Catalytic activity of human guanylate-binding protein 1 coupled to the release of structural restraints imposed by the C-terminal domain

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Human guanylate-binding protein 1 (hGBP-1) shows a dimer-induced acceleration of the GTPase activity yielding GDP as well as GMP. While the head-to-head dimerization of the large GTPase (LG) domain is well understood, the role of the rest of the protein, particularly of the GTPase effector domain (GED), in dimerization and GTP hydrolysis is still obscure. In this study, with truncations and point mutations on hGBP-1 and by means of biochemical and biophysical methods, we demonstrate that the intramolecular communication between the LG domain and the GED (LG:GED) is crucial for protein dimerization and dimer-stimulated GTP hydrolysis. In the course of GTP binding and γ-phosphate cleavage, conformational changes within hGBP-1 are controlled by a chain of amino acids ranging from the region near the nucleotide-binding pocket to the distant LG:GED interface and lead to the release of the GED from the LG domain. This opening of the structure allows the protein to form GED:GED contacts within the dimer, in addition to the established LG:LG interface. After releasing the cleaved γ-phosphate, the dimer either dissociates yielding GDP as the final product or it stays dimeric to further cleave the β-phosphate yielding GMP. The second phosphate cleavage step, that is, the formation of GMP, is even more strongly coupled to structural changes and thus more sensitive to structural restraints imposed by the GED. Altogether, we depict a comprehensive mechanism of GTP hydrolysis catalyzed by hGBP-1, which provides a detailed molecular understanding of the enzymatic activity connected to large structural rearrangements of the protein.

Database
Structural data are available in RCSB Protein Data Bank under the accession numbers: 1F5N, 1DG3, 2B92.

Abbreviations
AlFX, aluminum fluoride; DSC, differential scanning calorimetry; DSP, dynamin superfamily protein; FRET, Förster resonance energy transfer; GDP, guanosine diphosphate; GED, GTPase effector domain; GMP, guanosine monophosphate; GppNHp, 5’-guanylyl imidodiphosphate (nonhydrolysable GTP analog); GTP, guanosine triphosphate; GTPS, guanosine 5’-O-(gamma-thio)triphosphate; hGBP, human guanylate-binding protein; LG, large GTPase domain; LP, labeled protein; MD, middle domain; NLP, nonlabeled protein; SEC, size-exclusion chromatography.
**Introduction**

Human guanylate-binding proteins (hGBPs) with seven paralogs (hGBP-1 to hGBP-7) comprise a subgroup of interferon (IFN)-inducible large GTPases within the dynamin superfamily proteins (DSPs) [1–3]. Most abundantly induced by IFN-γ and other pro-inflammatory cytokines, hGBPs are found to mediate innate immune functions against a diverse array of intracellular pathogens including viruses, bacteria, and protozoa [3–14]. Their cellular activities further enclose antiproliferative effects on endothelial cells [15,16]. In colorectal cancer cells, particularly hGBP-1 is established as reliable marker, which inhibits tumor growth through both strong anti-angiogenic activity and its direct antitumorigenic effects [17–19]. However, hGBP-1 is also shown to render some cancer cell lines resistant to chemotherapeutics or radiation [20,21], which altogether indicate a cell- and disease-specific activity of hGBPs awaiting proper characterization of the underlying molecular mechanisms.

Human GBP-1 is the first identified and hitherto best characterized hGBP in terms of biochemical and structural properties [3,22]. As a member of the DSPs, hGBP-1 has a typical multidomain architecture and exhibits characteristic features such as low binding affinity for guanine nucleotides (micromolar range), nucleotide-dependent homo oligomerization, and a dimer-induced acceleration of the GTPase activity [2,23]. The 68-kDa protein consists of three subdomains (Fig. 1A), a globular large GTPase domain (referred to as LG domain from here on) at the N terminus, followed by a purely α-helical domain, which is subdivided into a middle domain (MD, α7-11), and an elongated C-terminal domain termed GTPase effector domain (GED, α12-13). Overall, the LG domain and MD are entirely flanked by the GED within which the α13 helix is positioned next to the LG domain by a coiled-coil type of interaction with helix α12 [24,25].

The LG domain of hGBP-1 represents the catalytic unit, which harbors all sequence elements for guanine nucleotide binding and hydrolysis. Binding of the substrate GTP induces dimerization of the LG domains in a protein concentration-dependent fashion [24,26]. Those head-to-head dimers serve to mutually reposition their internal catalytic residues into the active sites, and thereby to stabilize the transition state for efficient cleavage of the GTP γ-phosphate [26]. The self-stimulated GTPase activity of hGBP-1 achieved by dimerization reaches up to 100 min⁻¹ under physiological and substrate-saturated conditions [2]. Above that, hGBP-1 has the unifying feature to hydrolyze GTP to GDP and GMP in two steps accomplished through successive cleavage of the γ- and β-phosphate groups [26–28]. Mechanistically, this is achieved by a shift of the intermediate product GDP to the active site subsequent to the cleavage and release of the γ-phosphate. Thus, GDP’s β-phosphate occupies the position where the γ-phosphate was located previously, and the same set of residues acts again to catalyze the GDP hydrolysis [26]. Importantly, this sequence of events relies on the GTP-induced hGBP-1 dimers (Fig. 1B), while monomeric hGBP-1 can catalyze very slowly the first but not the second step of GTP hydrolysis yielding

![Fig. 1. Structure and enzymatic activity of hGBP-1. (A) Ribbon plot of hGBP-1 in the GppNHp-bound form (PDB ID: 1F5N) is colored according to the subdomains: the N-terminal large GTPase domain (LG domain) in blue, the middle domain (MD) in yellow, and the C-terminal GTPase effector domain (GED) in orange. GppNHp is depicted in stick presentation, and the C-terminal amino acids including the CaaX motif not being solved in the structure are indicated by a dotted line. The schematic above will be used hereafter to indicate mutational alterations. (B) Schematic illustration of the unique GTPase reaction catalyzed by dimeric hGBP-1 is adopted and modified from Refs [29,39], according to which two competitive steps are proposed: After binding to GTP and the first step of phosphate cleavage, the GDP-bound dimer either remains and catalyzes the second step of phosphate cleavage yielding GMP as final product, or, the GDP-bound dimer dissociates irreversibly and releases GDP as final product.](image-url)
only GDP as product [26,29,30]. With its release from the active site, GDP becomes a dead-end product, which—upon re-association to hGBP-1—does not induce dimers which are required for catalysis of the β-phosphate cleavage. Likewise, GDP from bulk solution is bound by hGBP-1 but in contrast to GTP cannot serve as direct substrate for hGBP-1 [24,27]. Consequently, GMP emerges only in the course of GTP hydrolysis, that is, the second step of β-phosphate cleavage occurs only in continuation of the first step of γ-phosphate cleavage, provided that the reaction is catalyzed by LG domain-mediated dimers of hGBP-1.

Interestingly, several mutants of hGBP-1 have been described that cause notable differences in the final product GDP share obtained from GTP hydrolysis [31–33]. These encompass not only positions along the nucleotide-binding pocket but also along the distant position on the LG domain adjacent to the GED. At the latter position, an intramolecular network of salt bridges is established in the LG domain and GED interface by individual contacts from both α12 and α13 to the LG domain [32]. Those salt bridges maintain a compactly ‘closed’, safety-pin-like conformation of hGBP-1 in the absence of nucleotide [24,32]. In the course of GTP binding and/or hydrolysis, conformational changes within the LG domain occur that lead to disruption of the salt bridge contacts, thereby releasing the GED to promote intermolecular interactions with the GED of another hGBP-1 molecule (GED:GED) [32]. A structure of a full-length hGBP-1 dimer has not been solved yet, and it was once reported that through GED:GED interactions, hGBP-1 builds tetramers from LG domain-mediated dimers [33]. However, the new finding that tetramers do not occur implicates that hGBP-1 GED:GED interactions occur within a bulky dimer [34].

The role of the LG domain within the GTPase cycle is well understood [26,35]. However, the roles for the remaining subdomains and in particular for the C-terminal GED remain elusive. Besides the putative relevance to the final product share of GTP hydrolysis, there is a growing list of different functions mediated by the GED, suggesting a multifaceted importance of this subdomain. For instance, once the protein carries a farnesyl moiety at the C-terminal CaaX-box, two additional features are added as compared to the non-farnesylated form: One is the ability to interact with membranes and the other to assemble to polymers, which are notably higher ordered than dimers [36,37]. The idea is that GTP binding leads to the release of the farnesyl tail previously embedded in a hydrophobic pocket between MD and GED [36,38]. The now available farnesyl tail enables membrane attachment and, alternatively, polymer formation of hGBP-1.

The present study highlights the special role of the GED in the enzymatic machinery of hGBP-1, especially with regard to the second hydrolysis step that provides GMP. To address this, we generated an array of suitable point and truncation mutants of hGBP-1 and characterized their enzymatic activity as well as their capability to undergo nucleotide-dependent structural changes and dimerization by means of reversed-phase HPLC, Förster resonance energy transfer (FRET), and size-exclusion chromatography (SEC). We performed all measurements at 25 °C where the hGBP-1-catalyzed GTPase reaction yields an almost balanced ratio of the products GDP and GMP. This condition is in particular suitable to demonstrate the contributions of individual amino acids and subdomains on the product share. To obtain valuable mechanistic insights into the enzymatic machinery of hGBP-1, we studied here exclusively the nonfarnesylated form, which on the one hand is sufficient to form the catalytic competent unit of a dimer and on the other hand is incapable of forming higher ordered oligomers, potent to distort the investigations.

Results

Retaining GTPase activity, the isolated LG domain of hGBP-1 favors GMP production and gains additional GDPase activity compared to the full-length protein

GTP-dependent dimerization of hGBP-1, which in turn stimulates the successive hydrolysis of GTP to a mixture of GDP and GMP, is mediated by head-to-head dimerization of the catalytic LG domains [26]. Since the earlier studies were performed under low salt conditions [26,34,40], we first confirmed and characterized the differences in enzymatic properties between full-length hGBP-1 and the isolated LG domain under physiological salt conditions in detail.

Experimentally, dimerization-dependent acceleration of the GTPase activity can be obtained by a typical GTPase assay, where rates of GTP hydrolysis catalyzed by varying protein concentrations are measured and further processed according to the multturnover assay [28,35]. In this assay, dividing the rate of GTP hydrolysis (Fig. 2A,B, blue squares) by the respective protein concentration yields catalytic GTPase activity of hGBP-1 (k_{cat}^{GTP}). An k_{cat}^{GTP} increase with increasing protein concentration indicates that the acceleration of GTPase activity of hGBP-1 depends on dimerization (Fig. 2C,D, blue squares). Furthermore, fitting the
data accordingly [28] yields two characteristic parameters, namely the maximum catalytic GTPase activity ($k_{GTP,\text{max}}^{\text{cat}}$), which is achieved by the dimeric hGBP-1, and the dimer dissociation constant ($K_d$), which gives a measure for the dimer affinity.

We performed this assay at 25 °C in buffer maintaining physiological salt conditions and determined a $k_{GTP,\text{max}}^{\text{cat}}$ value of 19.1 min$^{-1}$ and a $K_d$ value of 0.2 µM for full-length hGBP-1 (wt) (Fig. 2C, blue squares and solid line). Within this experiment, we also monitored formation of products GDP and GMP. Similar to the GTP consumption, both GDP and GMP formation followed linear initial reactions, the rates of which were derived from linear fits (Fig. 2A, orange triangles, black circles, and solid lines). Catalytic activities were obtained dividing the initial rates by the respective protein concentrations, and maximum catalytic activities of GDP and GMP formation were acquired from the same quadratic fit used to determine $k_{GTP,\text{max}}^{\text{cat}}$ (Fig. 2C, orange triangles, black circles, and solid lines).

Obtained maximum activities of GDP and GMP formation for wt were 12.2 and 6.9 min$^{-1}$, respectively. Notably, the sum of those two values equals $k_{GTP,\text{max}}^{\text{cat}}$ and this illustrates two important facts. Firstly, GDP and GMP are formed successively by cleaving primarily the $\gamma$-phosphate and then the $\beta$-phosphate. Secondly, there is no additional GDPase reaction under the GTP-saturated condition, meaning the produced GDP was not reused as substrate. Thus, dividing each of the maximum activities for product formation by the maximum activity of GTP consumption yields the share of final products being roughly 60% GDP and 40% GMP, which is in line with our previous data [32,35].

The same experiment was performed on the isolated LG domain of hGBP-1 (LG). We confirmed that the LG domain-catalyzed GTPase reaction is two times faster ($k_{GTP,\text{max}}^{\text{cat}} = 47.8$ min$^{-1}$) than the wt-catalyzed one, while having a similar dimer affinity ($K_d = 0.3$ µM) (Fig. 2D, blue squares). However, the
product development unraveled one major difference between the two GTPase reactions: The LG domain, other than wt, catalyzed the formation of GMP (35.1 min⁻¹) prominently faster than GDP (12.8 min⁻¹), consequently yielding GMP as predominant product, which accounted for roughly 70% of the total product (Fig. 2D, orange triangles and black circles). Here, also the LG domain did not reuse the product GDP as substrate in the presence of a saturated amount of GTP.

To have an alternative approach for determining the product ratio, we newly established the long-term hydrolysis assay. The setup is similar to the GTPase assay (Fig. 2C,D) with the only difference that we investigate not the kinetics of the enzymatic reaction but the final nucleotide composition after 24 h of incubation (Fig. 2E,F). Despite the long incubation time, the observation that GTP was almost not hydrolyzed at low protein concentrations below the \( K_d \) value emphasizes the very slow activity of monomeric protein. However, at higher protein concentrations where GTP was completely hydrolyzed due to the high activity of the dimer, the percentages of GDP and GMP gave a direct read-out for the product share. By this approach, approximately 60% GDP and 40% GMP were detected only within a limited protein concentration range between 0.1 and 0.5 µM (Fig. 2F). At concentrations above, another reaction set in as reflected by an additional increase of GMP up to 100% accompanied by the decrease of GDP to 0% (Fig. 2F). This assigns the second major difference of the LG domain’s enzymatic activity as compared to wt, namely the ability to utilize GDP as substrate.

By offering GDP instead of GTP in the long-term hydrolysis assay, we confirmed both the GDPase activity of the LG domain (Fig. 3D) and the inability of wt to utilize GDP as substrate in the tested concentration range of the proteins (Fig. 3C). Former studies identified GDP-induced dimerization of LG domains as the prerequisite for efficient and self-stimulated hydrolysis of GDP similar to hydrolysis of GTP (Fig. 3B) [26]. Accordingly, we further quantified a protein concentration-dependent increase of the GDPase activity using the same assay as for the GTPase activity, yielding an almost 30-fold weaker affinity of the GDP-bound dimers (\( K_d = 8.5 \mu M \)) (Fig. 3B) as compared to the GTP-bound ones (\( K_d = 0.3 \mu M \)) (Fig. 2D). Also, the maximum catalytic GDPase activity (\( k_{cat,\text{GDP,max}} \)) was more than 10-fold slower than the GTPase activity (\( k_{cat,\text{GTP,max}} = 43.8 \text{ min}^{-1} \) versus \( k_{cat,\text{GDP,max}} = 3.3 \text{ min}^{-1} \)). However, this is still a 1000-fold higher activity as compared to the full-length protein, which achieves roughly 0.0015 min⁻¹ at the highest protein concentration measured (20 µM, Fig. 3A).

In summary, we show that the LG domain catalyzes GTP hydrolysis two times faster than wt and that in contrast to wt, the deletion of the C-terminal helical domains rather facilitates the successive β-phosphate cleavage to occur. This is likely achieved through maintaining a larger fraction of GDP-bound intermediate dimers (see Fig. 1B). Furthermore, we showed that the gained GDPase activity is a result of GDP-induced dimerization. Altogether, these two significant features of the isolated LG domain of hGBP-1 indicate that the rest of the protein, namely the MD and GED domains, has impact on hGBP-1’s enzymatic activity.

The C-terminal GED of hGBP-1 influences the hydrolysis of GDP catalyzed in the N-terminal LG domain

Motivated by the enzymatic differences between wt and the LG domain, we investigated the influence of the C-terminal part of the hGBP-1 on its enzymatic properties. We generated two truncated mutants on the basis of full-length hGBP-1, a mutant Δx13, which lacks helix x13 and a mutant ΔGED, which lacks the entire GED (both x12 and x13, see Figs 4A and 5B). In order to distinguish the effects of x13 from the effects of the whole GED on GTPase and GDPase activity of hGBP-1, we compared the generated mutants with wt protein by means of the GMP formation during GTP hydrolysis (Fig. 4C) and the ability to utilize GDP as substrate (Fig. 4B). For this comparison, we used 5 µM of protein catalyzing hydrolysis of 500 µM GTP and 10 µM of protein catalyzing hydrolysis of 500 µM GDP.

Both mutants Δx13 and ΔGED catalyzed GTP hydrolysis to yield ~80% GMP and 20% GDP (Fig. 4B,C), suggesting that helix x13 has a critical impact on the β-phosphate cleavage within the GTPase reaction. Moreover, when offering GDP instead of GTP as substrate, a direct correlation was revealed between catalytic GDPase activity (\( k_{cat,\text{GDP}} \)) and the truncation steps. Starting with a very low GDPase activity of wt (\( k_{cat,\text{GDP}} = 0.00075 \text{ min}^{-1} \)), a roughly 10-fold higher activity was obtained with each truncation step. With \( k_{cat,\text{GDP}} = 0.0071 \text{ min}^{-1} \), the GDPase activity of Δx13 is still slow but 10-fold higher than wt. Moreover, ΔGED was capable to hydrolyze GDP more than 100-fold faster than wt (\( k_{cat,\text{GDP}} = 0.11 \text{ min}^{-1} \)). And
finally, deletion of both the GED and the MD, which corresponds to the isolated LG domain, shows a more than 1000-fold higher GDPase activity than wt \( (k_{cat}^{GDP} = 1.4 \text{ min}^{-1}, \text{Fig. 4B}) \).

Considering that the GED within a full-length construct may affect GTP/GDP hydrolysis through intramolecular interactions with the LG domain, we performed the same experiments with mutants generated previously affecting these intramolecular interactions [32] (see Fig. 5). We observed that artificial coupling of the GED to the LG domain by a chemical cross-linker, resulting in a tight GED conformation, did not affect the catalytic GTPase activity but almost abolished GMP formation as well as utilization of GDP as substrate (Fig. 4B,C). On the contrary, the mutant (R227E/K228E), which abolishes intramolecular salt bridges between the GED and the LG domain and presumably has a loose GED, performed nucleotide hydrolysis activities similar to \( \text{GED} \). In addition to predominating GMP formation in the GTPase reaction (Fig. 4C), the R227E/K228E mutant efficiently hydrolyzed GDP as substrate \( (k_{cat}^{GDP} = 0.29 \text{ min}^{-1}, \text{Fig. 4B}) \).

These data suggest that the \( \beta \)-phosphate cleavage capability of hGBP-1 strongly correlates with the strength of the LG:GED intramolecular interactions. We hypothesized that weakening of this intramolecular interaction by our mutations and truncations results in reduced thermal stability. Therefore, we performed temperature-induced unfolding studies in the absence of nucleotide using differential scanning calorimetry (DSC) (Fig. 4D, Table 1). The highest \( T_m \) was obtained for hGBP-1 (wt), indicating that the highest stability was given in the absence of any mutation. In comparison, a significant decrease in stability by at least 3 °C was observed with each truncation, which gives evidence for mutual stabilization of the subdomains as established through intramolecular interactions. The rather high \( T_m \) of the LG domain among all truncation mutants was assumed to be caused by its compact globular shape. Most interestingly, the loose GED mutant, in which only two amino acids are mutated (R227E/K228E) in \( a' \) helix of the full-length construct, showed a similar destabilization as the truncation mutants. This underscores a key role of the amino acids R227/K228 in establishing the intramolecular LG:GED interactions.

In sum, stepwise deletion of the C-terminal helices (\( \Delta a_13, \Delta \text{GED}, \) and \( \Delta \text{MD-GED} \)) or loosening the contact between the LG domain and the GED (R227E/K228E) leads to an enhanced production of GMP during GTP hydrolysis and the ability to utilize GDP as substrate. Disruption of the salt bridge network between the LG domain and the GED and therefore release of the GEDs within the hGBP-1 dimer enables hGBP-1 to perform the second hydrolysis step.

**A cluster of LG domain phenylalanines is critical for intramolecular GED arrangement**

Given the described impact of the GED on the catalytic activities, we further characterized newly
generated point mutants for their conformational state, that is, the arrangements of the GED relative to the rest of the protein. We hypothesized that the GED, particularly through intramolecular interactions to the LG domain, impacts the unique β-phosphate cleavage step of hGBP-1. Moreover, as the loose GED mutant (R227E/K228E), being similar to the DGED, has an influence on the GDPase activity, we suggest that loosening or opening the GED is the reason for the increased GDPase activity. To confirm this, we sought further residues, which are responsible for the contact between the LG domain and the GED.

Within the LG domain and in close proximity to the GED, we initially investigated a nonstructured loop region, which precedes helix α3'. This loop region has several negatively charged residues, which might interact with a set of positively charged residues from the very C terminus of hGBP-1 (Fig. 5C). However, neither neutralization nor charge reversal of these residues (see mutants LoopAAA and LoopRRR in Fig. 5B) led to notable changes as compared to the enzymatic activity of wt (Table 1), which is why we conceived these residues not important for LG:GED interactions.

In close proximity to that loop, we further identified a cluster of four phenylalanine residues, F229 from helix α4' next to the two critical residues R227/K228, being similar to the ΔGED, has an influence on the GDPase activity. By structural analysis of different nucleotide-bound states, these four residues were found to experience conformational changes along the GTPase cycle, and thus, we marked them as candidates to influence the intramolecular GED arrangement (Figs 5 and 6A). We replaced these residues by alanines, either through single mutations (F229A, F171A, and F175A) or a triple mutation of all α3'-located phenylalanines (F171A/F174A/F175A, termed TripleF). While having a similar high catalytic GTPase activity as wt, all four mutants showed a remarkable shift of the product ratio in favor of GMP (70–75%), as also confirmed by long-term hydrolysis studies (Fig. 6B, pie charts and Table 1). Since we have observed similar results for

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**Fig. 4.** Alterations in hGBP-1’s enzymatic activity and stability depending on C-terminal contributions. (A) Schematics of point mutants and truncation mutants of hGBP-1 depict altering arrangements of the GED as compared to wt (see text and Fig. 5; colored according to the subdomains in Fig. 1A). (B) GTPase (blue columns) and GDPase activities (gray columns) of respective proteins were determined separately using 5 µM of protein and 500 µM of GTP or 10 µM of protein and 500 µM of GDP. (C) Pie charts depict the share of nucleotide products GMP (red) and GDP (gray) resulting from GTP hydrolysis catalyzed by the respective mutant. (D) Thermal stability of wt and selected mutants is given by their melting temperatures $T_m$ derived from differential scanning calorimetry. Error bars represent mean ± SD of three independent experiments ($n$ = 3). All values from the figure are summarized in Table 1.
Δα13 (Fig. 4B,C and Table 1), this suggests that all positions have at least some impact on the C-terminal α13 arrangement. Above that, all mutants significantly gained the ability to utilize GDP as substrate, which indicates a wider impact of the GED arrangement. Among the four mutants, the mutant F229A presented the slowest catalytic GDPase activity (0.11 min⁻¹), although this GDPase activity still remained in the range of what had been obtained for the ΔGED or the loose GED (R227E/K228E) (Table 1). The mutants F171A and F175A showed roughly 10–20 times faster GDP hydrolysis as compared to the mutant F229A, and both catalytic GDPase activities were similar to the activity obtained for the LG domain (Fig. 6C) (Table 1). For the TripleF mutant, the catalytic GDPase activity was even twofold faster than that of the LG domain (Fig. 6C) (Table 1). By measuring a protein concentration-dependent increase of the GDPase activity, we determined the maximum catalytic GDPase activity of the TripleF mutant to be $k_{\text{cat, max}} = 4.1 \text{ min}^{-1}$ (Fig. 6D). Assuming the GDP hydrolysis is also stimulated by protein dimers, we obtained a dimer dissociation constant ($K_d$) of 0.1 μM for the TripleF mutant, which indicates a more than 100-fold higher affinity compared to the GDP-induced LG domain dimer (see Fig. 3B, $K_d = 8.9 \mu M$).

Based on these enzymatic properties and decreased thermal stabilities of these mutants (Table 1), we concluded that besides the intramolecular salt bridge network, particularly the α3’ phenylalanines play a
Table 1. Catalytic activity and thermal stability of hGBP-1 mutants. Turnover numbers of GTP hydrolysis ($k_{cat}^{GTP}$) were derived from a reaction with 1–5 μM of protein and 500 μM GTP. The share of GMP on total nucleotide product from GTP hydrolysis was either derived from the same experiment using the ratio $k_{cat}^{GMP}/k_{cat}^{GTP}$ or from long-term hydrolysis studies. Turnover numbers of GDP hydrolysis ($k_{cat}^{GDP}$) were determined with 10 μM protein and 500 μM GDP. Melting temperatures of proteins ($T_m$) were obtained from the DSC measurements using 5–15 μM of protein. N.d.: not determined. Actual mutations for either abbreviation are summarized in Fig. 5.

| Protein | $k_{cat}^{GTP}$ (min$^{-1}$) | GMP (%) | $k_{cat}^{GDP}$ (min$^{-1}$) | $T_m$ (°C) |
|---------|-----------------------------|---------|-----------------------------|------------|
| wt      | 11                          | 40      | 0.00075                     | 49         |
| Δσ13    | 23                          | 81      | 0.0071                      | 45         |
| ΔGED    | 43                          | 83      | 0.1076                      | 41         |
| LG      | 40                          | 71      | 1.4                         | 45         |
| Tight GED | 34                        | 1      | ≤ wt                        | n.d        |
| Loose GED | 39                       | 89      | 0.2881                      | 42         |
| LoopAAA | 14                          | 43      | ≤ wt                        | n.d        |
| LoopRRR | 15                          | 44      | ≤ wt                        | n.d        |
| F229A   | 15                          | 69      | 0.11                        | 45         |
| F171A   | 12                          | 70      | 1.9                         | 44         |
| F175A   | 19                          | 72      | 0.94                        | n.d        |
| TripleF | 15                          | 75      | 3.8                         | 44         |
| FL-H74A | 5.4                         | 3       | ≤ wt                        | n.d        |
| ΔGED-H74A | 20                      | 60      | n.d                         | n.d        |
| LG-H74A | 16                          | 40      | n.d                         | n.d        |
| TripleF-H74A | 22                | 87      | 0.90                        | n.d        |
| FL-K76A | 4.3                         | 3       | ≤ wt                        | 49         |
| ΔGED-K76A | 60                       | 1       | n.d                         | n.d        |
| LG-K76A | 20                          | 3       | n.d                         | n.d        |

critical role in establishing intramolecular interactions between the LG domain and the GED, which upon mutation render an ‘open’ type of conformation.

Intramolecular LG:GED interaction regulates the dimer formation of hGBP-1

Dimerization of hGBP-1 has been found to be an essential prerequisite to catalyze its unique β-phosphate hydrolysis step yielding GMP [29,30]. Since the demonstrated mutants deviate from the wild-type protein in exactly this enzymatic step, we further investigated putative alterations in their capability to dimerize.

According to our previous studies, we analyzed different protein constructs in the presence of different nucleotides with size-exclusion chromatography (SEC) where dimerization of proteins was indicated by a shift to lower elution volumes (Fig. 7A) [34,40]. In addition to wt, we investigated all C-terminal truncation mutants as well as the mutants tight GED and F171A. As shown in Fig. 7A, all constructs appeared monomeric when bound to GMP and dimeric when bound to GDP·AlF$_4^-$ (an analog mimicking the intermediate state of γ-phosphate cleavage during GTP hydrolysis). The latter confirms the GTP hydrolysis-induced dimerization of all constructs, which was expected because of the self-stimulated GTPase activity (Table 1). Interestingly, with GTP analogs (GppNHp and GTPγS), which mimic the state of GTP binding, we observed notable deviations among the constructs in correlation with the availability of the C-terminal GED. While the wt and the tight GED remained mainly in the monomeric state, both the ‘open’ type of mutant (here represented by F171A) and the mutants having the GED truncated (ΔGED, LG domain) shifted mainly to the dimeric state. Here, the mutant Δσ13, which has a partly truncated GED, shifted only partly to the dimeric state. As the dimeric protein conformation in the presence of GTPγS exclusively occurred with the released or truncated GED mutants, we suspected that the LG:GED intramolecular interaction might affect the GTP binding-induced dimerization of hGBP-1.

To explore this, we generated double-labeled wt and F171A and monitored intramolecular structural changes in the presence of different nucleotides by Förster resonance energy transfer (FRET) according to the previously described method [34]. Due to different accessibilities of cysteines, the acceptor fluorophore locates on the single cysteine of the GED (the very C-terminal CaaX cysteine, see Fig. 5), and the donor fluorophore locates on another cysteine on a subdomain of the same protein molecule (MD or LG domain). By addition of nucleotide, a decrease of the FRET signal reports release of the GED from the rest of the protein, that is, an intramolecular opening [34]. As observed, a maximum opening of wt was achieved only in the presence of GDP·AlF$_4^-$, which was also the only analog that successfully induced dimerization in the SEC method (Fig. 7B,A). In contrast, the GTP-binding analogs GTPγS and GppNHp, which on SEC retained wt in the monomeric state (Fig. 7A), induced minor or no intramolecular opening similar to GMP. For the mutant F171A, except for GMP, all the tested nucleotides induced intramolecular opening to the same maximal extent as marked by the trace of GDP·AlF$_4^-$ (Fig. 7B), and all these nucleotides also induced dimerization observed by SEC (Fig. 7A). Overall, this demonstrates that the intramolecular release of the GED is tightly related to the dimerization of protein.

Given the intramolecular release of the GED, we wanted to investigate further the GED:GED interaction, which was suggested in our previous study...
Therefore, we generated single-labeled proteins having either a donor or an acceptor fluorophore localized to the single cysteine of the GED, which allowed us to monitor GED:GED interactions by an increase of the FRET signal [34]. For wt or F171A, all nucleotides, which induced intramolecular release of the GED and dimerization observed with SEC, also consistently induced a significant increase of the intermolecular FRET signal (Fig. 7C). This indicates that GED:GED interactions indeed occur during dimerization of the tested hGBP-1 constructs.

Finally, as suggested by similar kinetics of the respective intra- and intermolecular FRET traces (Fig. 7B,C), we asked whether intramolecular release of the GED and dimerization observed with SEC, also consistently induced a significant increase of the intermolecular FRET signal (Fig. 7C). This indicates that GED:GED interactions indeed occur during dimerization of the tested hGBP-1 constructs.

Overall, we demonstrate that the LG:GED intramolecular interaction controls the intermolecular GED:GED interaction on the one hand and influences the catalytic activity on the other hand.
Notably, considering that the LG domain dimerizes upon GTP binding, it appears surprisingly that wt was unable to do so (Fig. 7A,C in the presence of GppNHp and GTP\textsubscript{cS}), as both constructs have the LG:LG dimer interface in common [26]. In fact, our previous study did illustrate a growth of dimeric wt peak in the presence of GppNHp and GTP\textsubscript{cS} with increasing protein concentration [34]. Together with the detected molecular mass, this suggested that GppNHp- and GTP\textsubscript{cS}-bound hGBP-1 are in a rapid exchange between the monomeric and dimeric forms [34]. In the intra-FRET measurements, we observed a slight release of the GED only induced by GTP\textsubscript{cS} but not by GppNHp (Fig. 7B wt). This may be explained by a slight difference of the two nucleotides. GppNHp is not hydrolysable, whereas GTP\textsubscript{cS} is shown to be slowly turned over by hGBP-1 [40]. However, GppNHp induced no release of the GED, and both GppNHp and GTP\textsubscript{cS} failed to induce the GED:GED interaction for the wt (Fig. 7C). Therefore, we conclude that the GTP binding-induced wt dimers, which are in rapid equilibrium with monomers, are only formed using the LG:LG interactions.

Intramolecular opening between the LG domain and GED is triggered by the distant switch position H74

The so-called switch regions, switch I and switch II, are conserved structural elements nearby the nucleotide-binding pocket of GTP-binding proteins. These switch regions, through nucleotide-dependent conformational
changes, allow the proteins to interact with their downstream effector proteins [41]. In hGBP-1, intriguingly, replacement of several amino acids within these regions by alanine turned out to almost abolish the product GMP formation during GTP hydrolysis [31]. Therefore, those mutants were awarded as interesting candidates for the present study.

As representatives, we selected two mutants from switch I, H74A and K76A, and performed the protein concentration-dependent catalytic GTPase activity assays (Fig. 8A). Albeit dimerization of the mutants appeared to be attenuated by approximately 15-fold when compared to wt (Fig. 8B), their maximum catalytic GTPase activity was affected by two to three times only (Fig. 8C) though yielding almost no GMP as product (Fig. 8D). Since we observed a visible increase of the catalytic GTPase activity with increasing protein concentration, we excluded that the lack of GMP production results from disability of dimerization. Noticing that the tight GED mutant had the same deficiency in producing GMP (Fig. 4C), we suspected that the mutants H74A and K76A are also trapped in a closed conformation. In this conformation, the GED is locked and disables the protein to form a tight dimer which under wild-type condition is established in order to further produce GMP.

To test our hypothesis, the GEDs of the mutants H74A and K76A were truncated to overcome the closed conformations of the full-length proteins. For K76A, the truncated mutant ΔGED-K76A did not improve the GMP formation in GTP hydrolysis, although the maximum catalytic GTPase activity was significantly higher than for the ΔGED construct (Fig. 8E,F). In contrast, the deficiency in GMP formation caused by H74A was notably restored when the GED was truncated (ΔGED-H74A), yielding GMP even as predominating product (Fig. 8F). Similar results were obtained when H74A was introduced into an open type of full-length hGBP-1 mutant (TripleF-H74A) (Fig. 8F). These results therefore indicate distinct roles of the residues H74 and K76.

The results above suggest that H74 plays a crucial role in opening; therefore, the alanine mutation hinders the formation of the GED:GED contact. We further investigated these properties with intra- and intermolecular FRET studies. We detected that intramolecular opening and dimerization of H74A, as exclusively induced by GDP·AlF₄⁻, were notably slower than that of wt (Fig. 8H,I; left and middle panels). Also, the observed minor intramolecular rearrangements of wt induced by GTPyS were not visible for H74A (Fig. 8I; left and middle panels), indicating a somehow restricted flexibility of the mutant’s GED.

This was further supported by a prominent monomeric fraction of GDP·AlF₄⁻-bound H74A monitored by SEC (Fig. 8G; left panel). These properties suggest that intramolecular conformational changes between the LG domain and the GED are notably attenuated by the H74A mutation, that is, the GED release of H74A is restricted throughout the cycle of GTP binding and hydrolysis when compared to wt.

Consequently, in TripleF-H74A—wherein the contribution of α3’ phenylalanines to the LG:GED contacts is abolished—these restrictions were reversed (Fig. 8G–I, right panels). The observed pattern of nucleotide-dependent inter- and intramolecular interactions moreover resembled all characteristics of an open mutant (see Fig. 7A–C, F171A). Overall, the open conformation of TripleF-H74A turned out capable to bypass the intramolecular restrictions created by H74A, in turn yielding tight dimers producing GMP in GTP hydrolysis.

Altogether, we conclude that the residue H74, yet located at distant position from the LG:GED interface, has still a critical role in the intramolecular interaction. As to its particular localization within the LG domain, it is conceivable that H74 mediates a chain of conformational changes toward the GED. These conformational changes are induced by nucleotide binding to initiate the GED release for further GED:GED interactions which is necessary to build tight dimers, which is important for GMP production.

**Discussion**

We found that the GED affects two features of GTP hydrolysis. Firstly, the GED affects GMP production during the GTP hydrolysis. We identified the presence of the C-terminal end of the GED (α13 helix) to influence the ratio of the products GDP and GMP. When this region was deleted from the wt, a significant increase in the GMP production was observed. Secondly, we discovered that the GED contributes to the substrate specificity of hGBP-1. While the wt only employs GTP as substrate from solution but not GDP, the GED-truncated mutant gains the feature to effectively utilize also GDP as substrate.

The catalytic properties of hGBP-1 depend highly on dimerization of the protein [24,26]. Our previous crystallography studies revealed LG:LG interactions as the initial dimeric interface [24,26]. However, in a more recent study, intra- and intermolecular FRET measurements identified the GED to participate in the dimerization of hGBP-1 [34]. Therefore, both the LG:LG and GED:GED interactions contribute to the formation of a catalytically efficient dimer. In this work,
by applying SEC and FRET methods to a panel of suitable hGBP-1 mutants, we showed that nucleotide hydrolysis-driven opening of the GED is a prerequisite for the formation of the GED:GED contact.

We depict a detailed mechanism of GTP hydrolysis by hGBP-1 in Fig. 9. (i) Binding of GTP induces the formation of a hGBP-1 dimer with the LG:LG contact but is not sufficient to release the GED. Here, a
structural restraint evoked by the LG:GED contact is illustrated by flattened areas of the LG domains, and the GTP-bound dimer is in a rapid exchange with the monomers (Fig. 9). (ii) Upon catalyzing γ-phosphate cleavage of the bound GTP, an intramolecular opening of the GED is triggered to allow establishment of the GED:GED interface that reversely stabilizes the intramolecular opening. More importantly, this separation of the GED from the LG domain removes the structural restraint evoked by the LG:GED contact and may consequently add structural changes in the LG:LG interface including the nucleotide-binding pocket. In Fig. 9, these structural changes are symbolized by an increase of the area at the LG:LG interface when comparing the two GDP-bound dimers. (iii) GDP-bound dimers with the rearranged LG:LG interface and catalytic center are able to catalyze the second phosphate cleavage step, that is, the formation of GMP, while the GDP-bound dimers without the rearranged LG:LG interface are not able to catalyze the second phosphate cleavage. As for almost all GTP-binding proteins, GTP binding activates the protein—for example, enables effector binding—or in the case of hGBP-1 triggers dimerization and thereby positioning of the catalytic machinery [41,26]. Notably, these actions are not achieved when hGBP-1 binds GDP instead of GTP.

Of note, in the first step of the hydrolysis cycle, GTP binding-induced dimers of the GED-truncated mutants (LG domain and ΔGED) were visible in SEC. However, for the wt, GTP binding-induced protein dimers were shown to be in fast exchange with monomers. Thus, we deduce that existence of restraint GEDs on the wt might even weaken the LG:LG contacts. The scheme in Fig. 9 also reflects why the ‘open’ mutants yielded prominently GMP as the product of GTP hydrolysis and even gain substrate GDPase activity. When the GED is deleted or released by mutations such as the R227E/K228E, the structural restraint evoked by the LG:GED contact is eliminated. Therefore, compared to the wt, these mutants favor the formation of the dimers with the enhanced LG:LG interface (as demonstrated by the GDP-bound dimer with the increased area at the LG:LG interface in Fig. 9) that are active for intermediate or even substrate GDPase cleavage. Quite the contrary, when the GED is locked to the rest of the protein (tight GED and H74A), the restraint on the LG:GED contact is not or hard to overcome. Consequently, dimers cannot further adapt their conformational changes after the γ-phosphate cleavage and thus yield GDP as the final product.

This model, which is based on the observations described in the results, explains contributions of the GED to the catalytic activity of hGBP-1. We further identified amino acids located on the LG domain, which lead to the dynamic motion of the GED in the GTP hydrolysis. For instance, the α3′ phenylalanines located on the LG:GED interface together with the residues R227 and K228 directly contribute to the intramolecular interaction, while the H74 located on the switch region triggers a chain of conformational changes to transfer the nucleotide binding and hydrolysis signals to the distant LG:GED contact. In total, we revealed the roles of the GED to provide more comprehensive understandings of the unique GTP hydrolysis cycle of hGBP-1.

Most probably, the guanine nucleotide hydrolysis of hGBP-1 is designed to trigger further biological functions of hGBP-1, which virtually can be dictated by a timed sequence of events: the GTP binding, the γ-phosphate cleavage, as well as the following β-phosphate cleavage. As for almost all GTP-binding proteins, GTP binding activates the protein—for example, enables effector binding—or in the case of hGBP-1 triggers dimerization and thereby positioning of the catalytic machinery [41,26]. Notably, these actions are not achieved when hGBP-1 binds GDP instead of GTP.
of mutants (e.g., F171A, looseGED, or TripleF), buffer C was supplemented with 2 mM DTT in the second step of purification.

Protein concentrations were determined according to Ref. [34] using the molar absorption coefficients of ε_{280} = 43,240 M⁻¹·cm⁻¹ for all full-length constructs and Δε_{13}, ε_{280} = 41,960 M⁻¹·cm⁻¹ for ΔGED, and ε_{280} = 34,280 M⁻¹·cm⁻¹ for all LG constructs.

### Catalytic GTPase/GDPase activity assay

Protein and substrate (either 500 μM GTP or GDP) diluted in buffer C (50 mM Tris/HCl, pH 7.9, 150 mM NaCl, and 5 mM MgCl₂) were mixed and incubated at 25 °C. At different time points, nucleotide composition was analyzed by reversed-phase HPLC (Chromolith RP18e HPLC column; Merck, Darmstadt, Germany) in HPLC buffer (100 mM potassium phosphate, 10 mM tetrabutylammonium bromide, and 4% (v/v) acetonitrile), detecting the absorption of nucleotides at 254 nm with a MD-2015 diode array detector (Jasco, Gross-Umstadt, Germany). Initial rates of substrate (GTP or GDP) turnover or product (GDP or GMP) formation were obtained from linear fits to nucleotides’ concentration change over time in steady-state hydrolysis (remaining GTP > 60%). Dividing the initial rates by protein concentrations, the catalytic activities were obtained and further plotted against the protein concentrations. Data were further analyzed as described previously, with a quadratic binding equation that gives two parameters: the apparent dissociation constant of hGBP-1 dimers (K_d), and the maximum catalytic activity (k_{cat, max}) [28,35].

### Long-term GTP/GDP hydrolysis

As for the GTPase/GDPase assay, varying concentrations of protein and 500 μM of the respective substrate, either GTP or GDP, were mixed in buffer C and incubated at 25 °C for 24 h. The resulting nucleotide composition was analyzed by reversed-phase HPLC as for the catalytic GTPase/GDPase activity assay.

### Differential scanning calorimetry

Melting temperatures of different hGBP-1 constructs were obtained by differential scanning calorimetry (MicroCal VP-Capillary DSC, Malvern Panalytical, Worcestershire, UK). Protein samples were diluted to a concentration of 0.5 g L⁻¹ in buffer P and measured against the same buffer in the reference cell during a temperature ramp-up from 30 to 55 °C, yielding a difference in the amount of heat required to increase the temperature of the sample and reference. The heat capacity was plotted against the increasing temperature. Fit of the peak value according to the manufacturer’s program gave the melting temperature $T_m$. [45].
Analytical size-exclusion chromatography

Dimerization of hGBP-1 mutants (20 μm) in the presence of different nucleotides (buffer C supplemented with 250–320 μm of GMP, GTPγS, or GppNHp; purchased from Jena Bioscience, Jena, Germany) was analyzed by analytical size-exclusion chromatography (SEC) using the column Superdex 200 PC 3.2/30 (2.4 mL, GE Healthcare) as described previously [34]. Due to almost nonreversible behavior, exceptionally, SEC of GDP-AlF₄⁻-induced complexes was run with buffer C in the absence of any supplemental nucleotide. Prior to that, the protein was diluted to a total concentration of 20 μm in buffer C containing 10 mM NaF and 300 μm AlCl₃. After the addition of 250 μm GDP, the mixture was incubated for 10–30 min and injected to the column.

Fluorophore labeling of proteins

Following the labeling strategy described previously [34], proteins were labeled with the donor and acceptor fluorophores Alexa Fluor 488-C5-maleimide and Alexa Fluor 647-C2-maleimide (Thermo Scientific), respectively. Both single-labeled proteins and double-labeled proteins were generated: The single-labeled proteins carried maximal one fluorophore, either a donor or an acceptor dye, which in combination were used for intermolecular FRET measurements. The double-labeled proteins carried both a donor and an acceptor dye within one molecule and served to measure intramolecular FRET.

Forster resonance energy transfer

Inter- and intramolecular FRET studies were carried out according to Ref. [34] in buffer C and at 25 °C. FRET was monitored at 664 nm while exciting the donor at 498 nm. If not indicated otherwise, for intermolecular FRET measurements 1 μm donor-labeled and 1 μm acceptor labeled protein were mixed and incubated for 2–5 min before nucleotide addition. In analogy, for intramolecular FRET measurements protein concentration was adjusted to 2 μm, but containing 0.1 μm of double-labeled protein and 1.9 μm of nonlabeled protein to abolish intermolecular effects.

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Conflicts of interest

The authors declare no conflict of interest.

Author contributions

SI planned and performed experiments; analyzed data; discussed results; and wrote paper. PZ planned and performed experiments; analyzed data; discussed results; and wrote paper. MK planned experiments, analyzed data, and discussed results. OK supported experiments and analyzed data. SS supported experiments and discussed results. CH planned experiments, analyzed data, discussed results, and wrote paper.

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** Supporting information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Appendix S1.** Oligonucleotide sequences for hGBP-1 mutants.