Biochemical and Structural Characterization of the Gem GTPase

RGK proteins, encompassing Rad, Gem, Rem1, and Rem2, constitute an intriguing branch of the Ras superfamily; their expression is regulated at the transcription level, they exhibit atypical nucleotide binding motifs, and they carry both large N- and C-terminal extensions. Biochemical and structural studies are required to better understand how such proteins function. Here, we report the first structure for a RGK protein: the crystal structure of a truncated form of the human Gem protein (G domain plus the first part of the C-terminal extension) in complex with Mg-GDP at 2.1 Å resolution. It reveals that the G-domain fold and Mg-GDP binding site of Gem are similar to those found for other Ras family GTPases. The first part of the C-terminal extension adopts an α-helical conformation that extends along the α5 helix and interacts with the tip of the interswitch. Biochemical studies show that the affinities of Gem for GDP and GTP are considerably lower (micromolar range) compared with H-Ras, independent of the presence or absence of N- and C-terminal extensions, whereas its GTPase activity is higher than that of H-Ras and regulated by both extensions. We show how the bulky DXWEX motif, characteristic of the switch II of RGK proteins, affects the conformation of switch I and the phosphate-binding site. Altogether, our data reveal that Gem is a bona fide GTPase that exhibits striking structural and biochemical features that should impact its regulation and cellular activities.

Gem is an atypical protein of the Ras superfamily that belongs to the recently described RGK (Rad, Kir/Gem) subfamily encompassing the Rad, Gem (and its mouse ortholog Kir), Rem1 (also known as Rem or Ges), and Rem2 proteins (1). These proteins are thought to control the activity of L-type Ca2+ channels by interacting with their β-subunit (2–4) as well as to intervene in cytoskeletal dynamics. Indeed, Gem interacts with the microtubule network through the kinesin-like protein KIF9 (5) and regulates actin dynamics downstream from RhoA by inhibiting Rockβ (6) and interacting with the Rho GTPase-activating protein Gmip (7).

GTPases of the RGK family exhibit several specific features in their sequence that distinguish them from other members of the Ras superfamily (see Fig. 1). First, RGK members possess large N- and C-terminal extensions beyond the Ras-homologous G domain. The function of the N-terminal extension of 44–88 residues (71 residues in the case of Gem) is presently unknown. The C-terminal extension of 29–37 residues (31 residues for Gem) is devoid of consensus lipid modification sequences (prenylation or palmitoylation) despite having a conserved cysteine residue at the seventh position from the C terminus, but it carries a functional Ca2+/CaM binding site. There is evidence that the C terminus of Gem is necessary for its interaction with membranes (8) and that its association with Ca2+/CaM maintains Gem in the cytosol (9). Last, RGK proteins exhibit substitutions at key positions for GDP/GTP binding and hydrolysis: (i) within the PM1 motif (P-loop, GXXXGXG(S/T)) that is involved in phosphate binding, the residue equivalent to Gly12 in H-Ras, whose mutation causes the constitutive activation of Ras and Rho family proteins, is changed to glutamine in Gem, serine in Rad and Rem2, and serine in Rem1; (ii) within motifs that belong to the switch I and II regions, which are sensors for the presence of the γ-phosphate and thus drive the conformational switch of Ras family proteins during the GDP/GTP cycle, the highly conserved threonine residue from the PM2 motif in switch I (Thr35 in H-Ras) is lacking in the RGK family, whereas the conserved PM3 motif (DTAGQ motif) in switch II is changed to a bulky DXWEX motif. Beyond its γ-phosphate sensor/binding role, the DTAGQ motif is also critical for GTP hydrolysis, due to the important role that the glutamine residue (Gln41 in H-Ras) plays in the catalytic mechanism. This residue is conserved in Rad and Rem2 but is replaced by an alanine in Rem1 and an asparagine in Gem. Because of these changes, the structural

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4 The abbreviations used are: CaM, calmodulin; TEV, tobacco etch virus; rTEV, recombinant His*-tagged TEV protease; GMP-PNP, guanylyl-5’-[(β,γ-imido)-triphosphate; GTPγS, guanosine 5’-O-(3-thiotriphosphate); αCter helix, C-terminal helix of GemΔNΔCaM; GST, glutathione S-transferase.

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properties of the regions of RGK proteins involved in interacting with nucleotides as well as the biochemical features of GDP/GTP binding and GTP hydrolysis may be quite different from those of other Ras family proteins.

In order to investigate these questions, we have solved the crystal structure of a truncated form of human Gem, encompassing the G domain plus the first part of the C-terminal extension (Met73–Arg269) in complex with MgGDP at 2.1 Å resolution, and characterized its biochemical activities. In addition, we have investigated the influence of the N- and C-terminal extensions on the biochemical properties of Gem, including the association of CaM with its C-terminal region.

EXPERIMENTAL PROCEDURES

Bacterial Expression Constructs—Sequences encoding various portions of the human Gem protein were cloned in the bacterial expression vector pGST//1, a derivative of pGEX-4T1 in which the thrombin cleavage site has been replaced by a TEV protease cleavage site (10). To express the portion of Gem devoid of the full N-terminal extension and of the last part of the C-terminal extension (GemΔNΔCaM) that we consider as homologous to Ras, sequences encoding residues Met73–Arg269 were amplified by PCR and cloned between the EcoRI and Sall sites of pGST//1, leading after proteolytic cleavage to a protein containing a 7-residue linker (GAMDPEF) upstream from residue Met73. GemΔNΔCaM and GemΔC, encompassing residues Met73–Ser289 and Met1–Ser289, respectively, were similarly cloned into Ncol-Sall-digested pGST//1, thus leading to proteins with a 3-residue N-terminal linker (GAM) after TEV protease cleavage. The 5′-primers used were 5′-T GCT ACC ATG GGA ACC TAC TAC CGA GTG GTG CTC-3′ for GemΔNΔCaM and 5′-T GGT GCC ATG GGA ACT CTG AAT for GemΔC. The 3′-primer used was 3′-GCC ATG GGA ACC TAC TAC CGA GTG GTG CTC-3′. The purified protein was cleaved with rTEV protease on the beads in the last washing buffer (40 mM Tris-HCl, pH 8, 300 mM NaCl, 2 mM DTT, 10 mM MgCl2) and twice with the same buffer containing only 100 mM NaCl and protease inhibitors. Gem was cleaved from GST with rTEV protease on the beads in the last washing buffer overnight at 4 °C; the effluent was passed through successive Ni2+-nitrilotriacetic acid-agarose (Qiagen) and glutathione-Sepharose 4B columns to remove the rTEV protease and GST tag. Gem was concentrated using Centricube columns (Millipore), snap-frozen in liquid nitrogen, and kept at −80 °C. The purity of the preparations was assessed by SDS-PAGE (supplementary Fig. S1). Protein concentration was measured with a dye-binding assay (Bio-Rad) using BSA as a standard; as determined by [8-3H]GDP binding assays, our purified preparations contained 50–70% active protein. CaM was dissociated from GemΔNΔCaM by treatment with 2 mM EGTA for 10 min on ice; CaM was adsorbed onto Q-Sepharose (Amersham Biosciences) by a 15-min incubation at 4 °C and separated from GemΔNΔCaM by centrifugation. To form the GemΔC7-CaM complex, 100 μg of GemΔC7 was incubated with a 10-fold excess of purified CaM (11), and the complex was purified by gel filtration on a Superdex 75 column (Amersham Biosciences). Alternatively, the GemΔC7-CaM complex was directly purified from bacteria co-expressing both proteins as described above for the GemΔNΔCaM-CaM complex followed by gel filtration on a Superdex 75 column.

For crystallization purposes, GemΔNΔCaM was purified as follows. Frozen bacteria were resuspended in 50 mM Tris-HCl, pH 8, containing 250 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 0.1 mM GDP, 10% glycerol, 0.5 mg/ml lysozyme, and 5 mM DTT. They were disrupted by sonication, the lysate was ultracentrifuged at 100,000 × g for 30 min at 4 °C, and the supernatant was incubated at 4 °C with glutathione-Sepharose 4B beads for 2 h. The GST fusion protein was eluted by glutathione and cleaved with rTEV protease overnight at 4 °C, and rTEV and GST were removed as above. Gem was further purified by anion exchange chromatography on a MonoQ 5/5 column (Amersham Biosciences), concentrated to 12 mg/ml, frozen to liquid N2, and stored at −80 °C in 25 mM Tris-HCl, pH 8, 150 mM NaCl, 5 mM MgCl2, 1 mM GDP, and 1 mM DTT.

Nucleotide Binding and Dissociation—Gem protein in its various forms (0.5 μM) was incubated for increasing amounts of time at 30 °C in binding buffer (20 mM Hepes, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 10 mM MgCl2) containing 50 μg/ml BSA and either 10 μM [8-3H]GDP (11.7 Ci/mmol, Amersham Biosciences; 1:10 isotopic dilution) or 40 μM [5-γ-32P]GTP (1000 Ci/mmol, Amersham Biosciences; 1000 cpm/pmol) in a total volume of 250 μl. 10-μl portions were removed at the indicated times, diluted in 2 ml of cold binding buffer, filtered through nitrocellulose filters (Schleicher and Schuell NC45; pore size 0.45 μm), and washed three times with 2 ml of the same buffer. Radioactivity on the filter corresponding to protein-bound nucleotide was determined by liquid scintillation counting. Measures were performed in duplicate or triplicate. To determine kinetics of nucleotide dissociation, a 1 mM excess of unlabeled GDP or GTP was added to the binding reactions described above after 2 h of nucleotide binding. 10-μl portions were removed at the indicated times and treated as above to measure the amount of radiolabeled nucleotide remaining bound to the protein. The affinity of Gem for nucleotides was determined by incubating 4 pmol of Gem for 2 h at 30 °C in binding buffer with 0–30 μM [8-3H]GDP or 0–300 μM [5-γ-32P]GTP.
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Adaptation of various nucleotides for GDP binding to Gem was assessed by measuring the binding of 10 μM [8-3H]GDP to 0.5 μM Gem in the presence of 0–100 mM unlabeled competitors (GMP, GDP, GTP, GTPγS, GMP-PNP). GTP was repurified by ion exchange chromatography (Mono Q); all GTP analogues (labeled and unlabeled) were freed from contaminating GDP by treatment with agarose-bound alkaline phosphatase (Sigma) immediately prior to use. Contamination of labeled GTPγ35S by GDP35S was assessed to be less than 0.03% by thin layer chromatography on polyethyleneimine-cellulose.

**GTPase Activity**—Gem proteins (0.5 μM) were incubated with 20 μM [32P]GTP (3000 Ci/mmol; Amersham Biosciences; 4000 cpm/pmol) in 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 10 mM MgCl2 at 30 °C. 1-μl portions were removed at the indicated times and immediately mixed at 4 °C with 2 μl of a solution containing 0.2% SDS, 5 mM EDTA, 5 mM GDP, and 5 mM GTP. Samples were heated to 70 °C for 2 min to dissociate protein-bound nucleotides, and 1-μl aliquots were spotted onto polyethyleneimine-cellulose-covered thin layer chromatography plates (Merck). They were developed in 0.6 M sodium phosphate buffer, pH 3.4, for 30 min, dried, and autoradiographed. The amounts of labeled nucleotides were measured on the plates using a Storm 860 PhosphorImager (Amersham Biosciences), and the relative proportion of hydrolyzed GTP was calculated using ImageQuant.

**Crystallization and Structure Determination**—Crystallization assays were performed with the ΔNΔCaM portion of Gem (residues Met73–Arg694) using the hanging drop vapor diffusion method at 16 °C. Crystals were grown in a solution containing 0.9–1.1 M sodium acetate, 100 mM sodium cacodylate, pH 6.5, 5 mM MgCl2, and 2 mM DTT. Crystals appeared by self-nucleation in 1–2 h and reached their final dimension when deleted from its seven C-terminal residues (GemC7, Met1–Ser289); removal of the N-terminal extension (GemC7, Met1–Ser289) led to an insoluble protein that became soluble when co-produced and purified with CaM.

**RESULTS**

Production and Purification of Various Forms of the Gem Protein—Various forms of Gem were produced in recombinant bacteria as GST fusion proteins, purified, and cleaved from GST with rTEV protease. The resulting preparations contained 50–80% active protein (as assessed by their capacity to bind nucleotides; see below). We initially focused on the portion of Gem encompassing the G domain plus the first part of the C-terminal extension, hence devoid of the N-terminal extension, the CaM-binding region, and seven C-terminal residues of the C-terminal extension (GemΔNΔCaM, residues Met73–Arg694; see Fig. 1), to compare its biochemical and structural properties with those of H-Ras. In order to establish the influence of the N- and C-terminal extensions on the biochemical properties of Gem, we purified two longer forms of the protein. In our hands, full-length Gem was only soluble when deleted from its seven C-terminal residues (GemΔC7, Met1–Ser289); removal of the N-terminal extension (GemΔNΔC7, Met73–Ser289) led to an insoluble protein that became soluble when co-produced and purified with CaM.

**Nucleotide Binding and Dissociation**—As is the case with most small GTPases, it is likely that purified Gem contains a stoichiometric amount of bound nucleotide. Hence, we measured the exchange of this endogenous nucleotide for exogenously added [3H]GDP or GTPγ35S and maintained a large excess of exogenous nucleotide relative to protein in order to minimize the possible interference from the endogenous nucleotide released during the exchange reaction. As shown in Fig. 2A, GemΔNΔCaM exchanged its endogenous nucleotide for [3H]GDP and GTPγ35S with similar saturable kinetics. In both cases, binding of exogenous nucleotide was strictly dependent
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on the presence of high concentrations of free Mg$^{2+}$, with maximal binding reached above 5 mM (not shown). The competition experiment depicted in Fig. 2B shows that GemΔNΔCaM bound GDP with a higher affinity than GTP; among GTP analogues, GTPγS was almost as effective as GTP, whereas GMP-PNP exhibited a somewhat lower affinity. As is the case with other G proteins, GMP did not compete with GDP for binding to Gem. Binding of labeled GDP or GTP to Gem was saturable as a function of nucleotide concentration (Fig. 2, A and B), exhibiting affinities in the micromolar range, with a $K_d$ for GDP of 2.5 μM and a $K_d$ for GTPγS of 19 μM (Fig. 2E and Table 1), values that are several orders of magnitude larger than those reported for H-Ras (0.02 nM for GDP and 0.005 nM for GTP) (20). These values did not change when a 10-fold lower protein concentration was used in the assay, enabling us to measure the influence of the C-terminal extension alone and CaM binding to GemΔNΔCaM; however, GTP dissociated with a 3-fold slower half-time. For reasons detailed above, the form of Gem devoid of its N-terminal extension but comprising its C-terminal extension was purified as a complex with CaM; treatment with 2 mM EGTA released the interaction between the two proteins, as evidenced by size exclusion chromatography (not shown), which enabled us to measure the influence of the C-terminal extension alone and CaM binding to GemΔNΔCaM on its biochemical properties. As above, the only significant difference from GemΔNΔCaM was the moderately slower GTP dissociation kinetics, comparable with those measured for GemΔC7; no significant variation attributable to the binding of CaM to the C terminus of Gem was detected. Hence, in the full-length Gem molecule (GemΔC7), the C-terminal extension may act to moderately stabilize the interaction with GTP.

GTPase Activity—Because of the important changes in the switch II region between Gem and H-Ras, assessing whether Gem carries an intrinsic GTPase activity was of particular interest. As shown in Fig. 3, GemΔNΔCaM indeed hydrolyzed GTP to GDP as assessed by thin layer chromatography. That this reaction genuinely represented the GTPase activity of Gem, as assessed by thin layer chromatography (not shown), which enabled us to measure the influence of the C-terminal extension alone and CaM binding to GemΔNΔCaM on its biochemical properties. As above, the only significant difference from GemΔNΔCaM was the moderately slower GTPase activity, comparable with those measured for GemΔC7; no significant variation attributable to the binding of CaM to the C terminus of Gem was detected. Hence, in the full-length Gem molecule (GemΔC7), the C-terminal extension may act to moderately stabilize the interaction with GTP.

FIGURE 1. Sequence alignment for RGK proteins. Conserved residues are indicated by dots, conserved motifs are highlighted in gray, and their names are given below. *, position of documented phosphorylation sites. Note that multiple conserved serines are found in the N- and C-terminal extensions. A schematic diagram of the secondary structures of Gem is shown above the sequence. The sequence of H-Ras is aligned along those of RGK proteins for comparison. SH3, Src homology 3.

- N-terminal extension -

| Gem | MTJN | Red1 | N2707c | Red1 | Gem | N2707c |
|-----|------|------|--------|------|-----|--------|
| Motifs | Gxin | Gxin | Gxin | Gxin | Gxin | Gxin |

- C-terminal extension -

| Gem | DEEXE| KETAFPP | DFLKQH | DEEXE| KETAFPP | DFLKQH |
|-----|------|--------|--------|-----|--------|--------|
| Motifs | Gxin | Gxin | Gxin | Gxin | Gxin | Gxin |

and 8 min, respectively (Table 1). These values are 1 (for GDP) to 2 (for GTP) orders of magnitude smaller than for H-Ras, from which both GDP and GTP exhibit dissociation half-times of ~45 min (20).

The presence of the C-terminal extension alone or both the N- and C-terminal extensions of Gem only moderately affected its nucleotide binding characteristics (Table 1). Indeed, GemΔC7 (whether alone or in complex with CaM; data not shown) exhibited very similar GTP dissociation kinetics as well as affinities for both GDP and GTP as GemΔNΔCaM; however, GTP dissociated with a 3-fold slower half-time. For reasons detailed above, the form of Gem devoid of its N-terminal extension but comprising its C-terminal extension was purified as a complex with CaM; treatment with 2 mM EGTA released the interaction between the two proteins, as evidenced by size exclusion chromatography (not shown), which enabled us to measure the influence of the C-terminal extension alone and CaM binding to GemΔNΔCaM on its biochemical properties. As above, the only significant difference from GemΔNΔCaM was the moderately slower GTP dissociation kinetics, comparable with those measured for GemΔC7; no significant variation attributable to the binding of CaM to the C terminus of Gem was detected. Hence, in the full-length Gem molecule (GemΔC7), the C-terminal extension may act to moderately stabilize the interaction with GTP.
activity representing several cycles of hydrolysis was measured for Gem. Nevertheless, rate constants for the initial velocity of the hydrolysis reaction may be compared; the $k_{cat}$ of the reaction measured for GemNΔCaM was ~30-fold higher than that of H-Ras (Table 1).

Despite their moderate effect on the interaction with GTP, the N- and C-terminal extensions of Gem greatly impacted its intrinsic GTPase activity. Indeed, when the C-terminal extension alone was present, no more intrinsic GTPase activity could be measured, although GTP still bound to the protein. Treatment of the complex with 2 mM EGTA, to release CaM from Gem, and further elimination of CaM by ion exchange chromatography had no effect (data not shown), suggesting that the presence of the C-terminal extension inhibited the GTPase activity of Gem. In contrast, GemNΔC7, containing both the N- and C-terminal extensions, exhibited a 20-fold higher GTPase activity than GemNΔCaM. Reconstitution with CaM and purification of the complex by size exclusion chromatography (or direct purification of the GemNΔC7-CaM complex; data not shown) did not affect the GTPase activity (Table 1). Therefore, the N- and C-terminal extensions influence the GTPase activity of the full-length Gem molecule, but CaM binding to the C terminus of the molecule exhibits no detectable effect.

**Overall Structure of Gem and Its Nucleotide Binding Site** —The crystal structure of GemNΔCaM was determined in complex with

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**FIGURE 2. Biochemical characteristics of GemNΔCaM.**

- **A.** nucleotide binding kinetics. 0.5 μM protein was incubated for the indicated time intervals with 10 μM [3H]GDP (▲) or 40 μM GTPγS (■), and bound nucleotide was measured as detailed under “Experimental Procedures.” 8, competition of various nucleotides for GDP binding to Gem. ▲, GDP; ●, GTP; ■, GTPγS; ●, GMP-PNP; ▼, GMP. C, binding of [3H]GDP to Gem as a function of GDP concentration (▲). D, binding of GTPγS to Gem as a function of GDP concentration (■). E, Scatchard plot of the data from C (▲) and D (■). F, dissociation of nucleotides from Gem. Dissociation of previously bound [3H]GDP by excess unlabeled GDP (▲) and previously bound GTPγS by unlabeled GTPγS (■) are shown.

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**TABLE 1**

| Biochemical characteristics of the various forms of Gem | GemNΔCaM With 5 mM CaCl₂ | GemNΔC7-CaM With 2 mM EGTA | GemNΔC7 | H-Ras* |
|-------------------------------------------------------|--------------------------|----------------------------|----------|--------|
| $K_{d}$ (GDP) (μM)                                      | 2.5 ± 0.9                 | 1.8 ± 0.1                  | 4.6 ± 2   | 2 × 10⁻⁶ |
| $K_{d}$ (GTP) (μM)                                     | 19.6 ± 10                 | 15.5 ± 2.4                 | 15 ± 3.6  | 5 × 10⁻⁷ |
| $k_{cat}$ (GDP) (min⁻¹)                                | 0.087 ± 0.018             | 0.087                      | 0.069     | 0.087  |
| $k_{cat}$ (GTP) (min⁻¹)                                | 0.83 ± 0.137              | 0.31 ± 0.079               | 0.146 ± 0.05 | 0.23   |
| $t_{1/2}$ (min)                                        | 8 ± 2                     | 10                        | 0.7 ± 0.12 | 23     |
| $t_{1/2}$ (min)                                        | 0.83 ± 0.15               | 2.25 ± 0.75               | 4.75 ± 2.5 | 0.59 ± 0.027 |
| $k_{cat}$ (min⁻¹)                                      | 0.027 ± 0.005             | ND                        | ND        | 0.001  |

* Data concerning H-Ras from Ref. 20 are shown for comparison.

* ND, not detected.
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Mg-GDP at 2.1 Å resolution (Table 2 and Fig. 4A). Two molecules (A and B) of Gem are present in the asymmetric unit and are virtually identical, yielding a root mean square deviation value of 0.6 Å (on 158 Ca excluding the switch regions). The overall structure of the G domain of Gem resembles that of other small GTPases with the classical six-stranded β-sheet, surrounded by five α-helices (a root mean square deviation value of 0.79 Å on 132 Ca is obtained when compared with H-Ras-GDP (Protein Data Bank code 4Q21) (22). In addition to the G domain, the structure reveals that the first part of the C-terminal extension (Asp244–Arg264) (Fig. 1) folds into an α helix, which contacts the tip of the interswitch (strands β2–β3 between switch I and switch II) and extends antiparallel along the α5 helix (Fig. 4A). The electron density maps unambiguously show the presence of a tightly bound GDP molecule and a Mg2+ ion at the active site (supplemental Fig. S2). The bound nucleotide has extensive hydrophobic and hydrophilic interactions with the surrounding residues (Fig. 4B and supplementary Table S1) that are conserved in other small GTPases. In the GemΔNΔCaM-GDP structure, the Mg2+ ion is coordinated in a classical manner by six oxygens in a virtually perfect octahedral geometry with the hydroxyl group of the P-loop Ser89 Oγ (2.2 Å), the β-phosphate of GDP (2.0 Å), and four water molecules (2.0–2.3 Å) (Fig. 4B).

**The C-terminal Helix**—The C-terminal helix (αCter helix) of GemΔNΔCaM, extending immediately beyond the G domain, has a highly charged surface. This helix makes two patches of interaction with both the tip of the interswitch and the C-terminal part of the α5 helix (Fig. 4C). First, a network of hydrogen bonds takes place near the N terminus of the α5 helix (Fig. 4C). A network of hydrogen bonds with the carboxylate of Asp244 from the α5/αCter loop and the carboxamide of Asn249 from the αCter helix. Asn249 is stabilized by another hydrogen bond with the carbonyl main chain of Arg240 from the α5 helix. On the other side, Arg252 makes two hydrogen bonds with the carboxylate group of Glu233 from the interswitch. This network of interactions is supported by hydrophobic packing contacts between the aliphatic side chains of Arg252 with Arg240 from the α5 helix. Note that the Arg240 side chain is maintained in place via two hydrogen bonds with carboxyls from the interswitch backbone (Val216 and Glu233). Second, a network of van der Waals interactions takes place at the end of the αCter helix. Thus, the side chain of Glu256 makes contacts with the side chains of Arg237 from the α5 helix and Asp211 from the interswitch, which in turn contacts the main chain of Arg252 from the αCter helix. In addition, the aromatic side chain of Tyr255 packs against the tip of the interswitch (main chains of Asp241–Gly242). And last, Arg243 from the α5/αCter loop interacts with the β1 strand and the end of switch II. Its guanidium group makes two hydrogen bonds with the main chain carbonyl of Tyr252 and the carboxylate side chain of Asp151, respectively. Altogether, these interactions maintain the αCter helix packed against the G domain and may consequently direct the CaM-binding region in the vicinity of the switch I and nucleotide binding site.

**The Switch Regions**—Two distinct conformations are observed for the switch regions in molecules A and B with Gly97/Asp112 and Trp135/Val149 as hinge residues for switch...
I and II, respectively (Fig. 4A). Note that only the ends of both switches have interpretable electron densities and were modeled, whereas regions Met<sup>102</sup>–Asp<sup>105</sup> and Lys<sup>136</sup>–Asn<sup>139</sup> for molecule A and regions His<sup>99</sup>–Gly<sup>110</sup> and Lys<sup>136</sup>–Lys<sup>142</sup> for molecule B were omitted from the model, respectively, for switch I and II. The distinct conformations of both switch extremities are driven by crystal packing contacts. This suggests that in the absence of protein contacts, the switch regions are disorganized in the GDP-bound form of Gem<sub>ΔNΔCaM</sub>. However, in the absence of a structure for the GDP-bound form of full-length Gem, it is not possible to anticipate how the N- and C-terminal extensions will impact the conformation of the switch regions.

The N-terminal hinge residue of switch I, Gly<sup>97</sup>, exhibits a dramatic difference in its torsion angles with a ΔΦ of 179.6° between molecules A and B. This leads to a reverse turn of switch I in molecule A that opens the nucleotide binding site (Fig. 4A). Thus, the N terminus of switch I in molecule A (Val<sup>98</sup>–Ser<sup>101</sup>) is close to the β<sub>5</sub> strand of the interswitch. It is stabilized by a hydrogen bond between the carbonyl main chain of His<sup>99</sup> and the guanidium side chain of Arg<sup>116</sup> from the β<sub>2</sub> strand. In molecule B, the N terminus of switch I is only modeled for residue Val<sup>98</sup>, but it clearly points in the opposite direction than in molecule A. In both molecules, the C terminus of switch I ends with Asp<sup>112</sup>, where a bend occurs to lead to the β<sub>2</sub> strand (Fig. 4A). In molecule A, this region adopts a 3<sub>10</sub> helical

**FIGURE 4. Crystal structure of GDP-bound Gem-ΔNΔCaM.** A, an overall view of Gem-ΔNΔCaM-GDP is shown with switch I in green, the interswitch in yellow, switch II in pink, and the C-terminal helix in blue. Note that both conformations of switch I and II are shown superposed in dark for molecule A and in bright for molecule B. B, the scheme of Mg-GDP interactions is given for molecule A with distances (see supplemental Table S1 for distances in molecule B). An inset of the magnesium coordination sphere is shown separately for clarity. C, close view of the C-terminal helix (blue) and its interaction with the interswitch (yellow) and the α<sub>5</sub> helix (white) with secondary structure shown as coils. Hydrogen bonds are indicated by dashed lines. Note that for clarity, the orientation is not strictly conserved with the overall view (A). D, close view of the DMWEN motif in switch II (pink). The side chain of Glu<sup>134</sup> from molecule A is shown as transparent, indicating its partial occupancy. Orientation with the overall view is conserved.
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FIGURE 5. Comparison of the switch regions of GemΔNΔCaM-GDP with H-Ras-GDP/GTP. A, the H-Ras-GDP (Protein Data Bank code 4Q21; transparent light blue) and H-Ras-GTP (Protein Data Bank code 5P21; transparent dark blue) switch I-interswitch II regions are shown superposed on the GemΔNΔCaM-GDP structure (same colors as in Fig. 4) for comparison. B, close view of A with overall structure shown as coils and the C-terminal part of switch II deleted for clarity. The first residues from the DMWEN and DTAGQ motifs in Gem and H-Ras, respectively, are shown in ball-and-stick representations for comparison. Note the divergent backbone trace and side chain directions between Gem and H-Ras after the Asp131/Asp57 residues and the position of Met132 that shortens the β2 strand, pushing switch I away from the nucleotide binding site in Gem when compared with H-Ras.

The DXWEX Motif—The N terminus of switch II contains a DXWEX motif (Asp131–Asn135 in Gem) specific to RGK proteins in place of the highly conserved DTAGQ motif in other small GTPases (Fig. 1). In this region, all RGK proteins exhibit a conserved bulky tryptophan and negatively charged glutamate residues in place of the canonical small alanine and glycine residues in the DTAGQ motif. The second position of the DXWEX motif is occupied by a variable amino acid ranging from a large methionine residue in Gem to a smaller isoleucine residue in Rad/Rem2 and the canonical threonine in Rem1. In addition, at the position of the glutamate that plays a catalytic role in the GTPase activity of Ras, Rad and Rem2 also exhibit a glutamate residue (its catalytic role has not been assessed), whereas Gem and Rem1, respectively, have an asparagine and an alanine. Our structure reveals how such a bulky motif with two well defined distinct conformations (molecules A and B; Fig. 4D) impacts the overall structure and the nucleotide-binding site of Gem. A striking difference is observed for the position of the carbonyl of Trp133 that flips outward from the magnesium binding site in molecule A (Fig. 4D). This position is stabilized by a hydrogen bond with the side chain of His143 that belongs to the C-terminal part of switch II (Glu140–His145), modeled only in molecule A. Thus, although Met132 and Trp133 superpose well in molecules A and B, Glu134 and Asn135 positions are divergent (Fig. 4D). The side chain of Glu134 is clearly oriented outward in molecule B, whereas it points toward the nucleotide-binding site in molecule A. In this molecule, the carboxylate group of Glu134 makes a water-mediated interaction with the magnesium ion, thus participating like Asp131 in the water-mediated coordination sphere of the magnesium ion (Fig. 4D). Note that the canonical aspartate (Asp131) exhibits a conserved structural position with other small GTPases (Fig. 4, B and D).

Structural comparison of the DMWEN motif in Gem-GDP with the DTAGQ motif in H-Ras-GDP/GTP (22, 23) (Fig. 5) reveals that the bulky tryptophan (Trp133 in Gem) that is conserved in all RGK proteins and replaces an alanine residue in other small GTPases imposes a unique conformation on this region (Fig. 5A). Its bulky side chain is turned outward from the nucleotide binding site packing along a hydrophobic patch composed of the C terminus of the β1 strand (Ile81–Gly82), the α3 helix (Leu71), and switch II (Met47 in both molecules and locked by His145 in molecule A). This differs from the equivalent alanine residue (Ala59) of the DTAGQ motif that extends in the direction of the nucleotide-binding site (Fig. 5B). Such a position for Trp133 forces the backbone of the preceding residue (Met132) to protrude toward the N terminus of the β2 strand at 2.3 Å (Cα atoms) from the position found for the equivalent threonine residue (Thr58) in H-Ras. A consequence of this protrusion is that Asp112 makes a bend to avoid a steric clash with the Met132 backbone, thus shortening the β2 strand by 1 residue at its N terminus when compared with H-Ras (Fig. 5).

DISCUSSION

Structural comparisons between the G-domain of Gem and those of other Ras superfamily GTPases reveal that Gem adopts their conserved fold and GDP binding site. Accordingly, Gem also binds GDP and GTP and exhibits an intrinsic GTPase activity. Hence, Gem exhibits the hallmarks of a bona fide GTPase of the Ras superfamily. These structural properties should be shared among all proteins of the RGK family, consistent with the rather high sequence conservation within their G domains (48–64% identity versus 27.5% identity with the G domain of H-Ras). Nevertheless, our study shows that some striking structural elements distinguish Gem from other Ras family proteins, which may be responsible for its distinctive biochemical properties.

The C-terminal Extension—The first part of the C-terminal extension of Gem (Asp244–Arg264; Fig. 1), in the absence of the rest of this region, folds into an α-helix (called here αCter) that packs at the tip of the interswitch lining antiparallel to the α5
helix (Fig. 4, A and C). The position of this helix relative to the G domain is reminiscent of that of the N-terminal helix in Arf proteins in their GDP-bound form, except that in Arf proteins, the interswitch is retracted, the N-terminal helix lies parallel to the α5 helix, and its interactions with the G domain are mainly hydrophobic (supplemental Fig. S3A). However, despite some structural similarities, no data are available to envision that the C-terminal helix of Gem could be released from the G domain and move away as is observed for the N-terminal helix of Arf proteins during their GDP/GTP cycle (for a review, see Ref. 24). Although the last part of the C-terminal extension containing the CaM-binding site and the C7 motif (seven C-terminal residues; see Fig. 1) is conserved in sequence among proteins of the RGK family, the first part of the C-terminal extension (αCter of Gem) is not (Fig. 1). This region in Rad, which shares 60% sequence identity with the homologous domain of Gem, should also exhibit an α-helical conformation. However, Rem1 and Rem2, respectively, possess one and two consensus Src homology 3-binding motifs (PXXP) within this region (Fig. 1) and therefore cannot adopt an α-helical conformation. Whether Rem1/Rem2 are actually able to bind Src homology 3 domain proteins through this region has not yet been reported. Thus, it appears that the first part of the C-terminal extension in the RGK family is the site of structural and probably functional distinction between the Gem/Rad and Rem1/Rem2 subgroups. Note that the first part of the C-terminal extension of Ran, which is unrelated in sequence to that of Rem1/Rem2, also is a proline-rich region. In Ran, this region adopts a nonstructured polypeptide stretch that also binds into the groove formed between the tip of the interswitch and the α5 helix (25) (supplemental Fig. S3B). Altogether, these observations highlight that the interface formed by the tip of the interswitch and the α5 helix is a site with which the N- or C-terminal extensions of small GTPases frequently interact.

Is the Switch I of Gem a Classical Switch Region?—In G proteins, the switch regions undergo drastic conformational changes upon the GDP/GTP cycle. To strictly delineate these regions for a given G protein, structures of its GDP- and GTP-bound forms are required. Here, despite the lack of structural data for the GTP-bound form, we have delineated the putative switch regions of Gem based on the distinct conformations observed in molecules A and B as residues Gly98–Asp112 and Trp133–Val149 for switch I and II, respectively. These distinct conformations, which are induced and stabilized by different crystal packing contacts, carry interesting information concerning the structural features of the switch regions. They also underline that in absence of crystal packing contacts, the switch regions of Gem-GDP (in the absence of the full N- and C-terminal extensions) are flexible, except for the C-terminal part of switch II (His145–Val149). Note that in the absence of a three-dimensional structure for the full-length Gem protein, we cannot anticipate how these two extensions as well as CaM binding will impact the switch I and II conformations of the GDP-bound form.

However, independent of the presence or absence of the N- and C-terminal extensions, the bulky DXWEX motif exerts a striking structural impact on the conformation of switch I within the G domain of Gem. Indeed, as compared with the structures of other small GTPases, Gem exhibits a β2 strand that is shortened at its N terminus, with residue Asp112 making a bend that opens the conformation of switch I far away from the nucleotide binding site and switch II (Fig. 4A). Arf and Ran GTPases in their GDP-bound forms also exhibit an open switch I conformation, but the N terminus of their β2 strand remains close to the nucleotide-binding site and to switch II (26–30) (supplemental Fig. S3, C–F). The bend at Asp112 in Gem is induced by the presence of the bulky DXWEX motif, whose conformation diverges from that of the classical DTAGQ motif in order to accommodate the bulky tryptophan residue (Fig. 5B). This residue is turned away from the nucleotide-binding site and packed against a hydrophobic patch. The main chain trace of the preceding residue, Met132, is forced to make a kink toward the β2 strand, which is therefore prevented from starting at the same position as observed in H-Ras (Fig. 5). Since the tryptophan residue from the DXWEX motif is conserved in RGK proteins and the main chain trace of the preceding residue is sufficient to drive the bend and thus shorten the N terminus of the β2 strand, we expect this striking feature to be shared by all RGK proteins in their GDP-bound form.

Is this position of switch I, far away from the nucleotide binding site, conserved during the GDP/GTP cycle? If such were the case, switch I may not play a role in the GDP/GTP cycle conformational change. This hypothesis is supported by the fact that the strictly conserved threonine residue of the PM2 motif (Thr35 in H-Ras) (Fig. 1), which binds the γ-phosphate of GTP thereby driving the switch I conformational change during the GDP/GTP cycle, is absent in RGK proteins. This suggests that switch I may have lost its ability to bind GTP and thus to switch during the GDP/GTP cycle. An additional consequence of a switch I open conformation along the GDP/GTP cycle could be that the nucleotide binding site may become accessible to the N- and/or C-terminal extensions, which may thereby exert an effect on the GTPase activity of the protein (see below).

Structural Insights for the Weak Nucleotide Binding Affinity—Gem exhibits affinities for GDP and GTP in the micromolar range, several orders of magnitude weaker than those measured for H-Ras (see Table 1). However, from a physiological point of view, this should not prevent Gem from being constitutively bound to nucleotides in cells, since the average intracellular concentrations of GDP and GTP are thought to be 100 μM and 1 mM, respectively. The weak nucleotide affinity measured for Gem, relative to H-Ras, is not the consequence of atypical interactions with Mg-GDP, since all of the interactions observed in other Ras superfamily GTPases are conserved. The biochemical data presented here reveal that the presence or absence of the N- and C-terminal extensions and CaM do not affect the affinity of Gem for nucleotides, implying that the structural differences that may explain the weaker nucleotide binding properties of Gem are contained within its G domain alone.

RGK proteins do not possess the G1 motif, consisting of a phenylalanine (equivalent to Phe28 in H-Ras; Fig. 1) that caps the guanine ring and thus contributes to the high affinity of the nucleotide for the binding site. Indeed, it was shown that mutating this residue to leucine in H-Ras and Cdc42 greatly
enhances their nucleotide dissociation rates, without affecting their nucleotide association kinetics or GTPase activity (31, 32). At this position in Gem, an alanine residue, Ala223 from the G3 motif (Figs. 1 and 4B), is found above the guanine ring. This shorter residue cannot substitute for the phenylalanine. This sequence difference probably contributes to the relatively weak nucleotide affinity and rapid nucleotide dissociation kinetics observed for Gem. Although Gem, Rad, and Rem2 possess an alanine residue in this position, Rem1 has a threonine residue, which suggests that it may have a slightly higher affinity for nucleotides than other RGK proteins. A striking observation in our structure is that in molecule A, a phenylalanine residue from a crystal symmetry molecule caps the guanine ring in a position similar to that of the phenylalanine G1 motif in other small GTPases (supplemental Fig. S4). This additional interaction in molecule A appears to be responsible for the difference in temperature factors observed between Mg·GDP from molecule A and molecule B (Table 2), thereby supporting the hypothesis that in the presence of such an interaction, Mg·GDP would have a higher binding affinity.

We anticipate that the presence of the atypical DXWEX motif at the beginning of switch II, close to the position of the γ-phosphate, also reduces the affinity of RGK proteins for nucleotides. First, and as discussed above, the DXWEX motif with the bulky Trp133 imposes a bend at the beginning of the β2 strand that directs switch I away from the nucleotide-binding site. Thus, switch I in this open conformation cannot cover the nucleotide, a feature that should contribute to the weaker measured nucleotide affinity. Second (and also due to the bulky Trp133), the carbonyl main chain of Met132, the residue of Gem equivalent to the threonine in the DTAGQ motif, can no longer make a hydrogen bond with the water molecule that binds the magnesium ion and Asp131 (Figs. 4D and 5B). Thus, the magnesium ion coordination lacks a water-mediated contact in the GDP-bound form (probably also in the GTP-bound form), which should further reduce the nucleotide affinity. Finally, the presence of a glutamate residue in the DXWEX motif close to the phosphate binding site, conserved in all RGK proteins, should contribute to diminish the nucleotide affinity by approaching its negatively charged side chain close to the γ- and β-phosphates inducing repulsion forces. This should be even more pronounced for the GTP-bound form of the protein, since the carboxylate side chain group of Glu134 will partially overlap with the γ-phosphate (supplemental Fig. S5), which may explain the faster dissociation kinetics and lower affinity of Gem for GTP as compared with GDP.

An Unexpected High GTPase Activity—The following biochemical observations came quite unexpected. (i) Gem exhibits an intrinsic GTPase activity, (ii) it is high relative to H-Ras (see Table 1), and (iii) it is greatly affected by the presence or absence of the N- and C-terminal extensions. The first observation is intriguing if one considers that Gem does not exhibit the consensus DTAGQ motif that is critical for GTP binding and hydrolysis in other small GTPases. In addition, the N- and C-terminal extensions of Gem are required for full GTPase activity but are not essential for the G domain of Gem to exhibit a GTPase activity. Altogether, these observations suggest that the molecular mechanism of the intrinsic GTPase activity of Gem is probably different from that of other small GTPases.

The GTPase activity of Gem is reduced when both the N- and C-terminal extensions are deleted and is severely impaired when the N terminus alone is deleted. Note that Ca2+/CaM has no effect on the GTPase and GTP binding activities of Gem. This discrepancy with an earlier report (33) may be related to the fact that we took care to remove the N-terminal GST tag from Gem during its purification. However, this raises an intriguing question concerning the observed inhibition of the GTPase activity by the C-terminal extension as to how this inhibition can take place without being affected by the presence or absence of CaM, which should cover a large part of the C-terminal extension and thus impact its structural properties. One attractive hypothesis is that the C-terminal extension folds back onto the nucleotide-binding site that is uncovered by the open conformation of switch I and interferes by steric hindrance with the GTPase machinery. Such a nonspecific interaction would act to inhibit the GTPase activity regardless of the presence or absence of CaM. In full-length Gem, the N-terminal extension may somehow interact with the C-terminal extension and abolish its inhibitory effect by affecting, for instance, the position of the C-terminal extension relative to the nucleotide-binding site. Such an interaction between the N- and C-terminal extensions could also be responsible for the 30-fold higher GTPase activity of GemΔCaC7 as compared with that of GemΔNΔCaM by positioning the C-terminal extension favorably for the GTPase machinery. Furthermore, binding of cellular partners to the N-terminal extension could release the C-terminal extension and thereby enable it to fold back onto the nucleotide binding site to inhibit the GTPase activity of Gem. This would represent a novel mechanism for regulating the GTPase activity of a Ras superfamily protein.

CONCLUSION

In the absence of the conserved PM2 (Thr35 in H-Ras) and PM3 (DTAGQ) motifs, it was first thought that RGK proteins would not bind GTP and therefore exhibit no GTPase activity. We show here that Gem binds both GDP and GTP with micromolar affinities and exhibits a high GTPase activity relative to H-Ras. Furthermore, we show that the N- and C-terminal extensions of Gem regulate its GTPase activity. We therefore anticipate that the GDP/GTP conformational cycle and the GTPase reaction of Gem will present important differences from those of other small GTPases. To fully understand the molecular mechanisms that govern Gem, additional structures of the protein bound to GTP in the presence of full N- and C-terminal extensions and also complexed with CaM will be required.

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Addendum—Ugochukwu (E. Ugochuk, M. Soundararajan, J. Elkins, C. Gileadi, G. Schoch, F. Sobott, O. Federov, J. Bray, N. Pantig, G. Berridge, N. Burgess, W. H. Lee, A. Turnball, M. Sundstrom, C. Arrowsmith, J. Weigelt, A. Edwards, F. von Delft, and D. Doyle (Structural Genomics Consortium (SGC)), unpublished results) have determined the structure of the G domain of Gem bound to GDP (Protein Data Bank code 2G3Y). In contrast with our structure, their structure lacks the first part of the C-terminal extension. No major difference in the overall structures was observed. However, a striking observation is that Glu$^{134}$ from the DMWEN motif, which points inward toward the nucleotide binding site, is much closer to the magnesium ion and $\gamma$-phosphate position than our structure revealed. This position is similar to the one that we have modeled in the site.

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