1 mM TCEP. The NBD fluorophore was excited at 479 nm, and emission was observed at 560 nm. Fluorescence anisotropy $\langle r\rangle$ was determined as:

$$\langle r\rangle = \frac{I_{VV} - G \times I_{VH}}{I_{VV} - 2 \times G \times I_{VH}}$$

where I is the fluorescence intensity and the first subscript letter indicates the direction of exciting light and the second shows the emitted light; the "G-factor" G was defined as:

$$G = \frac{I_{HV}}{I_{HH}}$$

Primary data analysis was performed with Grafit 5.0 (Erithacus software) and the "Fluorescence" implementation (Jobin Yvon Inc., USA) of Origin 7.0 (Originlab Corporation, USA).

2.7. Steady-state enzyme kinetics and data analysis

After setting up a preincubation reaction mixture containing 0.1 μM GGTase-I and 1 μM NBD-GPP with or without RhoGDI in FA buffer with 20 μM $ZnCl_2$ (FAZn) at 37 °C for 2 min and measuring its fluorescence anisotropy, the reaction was started by the addition of RhoA. Initial velocities were calculated from the linear phase of the reaction. Then, apparent initial rates and the Michaelis–Menten (K_m) constant were calculated as described previously with some changes [17]. In brief, the apparent initial rates were defined as:

$$v' = \frac{\Delta\langle r\rangle}{\Delta t} \times A_0$$

where $\Delta\langle r\rangle$ is fluorescence anisotropy change over time Δt and A_0 is the initial concentration of a substrate (RhoA). K_m was obtained from a fit to the Hill Eq. (1):

$$v' = \frac{V'_{max} \times A_0^h}{K_m^h + A_0^h} \quad (1)$$

where V'_{max} is the apparent maximal prenylation velocity and h is the Hill coefficient.

2.8. SDS–PAGE-based NBD-geranylation assay

The assay was carried out as described previously with minor modifications [18]. In brief, RhoA (4 μM) was incubated with GGTase-I (0.4 μM) at RT, and NBD-GPP (4 μM) was in FAZn buffer in a final volume of 10 μl. Then, the reaction was quenched by the addition of 2× Laemmli SDS–PAGE sample buffer and analyzed by electrophoresis. Subsequently, the gels were scanned on a Typhoon Trio fluorescent scanner (GE Healthcare; λ_{ex} = 488 nm; cutoff filter 520 nm), followed by staining with SimplyBlue (Invitrogen) and documentation with Odyssey (LI-COR Biosciences, USA).

2.9. HPLC-based geranylgeranylation assay

GGTase-I mediated RhoA geranylgeranylation was carried out in a 96-well 2-ml plate in a water bath. The reaction mixture, containing 0.25 μM GGTase-I and 1–16 μM RhoA with or without RhoGDI in FAZn buffer, was preincubated at 37 °C for 2 min before the addition of 2 mM GGPP solution (Sigma–Aldrich) to a final concentration 100 μM and mixed; 50-μl aliquots were withdrawn from the reaction and mixed with 50 μl of ice-cold stop solution (20% glycerol, 5 mM EDTA, 0.05% TFA) and flash-frozen. Samples were analyzed via HPLC (Prominence, Shimadzu) on a 150 × 2 mm C4 Jupiter column (300-Å pore size, 5-μm diameter beads) and a 40–61.5% acetonitrile gradient in 0.05% TFA. Obtained chromatograms were analyzed in Shimadzu LabSolution software and Grafit 5.0 (Erithacus software).

2.10. Microscale thermophoresis measurements

Thermophoresis was used to measure the affinity between GGTase-I and RhoGDI-TMR or between GGTase-I and RhoA-GG∗RhoGDI-TMR complex in a setup similar to that reported elsewhere [19]. Protein mixtures were loaded into standard treated glass capillaries. Measurements were carried out at room temperature in a Monolith NT.115 instrument (Nanotemper Ltd., Germany). The TMR-labeled RhoGDI was illuminated with a green LED (540–560 nm), and fluorescence was detected with a 585–615-nm cut-off filter. Data analysis was performed with NT Analysis 1.5.37 software (Nanotemper Ltd., Germany) as well as with Dynafit 4.0 (Biokin, USA) [20].

2.11. Isothermal titration calorimetry measurements

Binding affinities of the unprenylated RhoA to RhoGDI and GGTase-I were determined using ITC200 (MicroCal, USA) as reported earlier [5].

3. Results

3.1. Unprenylated RhoA interacts with GGTase-I and RhoGDI

We conjectured that the high affinity of RhoGDI for unprenylated RhoA may drive the formation of RhoA∗RhoGDI complex prior to the prenylation event [5]. To test this possibility, we compared the affinities of RhoA for RhoGDI and GGTase-I using isothermal titration calorimetry (ITC). Binding of RhoA to RhoGDI has a K_d value of 180 ± 6 nM (Fig. 1A), while the RhoA:GGTase-I interaction displays a K_d value of 12.4 ± 0.7 μM (Fig. 1B). We then sought to establish whether the interaction of RhoGDI with unprenylated Rho GTPases could also occur in mammalian cells [21]. To this end, we used fluorescence lifetime imaging microscopy-Förster resonance energy transfer (FLIM-FRET) to follow the interaction of C-terminally EGFP- or mCitrine-tagged RhoGDI with N-terminally mCherry-tagged Rho proteins in transfected BHK cells.

In cells transfected with RhoGDI-mCitrine and mCherry-RhoB, the donor fluorescence lifetime was significantly reduced, suggesting that high FRET is due to the interaction of the proteins. FRET remained high, even after cells had been treated with an inhibitor of isoprenoid biosynthesis, compactin. This indicates that RhoGDI and unprenylated RhoB stably interact in mammalian cells (Supplementary Fig. S1A). To further validate these data, we performed FLIM-FRET experiments using a C-terminally truncated mutant of RhoA that cannot undergo prenylation. The observed FRET levels were comparable to those of prenylatable Cdc42 and RhoB (Supplementary Fig. S1B). These data confirmed our *in vitro* findings that RhoGDI can associate with non-prenylated RhoGTPases and further suggest that RhoGDI may interact with RhoGTPases following their synthesis and prior to their prenylation by GGTase-I.

3.2. RhoGDI, RhoA, and GGTase-I do not form a stable ternary complex

Since RhoGDI is able to associate with both unprenylated and prenylated Rho proteins, we wanted to establish whether prenylation of RhoA protein requires its dissociation from RhoGDI. To this end, we labeled RhoGDI with tetramethylrhodamine maleimide (RhoGDI-TMR), which conjugates to a single cysteine residue and was shown not to affect RhoGDI's functionality [13]. We then isolated complexes of geranylgeranylated RhoA with either GGTase-I or RhoGDI-TMR by size-exclusion chromatography (Supplementary Figs. S2 and S3). Next, we tested ternary RhoA∗RhoGDI∗GGTase-I complex formation by mixing 1 μM solution of geranylgeranylated RhoA complexed either to GGTase-I or to RhoGDI with a counter binding partner, RhoGDI-TMR or GST-GGTase-I, respectively. As a measure of complex formation, we monitored the change in fluorescence anisotropy of TMR dye (Fig. 2A). We observed an increase in anisotropy signal upon addition of the RhoA-GG∗GGTase-I complex to the solution of labeled RhoGDI. We interpreted this as relocation of prenylated RhoA to RhoGDI molecule due to the higher affinity of RhoGDI for prenylated RhoA [5]. This notion gets support from the observation that addition of GST-GGTase-I to RhoA-GG∗RhoGDI-TMR did not result in anisotropy changes.

To corroborate these observations using an independent method, we titrated increasing concentrations of GGTase-I to either RhoGDI-TMR or its complex with RhoA-GG and measured

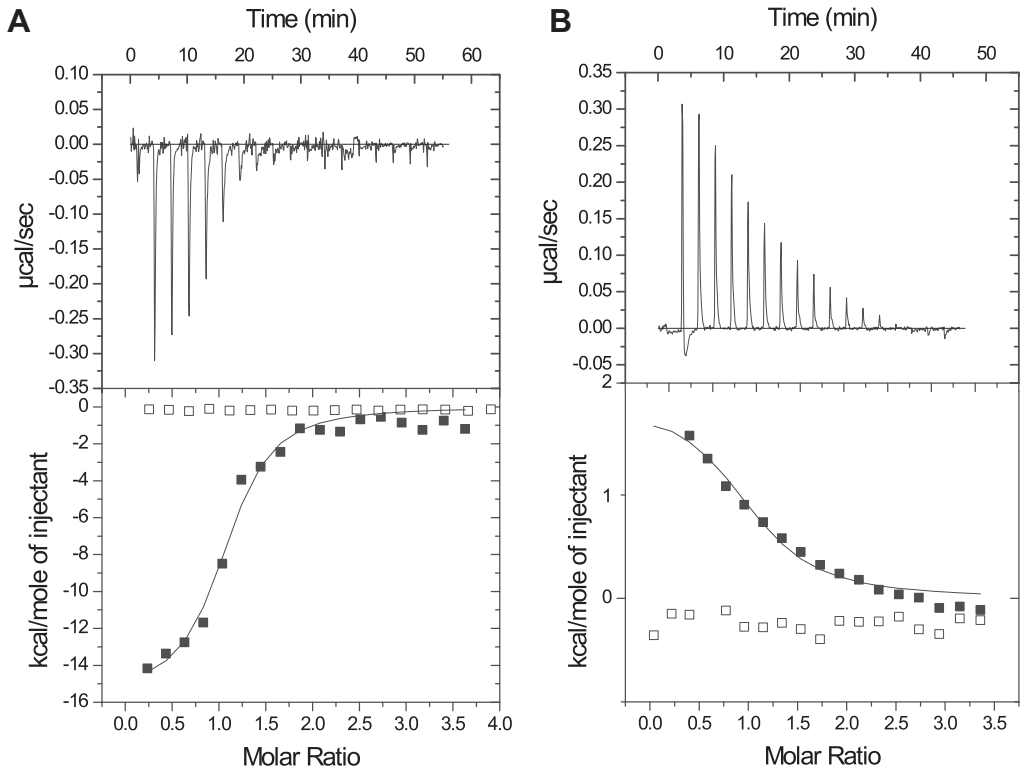

Fig. 1. Analysis of unprenylated RhoA interaction with RhoGDI and GGTase-I by ITC. (A) Representative ITC data for the titration of 10 μM RhoGDI with either 150 μM RhoA·GDP (filled squares) or buffer (empty squares). In the lower panel. the solid curve represents the fit of the data to a 1:1 stoichiometry binding model with a calculated K_d value of 180 nM. (B) Titration of 680 μM RhoA (filled squares) or buffer (empty squares) to 60 μM solution of GGTase-I. The data in the lower panel was fitted as in (A) giving a K_d value of 12 μM.

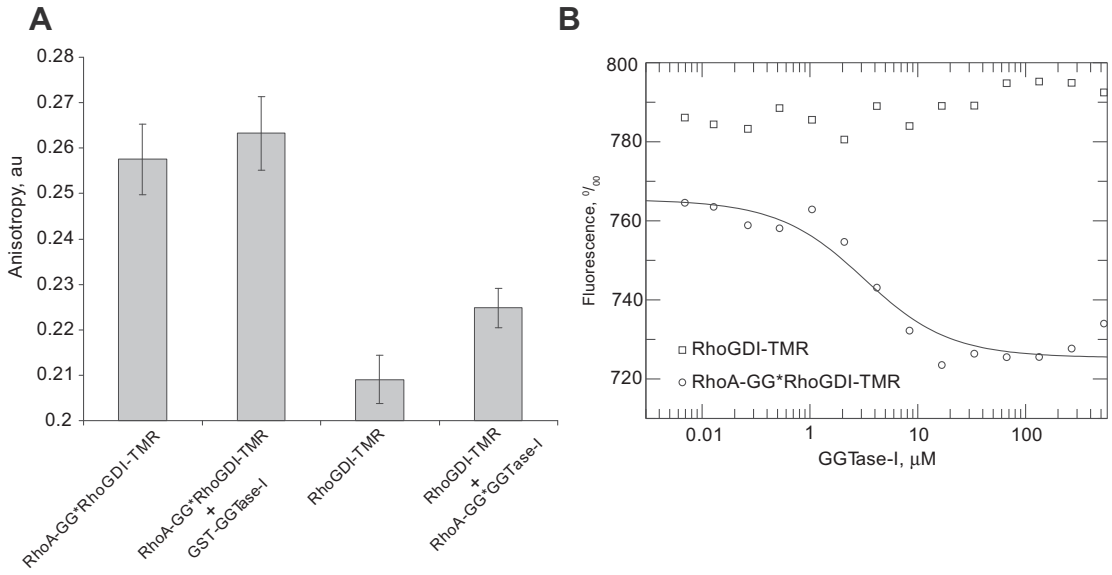

Fig. 2. Interaction analysis of RhoA-GG∗RhoGDI or RhoA-GG∗GGTase-I complexes with GGTase-I and RhoGDI proteins, respectively. (A) Equal amounts of RhoA-GG∗RhoGDI-TMR and GST-GGTase-I or RhoA-GG∗GGTase-I and RhoGDI-TMR were mixed, and changes in fluorescence anisotropy were monitored. Error bars represent standard deviation of the measurements. (B) Microscale thermophoresis titration of either RhoGDI-TMR (open squares) or RhoA-GG∗RhoGDI complex (open circles) with GGTase-I. Fitting of the latter data to a competition binding model (solid line) resulted in a K_d value of 1.2 nM for the RhoA-GG∗GGTase-I interaction, with 50 pM for the RhoA-GG∗RhoGDI interaction. The nonlinear fit of the data was performed in the Dynafit 4.0 program.

changes in RhoGDI thermal diffusion by microscale thermophoresis (Fig. 2B). Titration of RhoGDI-TMR with GGTase-I did not change the thermal diffusion of the former, indicating that there was no association between these proteins. On the other hand, titration of the RhoA-GG∗RhoGDI-TMR complex with GGTase-I led to dose-dependent changes in thermal diffusion of RhoGDI-TMR. This either could be the result of association of the enzyme with RhoA-GG∗RhoGDI-TMR complex or could be interpreted as sequestration of geranylgeranylated RhoA from its complex with RhoGDI by GGTase-I. The latter appears to be most likely explanation. The RhoA:GGTase-I interaction is expected to be driven by the GTPase C-terminus [22], which should become sterically inaccessible in the RhoA-GG∗RhoGDI complex. With the assumption that RhoGDI and GGTase-I interact with RhoA in a mutually exclusive manner, we fitted this titration data, yielding a K_d value of 1.2 ± 0.28 nM for the RhoA-GG interaction with GGTase-I, where the K_d value of RhoA-GG∗RhoGDI complex was fixed at 50 pM. This is reasonably close to the previously reported affinities [5].

3.3. Effect of RhoGDI on GGTase-I-mediated NBD geranylation of RhoA

We anticipated that RhoA must dissociate from RhoGDI for its posttranslational modification. In such a case, RhoGDI may act as an inhibitor of the prenylation reaction. However, a comparison of the k_{off} of the RhoA∗RhoGDI complex (0.55 s^{-1} [5]) with the k_{cat} values of the GGTase-I-mediated prenylation of GST-RhoA (0.08 s^{-1}) [18] and GST-Cdc42 (0.0057 s^{-1}) [23] demonstrates that dissociation of the RhoA∗RhoGDI complex is more than an order of magnitude faster than the rate of catalysis. Therefore, the presence of stoichiometric amounts of RhoGDI should not significantly hamper the availability of RhoA for its modification by GGTase-I. In contrast, the high-affinity interaction of RhoGDI with prenylated RhoA may drive the reaction equilibrium and free the GGTase-I for further prenylation cycles. In order to test this conclusion experimentally, we established a fluorescent *in vitro* RhoA prenylation assay in which we used a fluorescent isoprenoid analog, NBD-geranyl pyrophosphate (NBD-GPP). NBD-GPP was shown to be an efficient prenyl donor for GGTase-I and FTase [18]. We took advantage of significant changes in fluorescence anisotropy upon prenylation of RhoA with NBD-GPP (Supplementary Fig. S4A) to monitor the progression of the reaction. Using this technique, we determined the Michaelis–Menten constant for NBD-GPP (0.75 μM) (Supplementary Fig. S4B), which was 360-fold lower than for NBG-FPP but comparable to the K_m value of farnesyltransferase for NBD-GPP (0.5 μM) [18].

Next, we measured the steady-state kinetic parameters for RhoA as a protein substrate for the NBD-geranylation reaction. We found a K_m value for RhoA of approximately 3.3 μM (Hill coefficient 3.42) (Supplementary Fig. S4C and D), which is comparable to the value previously determined for the FTase-driven prenylation of GST-Ki-Ras with the same isoprenoid substrate [18]. We used this prenylation assay to monitor NBD geranylation of RhoA by GGTase-I in the presence of increasing concentrations of RhoGDI (Fig. 3A). The addition of RhoGDI to the reaction mixture resulted in an increase of the Michaelis constant. With that, we observed an even more profound increase in the values of the apparent catalytic constant of the reaction in a RhoGDI-concentration dependent manner. Overall, GGTase-I catalytic efficiency (k'_{cat}/K_m) rose more than 3-fold in the presence of RhoGDI. Also, the fit to a hyperbolic equation of the obtained K_m and k'_{cat} values (Fig. 3B) demonstrated that the half-maximum value of either enzymatic parameter was reached at an RhoGDI concentration of approximately 0.3 μM, which is significantly lower than the concentrations of RhoA in the reaction mixtures. To provide independent confirmation of the observed phenomenon, we repeated the experiments using an SDS–PAGE-based prenylation assay [18]. Here, we observed a clear RhoGDI concentration-dependent increase in the yield of RhoA NBD geranylation (Supplementary Fig. S5), supporting the data obtained in the solution-based assay.

3.4. Effect of RhoGDI on GGTase-I-mediated geranylgeranylation of RhoA

In the next step, we sought to confirm that the observed effect of RhoGDI on RhoA prenylation was similar when the native GGPP substrate was used. We took advantage of the significant increase

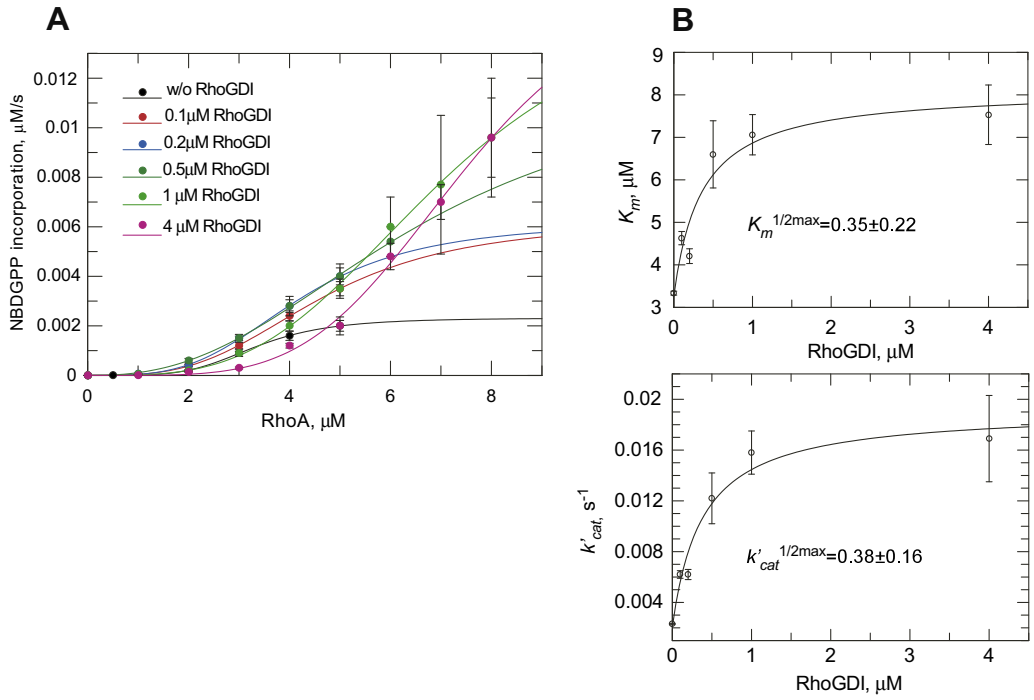

Fig. 3. Effect of RhoGDI on GGTase-I-mediated NBD-geranylation of RhoA. (A) Comparison of RhoA prenylation initial velocity rates in the presence of the indicated concentrations of RhoGDI. Data were fitted to the Hill equation (see Section 2). (B) Effect of RhoGDI on the steady-state enzymatic parameters of the prenylation reaction. Both K_m and apparent k'_{cat} values were fitted to the hyperbolic equation. Error bars represent error of the fit.

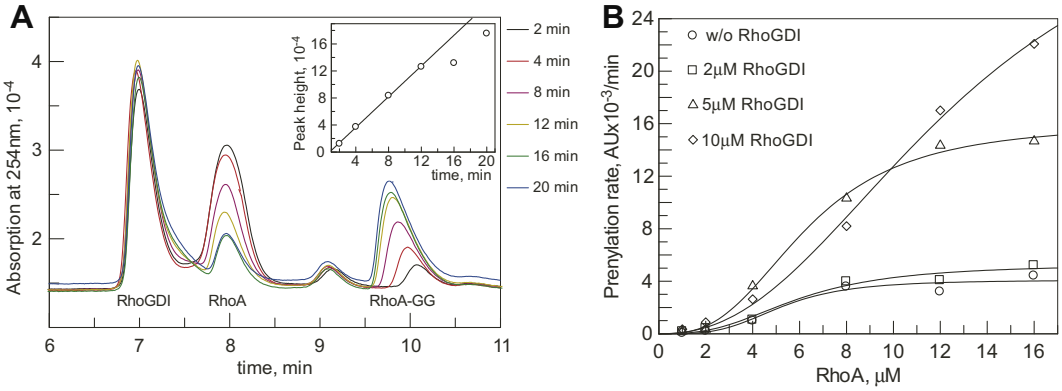

Fig. 4. Kinetics of RhoA geranylgeranylation by GGTase-I in the presence of RhoGDI. (A) A characteristic chromatogram of the prenylation mixture demonstrating accumulation of RhoA-GG and disappearance of RhoA at different time points. Embedded graph shows the plot of the height of the RhoA-GG peak against reaction time. The line is a linear fit of the first 4 points. (B) Measurement of K_m and V_{max} of RhoA geranylgeranylation by GGTase-I in the presence of different concentrations of RhoGDI. Data were fitted to the Hill equation.

in RhoA hydrophobicity upon geranylgeranylation, whereby prenylated product could be separated from the unprenylated substrate on a C4 HPLC column (Fig. 4A). We used this assay to monitor the linear phase of the prenylation reaction in the presence of different RhoGDI concentrations. Plotting the rates of the geranylgeranylation reaction clearly revealed a sigmoidal dependence of the prenylation rate on RhoA concentration, prompting us to fit the data to the Hill equitation (Fig. 4B). This may be explained by the relative concentration of RhoA (1–16 μM)—which, at lower concentrations, is only a little higher than the GGTase-I concentration (0.25 μM). For enzymes with two substrates, such sigmoidal behavior could be expected [24]. We next aimed to determine the effect of RhoGDI on the kinetics of RhoA prenylation. We calculated a K_m value for RhoA in this condition of 5.2 ± 1.1 μM (Hill coefficient 3.56), which is very close to the constant measured with NBD-GPP. As can be seen in Fig. 4B and Table 1, the apparent V'_{max} values were significantly increased in response to the addition of RhoGDI to the reaction mixture, whereas the K_m parameter changed comparatively little and the Michaelis constant only increased

Table 1
Steady-state enzymatic parameters of geranylgeranylation in the absence or presence of RhoGDI. Errors are errors of the fit.

RhoGDI (μM)	V_{max} (Au/min)	K_m (μM)	V_{max}/K_m
0	4093 ± 562	5.23 ± 1	782.9
2	5280 ± 806	5.93 ± 1.18	890.5
5	16014 ± 756	6.30 ± 0.37	2540.2
10	36248 ± 8720	13.04 ± 2.93	2778.7

at the highest RhoGDI concentration. Similar to the effect of RhoGDI on the kinetics of NBD-geranylation of RhoA, the addition of RhoGDI to the geranylgeranylation mixture resulted in a concentration-dependent increase of catalytic efficiency, with a 3.5-fold maximum change.

4. Discussion

RhoGDI, for a long time, has been given the role of a passive negative regulator of Rho GTPases. However, recently, the spectrum of RhoGDI-mediated regulation has been largely extended and now includes regulation of Rho protein crosstalk and shuttling between cellular membrane compartments [25]. In particular, it has been shown that prenylated Rho proteins that are not associated with RhoGDI in the cell are targeted by proteases [9]. Given this physiological requirement for Rho proteins to associate with RhoGDI, we conjectured that this protein may also be involved in the regulation of prenylation. This previously unconsidered role of RhoGDI in the regulation of the prenylation reaction lies in its ability to bind both the substrate and product of the prenylation reaction.

With many lines of evidence, we demonstrated that RhoGDI facilitates GGTase-I-mediated prenylation by increasing steady-state enzymatic parameters. First, we have shown that the apparent k'_{cat} of the NBD-geranylation reaction was increased by a factor of 7.4 as a result of RhoGDI presence in the prenylation mixture, whereas the Michaelis–Menten constant increased by 2-fold. Overall, GGTase-I 'catalytic efficiency' (k'_{cat}/K_m) increased by more than 3 times. More importantly, the same effect was observed with native isoprenoid geranylgeranyl pyrophosphate. We assumed that the increase in K_m value is due to the binding of RhoGDI to RhoA protein substrate, which lowers the effective concentration of the latter. On the other hand, the increase in k'_{cat} (or V_{max}) values is solely the result of tight RhoGDI binding to the prenylated RhoA. This drives the equilibrium of the GGTase-I interaction with prenylated protein towards dissociation of this complex. Thus, the effect of RhoGDI on the kinetics of RhoA prenylation is defined by the kinetics of RhoGDI interaction with either form of RhoA, where the increase in the rate of prenylation is coming solely from the kinetic trapping of modified RhoA by RhoGDI.

Overall, we have shown that RhoGDI accelerates the RhoA prenylation reaction. This effect might be relevant *in vivo*, where RhoGDI initially forms complexes with unmodified Rho proteins and subsequently may support the prenylation reaction by trapping the prenylated product. Importantly, our findings strongly support a recent hypothesis that the association of newly prenylated Rho GTPases with RhoGDI is a prerequisite for their proper cellular functioning [9]; hence, direct handover may play a physiological role [9,26,27]. It may be that the presence of free RhoGDI in the cell, the amount of which is normally limited due to its constant occupation by Rho GTPases, serves as a signal for acceleration of prenylation of newly translated Rho proteins.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.09.024.

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