Human Drg1 is a potassium dependent GTPase enhanced by Lerepo4.

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Abbreviations

TGS domain, ThrRS, GTPase, SpoT domain; DFRP domain, DRG family regulatory protein domain; NTPase, nucleoside triphosphatase; GDP-AlFx, GDP aluminium fluoride; HAS-GTPase, hydrophobic amino acid substituted for catalytic glutamine; TEES superfamily, TrmE-Era-EngA-YihA-Septin-like superfamily; GAP, GTPase activating protein; GEF, guanine-nucleotide exchange factor. BME, β -mercaptoethanol

Keywords

Lerepo4, Drg1, TGS domain, potassium dependent stimulation, ribosome binding GTPase.

Abstract

Human Drg1, a guanine nucleotide binding protein conserved in archaea and eukaryotes, is regulated by Lerepo4. Together they form a complex which interacts with translating ribosomes. Here we have purified and characterized the GTPase activity of Drg1 and three variants, a shortened mutant depleted of the TGS domain, a phosphomimicking mutant and a construct with the two combined mutations. Our data reveal that potassium strongly stimulates the GTPase activity, without changing the monomeric status of Drg1 and that this activity is notably reduced in the mutants. The nature of Lerepo4 association has also been investigated. Dissecting the role of the different domains revealed that Dfrp domain is the sole responsible for the Drg1 increase in thermal stability and the four fold stimulation over its catalytic activity. Lerepo4 action leaves Drg1 affinity for nucleotides unaffected, feasibly favoring a switch I reorientation, mainly via the TGS domain. Drg1 displayed a high temperature optimum of activity at 42°C, suggesting the ability of being active under possible heat stress conditions.

Introduction

Drg1 is a human GTPase belonging to the Obg family that is found in archaea and in eukaryotes but not in eubacteria [1, 2]. It was first discovered as a developmentally regulated GTPase, abundantly transcribed in growing cells in mammalian and frog embryos [1, 3, 4] and in actively growing tissues in plants [5, 6], being predicted to regulate cell growth [5, 7]. It associates with polysomes, playing a role in translation [8].

DRG proteins from nearly all organisms contain 365–370 residues, with several well differentiated domains, the N-terminal HTH domain, the canonical GTP-binding domain, the S5D2L insertion domain and a small C terminal TGS domain [9]. The G domain responds to the classical architecture of GTPases with the five G boxes and two switch regions [10], whereas the TGS domain has a predominantly beta-sheet structure sharing a common basic fold similar to ubiquitin and sumo proteins (PDB code 2EKI). TGS domain appears in other members of the family like YchF and Obg, and also in threonyl tRNA synthetases, suggesting a regulatory role [11].

GTPases are typically very inefficient enzymes, thus, many of them get associated with specific regulators, the GDP-GTP exchange factors (GEFs) to mediate their activation, and the GTP-hydrolysis activating proteins (GAPs) to terminate their activity [12]. GAPs usually provide a catalytic residue to the active center that functions *in trans*, while GEFs help to lower the affinity for the guanine nucleotides. Alternatively, there have been found GAP -independent ways of activation as dimerisation, association with ribosomal subunits or cation binding, usually a potassium ion [13]. Neither GAP nor GEF have been found for Drg1, which, according to its sequence has been predicted to be a potassium-dependent GTPase [13].

Nucleotide cleavage in GTPases is triggered by a water molecule conducting a nucleophilic attack on the gamma phosphate of GTP. There are different ways of positioning or aligning the attacking water molecule and stabilizing the transition state of the phosphoryl transfer reaction [10]. Apart from a catalytic residue generally provided by switch II, the catalytic machinery can be further reinforced by a positive charge introduced by a GAP *in trans* (usually the so-called arginine finger or asparagine thumb, as in Rho and Ran, respectively [14]). Alternatively, the same role can be taken by the binding of a monovalent cation, as potassium ion (chemical GAP), coordinated

by switch I. The potassium contribution has been recently unraveled thanks to the solved structures of MnmE and FeoB in their active and inactive states [15, 16]. Surprisingly, even though the main catalytic residue is usually found in a conserved position (Gln61 from Ras) in switch II, a novel group of GTPases with a hydrophobic residue instead, called HAS-GTPases [17], has been described in which either the catalytic residue has been replaced by a network of backbone contacts, as in FeoB [15], or it could be provided by new regions of the protein, as Glu282 from MnmE [16].

After sequence alignments and the solved structure of the yeast homolog, Rbg1 (PDB code 4A9A), DRG factors can be considered HAS-GTPases, provided that we observe that instead of the catalytic glutamine, there is a hydrophobic residue, Ile122, in switch II adopting a “retracted conformation”. On the other hand, although there is a considerable conformational variability in the switch regions in the absence of a nucleotide, and they can adopt different conformations according to the crystal packing, the arrangement of switch I resembles that of FeoB or MnmE complexed with GDP [15, 16].

Interestingly, although Drg1 is not essential, two and three paralog proteins highly similar can be found in animals and plants, respectively, suggesting an important role of these redundant proteins in fundamental biological processes [1, 8]. In this sense, microarray data and reporter gene assays have shown that DRGs from *Arabidopsis thaliana* are transcriptionally regulated, and the protein accumulates directly and specifically in response to heat stress and desiccation [18, 19]. Besides, Drg1 from *Candida albicans* plays a role in the control of invasive filamentation [20]. Lastly, no clear phenotype has been found for the Drg1 or Drg2 knockdown human cell lines [21] neither for the yeast gene deletion strains [22]. Taken together, these observations

suggest that Drg1 may require certain specific conditions to switch on its regulatory function through the protein synthesis machinery.

In this regard, the biological functions of the members of two subfamilies of the Obg family of GTPases which have been better characterized to date, Obg, OLA1 and YchF, have been related to stress response and ribosome assembly [23-28].

Drg1 is subjected to a tight regulation. It has been found to be a target of sumoylation, stimulated by the MEKK1 Map3kinase [29], of ubiquitination [29, 30], and of phosphorylation, undertaken by MPSK1 [31]. The physiological meaning of this latter action is still unclear, but other GTPases are down or upregulated by phosphorylation [32].

Lerepo4 (or Dfrp1) was first identified *in vitro* and further confirmed *in vivo* as a partner of Drg1 [1, 8, 30], and it has been demonstrated in yeast to be responsible for its recruitment to polysomes [9, 33]. Automated predictions have considered Lerepo4 as an intrinsically unstructured protein, and the structure of the C-terminal part of Tma46 solved in complex with Rbg1 corroborates this idea, as it adopts a non globular extended conformation, contacting the GTPase and TGS domains of Rbg1 [9].

Lerepo4, highly conserved among eukaryotes, has two unique CCCH-type N-terminal zinc fingers and a C-terminal Dfrp domain, being the second responsible for the interaction with Drg1 [9]. The two consecutive repeats of the Zn finger are similar to the C3HC4-type and C3H2C3-type RING fingers, characteristic of proteins involved in degradation through the ubiquitin proteasome pathway. Lerepo4 is somehow related to this pathway, since it diminishes Drg1 protein degradation by preventing its polyubiquitination [30], representing therefore a way of regulating Drg1 protein levels, which may be important for its function.

Additional roles of Lerepo4 are poorly understood. However, recent data have revealed that human cells increase Lerepo4 expression upon HIV infection [34], and it is also upregulated in neuronal rat cells by neurotrophic factor stimulation [35].

To gain further insights into the function of Drg1, the role of the TGS domain, the mode of interaction with Lerepo4 and its effects on GTPase activity, the kinetic characteristics of Drg1 have been addressed in the present work.

Results

Characterization of Drg1 enzymatic activity

In order to characterize human Drg1, the full length and the different variants were overexpressed in *E. coli* and purified nearly to homogeneity (Fig.1A,B). The kinetic parameters of GTP hydrolysis were analysed and the best fit of the data was obtained using the Michaelis-Menten equations. Although Drg1 catalyzes the production of equimolar amounts of GDP and Pi at a slow rate, it is greater than the one observed for other Obg-like GTPases [36, 37]. The K_{cat} is 0.147 min^{-1} ($V_{max} = 3.59 \pm 0.04 \text{ nmol/min/mg protein}$), and the K_m for GTP is 0.25 mM (Table 1). This enzyme is relatively stable in a wide range of pHs (from 6.5 to 9.0), with an optimal pH, extending from 8 to 9 (Fig. S1A). Surprisingly, Drg1 displays a thermophilic behavior. Its activity shows a sharp increase between 37°C and 50°C almost doubling the activity values regarding to temperatures out of this interval (Fig. S1B). The optimal temperature is consequently relatively high (42°C , Fig. S1B). Interestingly, Drg1 needs high amounts of potassium ions (Fig. S1C) with the enzymatic activity increasing almost steadily until the highest K^+ concentration tested (1M), whereas it appears to be sodium independent (data not shown). Drg1 transformation of ATP into ADP remained undetected pointing towards a GTP/GDP specific enzyme (data not illustrated), conversely to other NTPases from the Obg family, like the YchF subfamily, which are able to hydrolyze both GTP

and ATP molecules [38]. This is not surprising, as the guanine specificity is mostly conferred by the G4 motif which in Drg1 fits into the consensus sequence.

Lerepo4 enhances the activity of Drg1

Despite the fact that Drg1 is able to catalyze GTP hydrolysis without auxiliary factors, the activity can be modulated in several ways. Apart from the mentioned effect of K^+ , Lerepo4 is the most determining factor on catalysis. The activity of Drg1 is around four times higher in the presence of this partner (Fig. 2A). Kinetic assays demonstrate that the ratio between Lerepo4 and Drg1 is 1:1 (Fig. 2B). This is equivalent to the association of the yeast homologs Rbg1 and Tma46, which is a heterodimer made of a monomer of each protein, as has been illustrated by the recently solved crystal structure of the complex [9]. While the biophysical aspects of Drg1, as the optimum temperature and pH, remain the same, the kinetic constant for potassium improves when Lerepo4 is present (Fig. S1, Table1).

The N-terminal part of Lerepo4 has no effect on Drg1 activity whereas the C-terminal part is responsible for the GTPase stimulation.

No increase of activity was observed when the Drg1 GTPase was evaluated in the presence of the N-terminal part of Lerepo4, corresponding to the Zinc fingers, even when the molar ratio was increased ten times with respect to Drg1. However, Lerepo4 Dfrp domain is enough to induce the reported increase in Drg1 activity (Fig. 2A) to the same extent as full length Lerepo4. The ratio between the C-terminal part of Lerepo4 and Drg1 is also shown (Fig. 2B), indicating a 1:1 stoichiometry.

GDP inhibition is competitive

Drg1 is completely and hyperbolically inhibited by GDP when assayed at 1.5 mM GTP, with a K_i of 0.27 mM (Fig. S2A, Table 1). The inhibition constant did not improve in the presence of Lerepo4, showing no real contribution of Lerepo4 in GDP binding. Lineweaver-Burk graphs show that the inhibition was competitive, since its mainly detrimental effect on the activity of the enzyme was the increase on the K_m (Fig. S2B).

Mimicking phosphorylation of Thr100 decreases Drg1 activity

Drg1 was identified as substrate of the human protein kinase MPSK1 and shown to be phosphorylated on Thr100, an evolutionarily conserved residue located immediately after the G2 motif, as part of switch I [31]. To assure reproducible enzymatic assays, instead of doing *in vitro* phosphorylation of the wild type enzyme, phosphorylation was mimicked through a mutation to an aspartate residue that simulates the negative charges present on phosphorylated serines. T100D mutation triggers a drop on the activity, rendering a two thirds less active enzyme, suggesting that phosphorylation could be a mean of downregulating the enzyme (Table 1, Fig. S3). On the other hand, the mutation does not have a significant effect over GTP binding nor GDP inhibition (Fig. S2B).

TGS domain is contributing to Drg1 activity and binding to Lerepo4

Although the GTPase activity is comprised within the G domain, the TGS domain has a modulatory effect over it, as reflected by the decrease of the maximum velocity of the truncated form (Table 1, Fig. S3). As expected, the TGS domain on its own does not show any activity (data not illustrated). GDP inhibited identically the wild type and the TGS truncated enzyme form (Fig. S2B), demonstrating that the Drg1 TGS domain is not involved in GDP mediated inhibition. Similarly, GTP binding was unaffected.

Interestingly, the data shows that in the absence of Lerepo4, the activity of the TGS truncated form was greater than the activity of the phosphorylation mimicking mutant. However, the opposite holds true in the presence of Lerepo4, as the truncated form lacks a great percentage of the area used to interact with Lerepo4 (Fig. S3). Nevertheless, the TGS truncated Drg1 gets associated to the C-terminal part of Lerepo4 with a 1:1 ratio (Fig. 2B).

The TGS deletion when combined with the phosphorylation mimicking mutation produced an additive effect, making the enzyme less active and susceptible to Lerepo4 stimulation.

Drg1 is thermally stabilized by Lerepo4

The incubation of Drg1 with Lerepo4 at various temperatures prior to the enzymatic measurement of the activity, showed a protective effect of Lerepo4 over Drg1 GTPase activity. This effect was extremely evident at 60°C, in which after 5-min incubation around 70% of the activity was retained when Drg1 was accompanied by Lerepo4, but only 10% activity was maintained by Drg1 on its own (Fig. 3A).

The phosphorylation emulating mutant T100D displayed only a slightly decreasing effect on the Drg1 GTPase activity pre-incubated on its own (Fig. 3B). The TGS domain deletion, on the other hand, showed a reduction in the Lerepo4 mediated thermal protection that became evident at 60 ° C (Fig. 3C).

The combination of the phosphomimicking mutation plus the TGS deletion had a stronger effect over the enzyme, making Drg1 more unstable, noticeably at 37°C, and less susceptible to Lerepo4 protection, shown as a reduced relative activity at 45°C (Fig. 3D).

K⁺ dependent activity is affected by TGS and by phosphorylation

When Drg1 variants were assayed alone, they all showed low affinity for potassium. The truncated mutant showed a slight increase in K⁺ affinity suggesting a moderate negative influence of TGS domain over the K⁺ binding conformation of switch I. This effect was more notorious for the phosphomimicking mutant, which in fact is introducing a negative charge in place of Thr100, possibly affecting the positioning of the loop, in a favorable manner towards K⁺. However, when both mutations were combined, K⁺ affinity was similar to Drg1.

In the presence of Lerep4, the K_m for potassium of wild type Drg1 experienced a ten fold improvement; the phosphomimicking mutant reached the same value, while the truncated versions of the enzyme were unable to improve substantially the binding of potassium (Table 1, Fig. S4).

K⁺ does not promote the dimerisation of Drg1

The apparent state of Drg1 deduced by the elution volume of the size exclusion chromatography, 82 ml, with a buffer without potassium is the one of a monomer (data not illustrated). The same chromatogram was obtained when the running buffer included K⁺, or K⁺ plus GDP plus aluminium trifluoride, denoting that K⁺ does not play a role in Drg1 dimerisation as an additional way of activating the enzyme. This is opposite to other GTPases as MnmE, a tRNA modification GTPase belonging to the Era-related GTPases [16], but akin to YchF, an ATPase from its family, or to EngA [39, 40]. A similar result was achieved through differential ultracentrifugation techniques. The c(s) profile of the protein obtained in the velocity experiment analysis showed three peaks at 0.8 mg/mL, regardless of the buffer used: the predominant component, at 72.2 %, had a sedimentation coefficient of 2.6 ± 0.1 S, which is equivalent to a molecular weight of 56 kDa, close to the size of the monomer

of Drg1 (Fig. S5). A minor proportion of higher molecular weights with apparent molecular masses close to Drg1 dimer or tetramer were also detected (Fig. S5), however this was independent of the presence/absence of potassium and/or GDP plus aluminium trifluoride indicating that potassium is not regulating the oligomeric state of the protein.

Discussion

For the first time, we have accomplished the detailed biochemical study of a GTPase from the Drg1 subfamily, filling the gap between all the structural and functional knowledge gathered in the past years about DRG factors and the lack of data about its kinetic performance.

No need for GAPs and GEFs

Drg1 exhibits a reasonable intrinsic GTP hydrolysis rate of 0.15 min^{-1} (greater than the values for Ras proteins, which are $\sim 0.02 \text{ min}^{-1}$), suggesting that there is no need for a GAP, in contrast to the small GTP-binding proteins of the Ras superfamily [14]. The activity of Drg1 is not much lower than that of other GTPases from the TEES superfamily, as FeoB or EngA [15, 40]. Likewise, Drg1 has micromolar binding constants for GTP and GDP, which gives rise to a rapid exchange of the nucleotide and hence it does not entail the presence of GEFs.

These properties indicate that, *in vitro*, the GTPase cycle of Drg1 occurs efficiently without auxiliary factors. Although the catalytic mechanism remains unknown, our results substantiate the importance of K^+ , confirming previous bioinformatic predictions, albeit the presence of serine residues instead of the two conserved asparagines in the region of the G1 motif [13]. Furthermore, we have expanded the knowledge by demonstrating that sodium fails to stimulate Drg1 activity, what compel us to include it within group I of potassium-selective cation-dependent G proteins [13].

Proposed model of Lerepo4 stimulation of Drg1 activity

In addition, Lerepo4, the partner protein which interacts with Drg1 in translating ribosomes, was demonstrated to be a modulator of the enzyme in several ways. As presented previously, binding of Lerepo4 to Drg1 is essential to maintain normal levels of chicken and human Drg1 *in vivo* [30] and to prevent its ubiquitin-mediated degradation [30]. Here it has been shown that the physical interaction with the Dfrp domain of Lerepo4 protects Drg1, conferring a greater thermal stability. Additionally Lerepo4 also improves Drg1 hydrolysis rate, rendering a more efficient enzyme. YchF experiences a similar effect, as when interacting with ribosomal proteins has a 10 fold increase on activity [26].

The solved structure of the yeast homologs Rbg1 and Tma46 in complex, almost rules out a role of Lerepo4 homolog as a GAP since they bind to their GTPase partners in the GTP-bound state, whereas Tma46 binds to Rbg1 in the absence of nucleotide. Moreover, the Rbg1-Tma46 structure shows Tma46 grasping Rbg1 opposite to the GTP binding site, displaying an open accessible area around the active center. Thus, it seems very improbable that Tma46 could contribute with any residue on the active center (Fig. 4). However, it should be pointed out that binding of Tma46 might condition the Rbg1 switch I orientation, as the C-terminal end of Tma46 contacts sideways the hinge of this loop (Fig. 5).

Reaching this point, question could arise about the specific role of Lerepo4 on the catalytic machinery. Our findings show that Lerepo4 has no effect on nucleotide binding, improving instead at a high extent the affinity for potassium ions, which indicates that Lerepo4 positive effect on GTPase catalysis is, at least, partially mediated by the observed increase in K^+ affinity.

As shown in Figure 5, the superposition of FeoB structures in its inactive and active forms, with the apo form of Rbg1, shows that switch I from Rbg1 superposes close to

the GDP inactive state of FeoB and far away from the GTP binding state. This implies that it could also undergo a large structural change upon hydrolysis of GTP, acting as a lid to close the active center, highlighting the idea of a loop with a great conformational mobility that is influencing to a large extent the catalysis. In fact, the mechanism of the GTPases from the Obg family has been described to be produced by a conformational change of switches, triggering internal domain rearrangements leading to an overall shape change of the molecule [10].

Regulatory role of the TGS domain

According to expectations, the TGS domain is unnecessary either for activity or for the inhibition of the enzyme but its presence, although worsening potassium binding, favors Drg1 catalysis, probably through a stabilization of a GTP-induced conformational state. In addition, it has been functionally ratified in yeast that the Rbg1 TGS domain is important for the association with Tma46, the Lerepo4 homolog, and as a consequence, this domain plays a primary part in the anchoring of Dfrps [9].

Although Lerepo4 still binds the truncated Drg1 variant with a 1:1 stoichiometry, it exerts a smaller activation over the mutant and a less protective effect during the high temperature pre-incubation assay, suggesting that the increase in the activity of Drg1 mediated by Lerepo4 takes place mainly through the TGS domain. Moreover, in the structure, Tma46 wraps the TGS domain of Rbg1 and then establishes contacts along the G domain. The structure of Rbg1-Tma46 complex shows a direct connection between Rbg1, TGS and G domains mediated by helix 2 of Tma46, suggesting that TGS could affect the catalytic properties of Rbg1 through Tma46 ([9] and Fig. 4).

Phosphorylation affects switch I conformation

Mimicking the phosphorylation of Drg1 in Thr100 produces an important decay on the activity of the enzyme, suggesting that phosphorylation on Thr100 might be a way to downregulate the GTPase activity of Drg1, with a probable repercussion in its function. In this sense, there is a report of a member of the same family, YchF, in which the function of the protein is regulated by phosphorylation, affecting its stress response [25].

Residue Thr100 is part of switch I, and it is located two residues away from the equivalent Thr35 of FeoB, which has been shown to be important for nucleotide hydrolysis [15]. Nonetheless, in the structure of Rbg1, the equivalent residue (Val101) is pointing away from the active center, likely not participating in catalysis (Fig. 5), implying that the aspartate replacement could be distorting the loop conformation instead, explaining the drop on the catalytic activity. Notwithstanding, this effect can be mostly neutralized by the binding of Lerepo4, which means that Lerepo4 is able to mend the conformation of switch I.

Other relevant effect of this mutation concerns the potassium binding. In the absence of Lerepo4, the affinity for this cation is improved, suggesting a loop conformation in which the residues involved in the binding are better positioned. As a result of these observations, we can conclude that potassium binding is necessary but not enough for catalysis. Other residues and molecular mechanisms must be entangled.

Possible involvement in heat stress

While affecting its activity, Lerepo4 has proved not to interfere with the biophysical characteristics of Drg1, as its optimal pH or temperature. Nevertheless, the enzyme exhibits a maximum of pH particularly basic, between 8 and 9, and a high temperature maximum with a broad range of temperatures in which is mostly active (from 37 to 50°C), which could be a hint about the possible involvement of Drg1 in heat stress, as is

the case of the Drg1 plant homolog [19] or other NTPases from its family, as Obg and OLA1 [27, 28].

Conclusion

Taken together, our data show that Drg1 is a low efficient GTPase, with abnormal optimal temperature and pH, possibly downregulated by phosphorylation, and dependent on potassium ions, although they don't induce dimerisation. The kinetic studies establish a double role for Lerepo4 in the stability and the activity of the enzyme, an effect that appears to reside in the Dfrp domain.

Our results glimpse a parallelism between two different kinds of mutations, a mutation inside switch I and the deletion of the TGS domain. Both mutants dampen their activity and improve potassium binding, indicating that they are affecting catalysis through switch I. Lerepo4 interaction gets to rescue T100D mutant to almost normal values but not the truncated variant, pointing to TGS as a regulatory domain. Lerepo4 could be capable of affecting Drg1 activity indirectly by binding to the TGS domain, transducing the signal to the G domain, and also directly through contacts between its C-terminal part and switch I, as the solved structure from the yeast homologs hint at (Fig. 4).

In short, we have clarified to some extent the catalytic features of Drg1, and its interaction with Lerepo4, foreseeing a paramount involvement of Lerepo4 on the task performed by Drg1, apart from its already proved protection against ubiquitination and its presence together with Drg1 in the ribosomes.

It would be interesting to determine the crystal structure of Drg1 in complex with a GTP analog to validate the experimental results shown about the architecture of the binding site. Efforts in this direction are presently underway in our laboratory.

Experimental procedures

Cloning and generation of mutant forms

Lerepo4 was amplified from the Megaman human transcriptome library (Agilent technologies, Santa Clara CA, USA) and cloned into a modified version of pET28 vector containing a cleavage site for PreScission protease enzyme (ppET28, kindly donated by Dr. Ramón Campos). Drg1 was cloned into pProExHTA from an IMAGE clone library. The Drg1 gene was truncated by PCR-amplification following the QuickChange method from Stratagene (La Jolla CA, USA) immediately after an in-frame stop codon that replaced the normal codon number 293. The QuickChange method was also followed to mutate residue threonine 100 by an aspartate, in the full length protein or in the variant lacking the TGS domain. The TGS domain of Drg1 (289-367aa) was subcloned into pET24. The N-terminal part of Lerepo4 (1-226 aa) and the C-terminal part of lerepo4 (221-396aa) were subcloned into ppET28 by PCR.

After confirming the presence of the desired constructs by DNA sequencing, we used the plasmids to transform *E. coli* BL21 (DE3) or BL21-CodonPlus (DE3)-RIPL cells (Agilent technologies, Santa Clara CA, USA).. Figure 1B shows a chart with a schematic representation of the different constructs.

Protein over-expression and purification

Proteins were over-expressed overnight at 20°C using 1mM IPTG (Isopropyl β -D-1-thiogalactopyranoside). Cells were harvested by centrifugation and stored at -80°C until use. Cells pellets were resuspended in Hepes 20 mM pH7.5, Glycerol 5%, NaCl 0.5M, β -mercaptoethanol (BME) 2mM, 0.01% Triton X-100 for sonication with a protease inhibitor cocktail tablet (Complete EDTA-free, Roche, Basel, Switzerland). Samples were centrifuged during 35 minutes at 30600 g. Supernatants were loaded on 5ml Nickel loaded Chelating columns washed with 20 mM Hepes pH7.5, Glycerol 5%,

NaCl 0.5M, BME 2mM and 20 mM imidazole and eluted on a gradient up to 500 mM imidazole. Protein containing fractions were pooled and concentrated. Then, a Superdex 200 High load 16/60 column fitted into a FPLC system (both from GE Healthcare Life Sciences, Little Chalfont, United Kingdom) was used at a flow rate of 1 ml min⁻¹, at 4°C, monitoring the A280 of the eluate. The solution used for equilibrating and running the column was Hepes 20 mM pH7.5, Glycerol 15%, NaCl 0.5M, BME 2mM. The purified wild type and variant proteins were stored at -80 °C at high protein concentration (over 15mg/ml) with 15% glycerol.

All variants were abundantly expressed in soluble and stable form (10%–20% of the soluble protein in crude *E. coli* extracts) and purified to homogeneity. Protein purity was assessed visually on SDS 10% or 15% gels. Protein concentration was quantified in a spectrophotometer at 595 nm using Bradford reagent (Bio-Rad, Hercules CA, USA) and bovine serum albumin as standard.

Enzyme activity assays

GTP hydrolysis was measured by a colorimetric assay for determination of Pi release [41]. The purified proteins (2-50 µM) were incubated with increasing concentrations (0–4 mM) of GTP in 50 µl of binding buffer (100 mM Tris-HCl, pH 8, 300 mM KCl, 20 mM MgCl₂, 10% glycerol) at 37 °C during 60 min. Reactions were stopped by adding 200 µl of daily prepared malachite green reagent. This reagent contained 2 volumes of 0.0812% malachite green, 2 volumes of bidistilled water, 1 volume of ammonium molybdate (5.72% in 6 N HCl), and 1 volume of 2.32% polyvinyl alcohol. The samples were read within the next 10 min in a Labsystems Multiskan® Plus plate reader using a 690-nm filter. Blanks containing the corresponding nucleotide concentrations in binding buffer plus malachite green reagent were subtracted from each sample. To quantitate the amounts of enzymatically released Pi, the samples were

compared with a standard curve, which was prepared with dilutions of a 300 μM KH_2PO_4 solution in binding buffer, over a range from 0 to 7 nmol of inorganic phosphate. To determine values for V_{max} and K_m , the data were fitted to the Michaelis-Menten equation using non-linear regression with GraphPad Prism Software. Inhibition curves were fitted to hyperbolic inhibition kinetics according to the expression $V=V_o*[S]/(K_i+[S])$. All assays were conducted at least three times, using also different batches of protein purifications.

Analytical ultracentrifugation experiments

An Optima XL-I analytical ultracentrifuge (Beckman-Coulter, Brea CA, USA) was used to perform the analytical ultracentrifugation experiments. The result was monitored using Rayleigh interferometric detection. Experiments were conducted at 20 °C using an AnTi50 eight-hole rotor and Epon-charcoal standard double sector centerpieces (12-mm optical path). Sedimentation velocity experiments were performed at 48 krpm using 400- μl samples, at 0.4 and 0.8 mg/mL, in buffer consisting of 100 mM Hepes pH 7.5, MgCl_2 20 mM, with or without 300mM KCl, or 300mM KCl plus GDP 1 mM and AlF_3 1mM. Differential sedimentation coefficient distributions ($c(s)$) were calculated by least-squares boundary modelling of sedimentation velocity data using SedFit software (Version 12.44).

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web site:

Figure S1. Effects of pH (A), temperature (B) and potassium ions (C) on Drg1 enzymatic activity.

Figure S2. (A) Lineweaver-Burk plot of inhibition kinetics of Drg1 for GDP. (B). Dependency of activity on GDP of Drg1 and the three variants with or without the C-terminal part of Lerepo4.

Figure S3. Influence of the Drg1 mutations on the concentration dependency of the activity for GTP, based on the absence (A) or presence (B) of the C-terminal part of Lerepo4.

Figure S4. Influence of the Drg1 mutations on the concentration dependency of the activity for potassium ions.

Figure S5. Sedimentation velocity data for Drg1.

Table 1. Kinetic parameters for GTP hydrolysis of Drg1 and the mutant proteins, in the presence or absence of the C-terminal part of Lerepo4. Values for GTP, GDP and potassium were obtained from concentration-activity plots fitted to hyperbolae and hyperbolic inhibition kinetics (see Experimental procedures). Standard deviations are given.

	GTP		GDP	K ⁺
	Kcat (min ⁻¹)	Km (mM)	Ki (mM)	Km (M)
Wild-type	0.147±0.002	0.25±0.01	0.27±0.03	1.20±0.13 ^a
T100D	0.080±0.002	0.26±0.03	0.23±0.02	0.31±0.03
Drg1ΔTGS	0.090±0.006	0.22±0.06	0.22±0.03	0.65±0.17
Drg1ΔTGS+T100D	0.035±0.001	0.19±0.02	0.29±0.06	1.70±0.43 ^a
Wild-type +C-terminal part of Lerepo4	0.580±0.006	0.25±0.01	0.37±0.05	0.10±0.01
T100D+ C-terminal part of Lerepo4	0.465±0.006	0.25±0.01	0.34±0.02	0.11±0.01
Drg1ΔTGS +C-terminal part of Lerepo4	0.181±0.006	0.19±0.03	0.35±0.03	0.60±0.30
Drg1ΔTGS+T100D+C-terminal part of Lerepo4	0.070±0.002	0.19±0.02	0.28±0.06	0.60±0.16

^a Apparent value, not reliable. The curves do not reach the steady state, due to insufficient amounts of effector.

Figure legends

Figure 1. (A) Coomassie-stained SDS–PAGE (10% polyacrylamide) of the purified wild-type and mutant forms from human Drg1 and Lerepo4. St, molecular mass protein marker (Nippon Genetics, Tokyo, Japan) with the masses, in kDa, indicated at the side. The numbers stand for Drg1 (1); Drg1 T100D (2); TGS deleted Drg1 mutant (3); double mutant of Drg1 (4); C-terminal part of Lerepo4 (5); N-terminal part of Lerepo4 (6); full length Lerepo4 (7); TGS domain (8). (B) Schematic representation of the domain organization of the different constructs used in this work. Figures denote residue numbers.

Figure 2. (A) Influence of Lerepo4 full length (●), Lerepo4 C-terminal part (○) and Lerepo4 N-terminal part (*) on GTP saturation curves of Drg1 with respect the activity of Drg1 alone (□). Assay contained 20 mM MgCl₂, 300 mM KCl and increasing concentrations of GTP. (B) Dependency of the activity of Drg1 on molar amounts of Lerepo4 full length (●), Lerepo4 C-terminal part (○), Lerepo4 N-terminal part (*) with respect to the Drg1 activity alone, considered as the zero value; and that of the TGS truncated variant regarding the C-terminal part of Lerepo4 (▲).

Figure 3. Wild type Drg1 and the three variants were incubated at the indicated temperatures with or without a molar excess of the C-terminal part of Lerepo4. (A) wild type Drg1. (B) the phosphomimicking mutant; (C) the TGS truncated mutant; (D) the TGS truncated mutant with the phosphomimicking mutation. Samples were taken after the indicated periods of time for assays of enzyme activity at 37°C. This activity is represented as a percentage of the activity shown before incubation. Values at 37°C in absence of the C-terminal part of Lerepo4 are shown as full circles (●), at 45°C as full squares (■), at 60 °C as full triangles (▲), connected by a dotted line, whereas in the

presence of the C-terminal part of Lerepo4 at 37°C as open circles (○), at 45°C as open squares (□) and at 60°C as open triangles (Δ).

Figure 4. Overall structure of the Rbg1-Tma46 complex. Tma46 interacts mainly with an Rbg1 area opposite to the GTP binding site. From the N to C terminus, Tma46 interacts with the back of the Rbg1 molecule, all around the G domain, and then goes to wrap the TGS domain, to finally go down to the front of the molecule, ending close to the hinge of switch I. Although Rbg1 has been solved in complex with only the Dfrp domain of Tma46, the structure suggests that neither the zinc fingers of Tma46 nor the HTH and S5D2L domains of Rbg1 are likely to be involved in these binding. Rbg1 is depicted in blue, while Tma46 is depicted in green.

Figure 5. Superimposition of the GDP-AlFx (red) and GDP bound (orange) switch I structures of FeoB, together with the apo form of Rbg1 (blue). Residue Thr35, Ile57 from FeoB, and Val101 and Ile122 from Rbg1 are shown in sticks. The C-terminal end of Tma46 is also shown (in green), contacting Gly87 and Glu89 from Rbg1, the hinge from where switch I emerges.

Figure 1

A

Figure 1

B

Full length Lerepo4 and variants

Full length DRG1 and variants

Figure 2

A

B

Figure 3

A

B

Figure 3

C

D

Figure 4

Figure 5

A

B

C

Figure S1. Effects of pH (A) and temperature (B) on Drg1 enzymatic activity alone (●) and Drg1 plus the C-terminal part of Lerepo4 (○). The optimum pH was determined by incubating the enzyme under the standard assay condition where buffer of the mixture was 100 mM of BisTris pH6, Mes pH 6.5, Tris pH 7, Tris pH 7.5, Tris pH 8, Tris pH 8.5, Bicine pH9 or CHES pH 9.5; and the optimum temperature was determined by incubating the enzyme under the standard assay condition at each temperature. (C) Dependency of the activity of Drg1 (●) and Drg1 plus the C-terminal part of Lerepo4 (○) on potassium ions. Assay contained 1.5 mM GTP, 20 mM $MgCl_2$ and increasing concentrations of KCl.

A

B

FigureS2. (A) Lineweaver-Burk plot of inhibition kinetics of Drg1 for GDP, at various concentrations. (B). Dependency of activity of Drg1 and the three variants with or without the C-terminal part of Lerepo4 on GDP. Activities are given as a percentage of the activities shown in the absence of GDP. Drg1 is represented as full circles (●), mutant T100D as full squares (■), the TGS deleted mutant as full triangles (▲) and the double mutant as full rhombus (◆), and when the C-terminal part of Lerepo4 is present, the same symbols are used but in its open form.

A

B

Figure S3. Influence of the Drg1 mutations on the concentration dependency of the activity for GTP, based on the absence (A) or presence (B) of the C-terminal part of Lerepo4. Drg1 is represented as full circles (●), mutant T100D as full squares (■), the TGS deleted mutant as full triangles (▲) and the double mutant as full rhombus (◆), and when the C-terminal part of Lerepo4 is present, the same symbols in its open form are used.

Figure S4. Influence of the Drg1 mutations on the concentration dependency of the activity for potassium ions. For clarity, the double mutant when assayed alone and the TGS deleted mutant in the presence of the C-terminal part of Lerepo4 have been omitted. The values of Drg1 are represented as full circles (●), those of mutant T100D as full squares (■), and when in the presence of the C-terminal part of Lerepo4 as open circles (○) and open squares (□), respectively. The values of the TGS deleted mutant are represented as full triangles (▲) and those of the double mutant together with the C-terminal part of Lerepo4 as open rhombus (◇).

Figure S5. Sedimentation velocity data for human Drg1 full length at 0.8 mg/mL. The experiment was carried out at 48,000 rpm and in 100 mM Hepes (pH 7.5), 300 mM KCl, 20 mM MgCl₂, 1 mM AlF₃ and 1 mM GDP. The c(s) curve suggests that Drg1 presents a predominant species at 72.2 % with a sedimentation coefficient of 2.6 ± 0.1 S, a second peak (20.8 %) with 3.8 ± 0.1 S, and a third with 5.3 S (5 %). The apparent molecular masses are 56 kDa, 99 kDa and 161 kDa, respectively. They were calculated from the c(s) distribution, with a frictional ratio of 1.7.