Rem2, a new member of the Rem/Rad/Gem/Kir family of Ras-related GTPases

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Here we report the molecular cloning and biochemical characterization of Rem2 (for Rem, Rad and Gem-related 2), a novel GTP-binding protein identified on the basis of its homology with the Rem, Rad, Gem and Kir (RGK) family of Ras-related small GTP-binding proteins. Rem2 mRNA was detected in rat brain and kidney, making it the first member of the RGK family to be expressed at relatively high levels in neuronal tissues. Recombinant Rem2 binds GTP saturably and exhibits a low intrinsic rate of GTP hydrolysis. Surprisingly, the guanine nucleotide dissociation constants for both Rem2 and Rem are significantly different than the majority of the Ras-related GTPases, displaying higher dissociation rates for GTP than GDP. Localization studies with green fluorescent protein (GFP)-tagged recombinant protein fusions indicate that Rem2 has a punctate, plasma membrane localization. Deletion of the C-terminal seven amino acid residues that are conserved in all RGK family members did not affect the cellular distribution of the GFP fusion protein, whereas a larger deletion, including much of the polybasic region of the Rem2 C-terminus, resulted in its redistribution to the cytosol. Thus Rem2 is a GTPase of the RGK family with distinctive biochemical properties and possessing a novel cellular localization signal, consistent with its having a unique role in cell physiology.

Key words: GTP, GTP-binding proteins, signal transduction.

INTRODUCTION

The Ras superfamily of low-molecular-mass GTP-binding proteins is composed of a diverse group of structurally related proteins that have been grouped into six broad subfamilies, the Ras, Rab, Rho, Arf, Ran, and RGK (Rem, Rad and Gem/Kir) families [1]. Despite the differences between these subfamilies, all Ras-related GTPases contain five highly conserved domains (G1–G5) and function as nucleotide-dependent molecular switches, alternating between an active GTP-bound and an inactive GDP-bound conformational state [1]. They are involved in signal transduction and the regulation of a wide range of cellular processes, including cell growth, transformation, differentiation and morphogenesis, vesicular trafficking and secretion, nuclear transport, and apoptosis [2–5]. Guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) [6] influence the relative proportions of molecules in the active and inactive conformations. GEFs promote activation by inducing the release of GDP, whereas GAPs inactivate Ras-related proteins by stimulating their intrinsic GTPase activity [1,6].

The RGK proteins are the founding members of a new class of Ras-related GTP-binding proteins [7–10]. All members of the RGK subfamily share structural features that are distinct from those of other Ras-related proteins. These include several non-conservative amino acid substitutions within regions known to be involved in guanine nucleotide binding and hydrolysis, extended N- and C-termini, and a conserved C-terminal motif thought to mediate membrane association but lacking a prenylation site present in other Ras-like molecules [11]. Rem, Rad and Gem/Kir also differ from each other and from other Ras-related proteins in the putative effector (G2) domain, suggesting that they interact with distinct regulatory and effector proteins [1,6]. The members of this Ras subfamily are also subject to transcriptional regulation. In mice, Rem is most highly expressed in cardiac muscle and at modest levels in lung, kidney and skeletal muscle [7]. The administration of lipopolysaccharide, a potent activator of the inflammatory and immune systems, results in a general repression of Rem mRNA levels, making Rem the first Ras-like GTPase to be shown to be regulated by repression [7]. Rad expression has been shown to be increased in the muscle of some type II diabetes patients, perhaps in response to increased insulin levels [8], whereas Gem/Kir expression is induced in mitogen-stimulated T-cells, cytokine-activated endothelial cells [9,12], fibroblasts on the administration of serum [13] and in response to specific oncogenic kinases [10]. Although the cellular function of these proteins remains to be established, Rad has been implicated as a regulator of glucose uptake in a variety of cultured cell lines [14].

Here we describe the identification and characterization of a novel RGK family protein, Rem2, which exhibits specific structural differences in the highly conserved domains that determine both GTPase activity and prenylation. Despite these structural alterations, Rem2 specifically binds guanine nucleotides in a Mg^{2+}-dependent fashion and hydrolyses GTP. Interestingly, both Rem and Rem2 display a very low GDP dissociation rate while having the unique property of a rapid uncatalysed rate of guanosine 5′-[γ-thio]triphosphate (GTP[S]) dissociation ($t_{\frac{1}{2}}$ of 15 min). This contrasts with other Ras-like proteins and could be of functional significance. In addition we have determined the subcellular distribution of the Rem2 protein and analysed the

Abbreviations used: GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; GFP, green fluorescent protein; GST, glutathione S-transferase; GTP[S], guanosine 5′-[γ-thio]triphosphate; RGK, Rem, Rad, Gem and Kir Ras-related GTP-binding proteins.

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The nucleotide sequence data for Rem2 will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number AF084464.

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role of the conserved RGK family C-terminal domain in cellular localization. These studies suggest that Rem2 possesses novel cellular localization signals that, in contrast with most Ras-related proteins, do not reside in conserved C-terminal cysteine-rich motifs.

EXPERIMENTAL

General methods

Standard molecular biology techniques were used [15]. cDNA clones were subcloned to plasmid pBlueScript II vectors (Stratagene) and sequenced by the dideoxy chain termination method with the M13 universal primer or specific internal primers. Nick-translated probes were synthesized with a labelling kit (Gibco BRL). Northern blot analysis of Rem2 mRNA was performed as described previously [16] with a Clontech (Palo Alto, CA, U.S.A.) Rat Multiple Tissue Northern Blot.

Cloning of Rem2

During the isolation of the Rem cDNA, we identified a closely related human expressed sequence tag EST50903 (accession number H19231) that seemed to encode a unique RGK family member. The DNA sequence of EST50903 (approx. 1100 bp insert) was obtained and found to encode an open reading frame of 217 residues, which was incomplete at the 5′ end. To obtain a full-length cDNA clone, the partial human EST (expressed sequence tag) was used to generate a nick-translated probe (10^6 c.p.m./ml) that was used to screen a rat brain cDNA library (Clontech) from which several cDNA clones were isolated [7]. The largest of these cDNA clones (no. 7-1, approx. 1.9 kb insert) was subjected to DNA sequence analysis and found to encode the entire putative open reading frame of rat Rem2.

Recombinant Rem2 was expressed as a glutathione S-transferase (GST) fusion protein. In a series of preliminary experiments it was determined that short C-terminal deletions were necessary to allow the expression of large amounts of intact and soluble protein. The smallest deletion (deletion of the final 21 residues from the C-terminus) that allowed stable protein production was therefore introduced to Rem2. To construct the expression vector pGEX-KG-Rem2C, PCR was performed on clone 7-1 with primers that introduced a 5′ *Bam*HI site and 3′ *Eco*RI site and resulted in the C-terminal deletion of 21 residues from the Rem2 protein. The PCR product was sequenced to verify the fidelity of the amplified cDNA insert and subcloned to *Bam*HI/*Eco*RI-digested pGEX-KG [17].

Production of recombinant protein

Recombinant Rem was produced by thrombin cleavage of the GST–Rem fusion protein with the following modifications to the method described previously [7]. Mg^{2+} was adjusted to 1 mM; GDP (10 μM) was added to all buffers except the dialysis buffer. The cleaved protein was dialysed against 50 mM Tris/HCl (pH 7.5)/150 mM NaCl/1 mM Mg^{2+}/1 mM dithiothreitol/10 % (v/v) glycerol for 2 h at 4 °C and stored in multiple aliquots at −70 °C. The addition of Mg^{2+} and 10 μM GDP was found to stabilize the Rem protein during storage. Recombinant Rem2 (rRem2) was produced as a GST fusion protein in BL21DE3 cells harbouring the plasmid pGEX-rRem2C by induction with 0.5 mM isopropyl β-D-thiogalactoside for 3–4 h. The fusion protein was affinity-purified on glutathione agarose, dialysed and stored in multiple aliquots in storage buffer as described previously [7].

Optimization of $[Mg^{2+}]$ for guanine nucleotide binding

The optimal concentration of free Mg^{2+} for guanine nucleotide binding to Rem was determined by incubating Rem (1 μg) with 5 μM [^{35}S]GTP[S] (0.45 μCi per sample) under various Mg^{2+} concentrations. The calculation of $[Mg^{2+}]_{free}$ and GTP quantification by the rapid filtration assay were performed as described previously [7,18]. After incubation at 22 °C for the indicated period, samples were withdrawn, washed in buffer containing 10 mM Mg^{2+} and analysed by filtration. Alternatively, Rem was subjected to a 5 min preincubation in 1 mM EDTA at 22 °C, after which $[Mg^{2+}]_{free}$ was adjusted to 1 mM and [^{35}S]GTP[S] binding continued for the indicated period. Because optimal guanine nucleotide exchange was achieved by treatment with EDTA, this procedure was used in all remaining experiments.

Guanine nucleotide binding assays

To determine the specificity of Rem2 nucleotide binding, GST–Rem2C (10 μg) was incubated in 1 mM EDTA at 22 °C for 5 min with 20 μM [^{35}S]GTP[S] (1.25 μCi per sample) and either no competing ribonucleotides (control) or the indicated unlabelled competing ribonucleotides at 1 mM. The reaction mixture was then adjusted to 10 mM $[Mg^{2+}]_{free}$ and the incubation continued for 15 min at 22 °C before nucleotide binding was determined by rapid filtration [7]. The amount of GTP[S] bound in the absence of competitor (control) was set at 100 % binding value and used to compare the binding in the presence of specific ribonucleotide competitors.

The concentration dependence of nucleotide binding to Rem was determined by the preincubation of Rem (1 μg) in standard reaction buffer containing 1 mM EDTA for 5 min at 22 °C with increasing concentrations of [α-^{32}P]GTP (0.9 Ci/mmol). GTP binding was initiated by adjusting $[Mg^{2+}]_{free}$ to 1 mM. The reaction mixture was incubated at 22 °C for 10 min, filtered and counted as above. The concentration dependence of nucleotide binding to Rem2 was determined as described above except that GST–Rem2C (10 μg) was incubated with increasing concentrations of [^{35}S]GTP[S] (0.9 Ci/mmol) and binding initiated by adjusting $[Mg^{2+}]_{free}$ to 10 mM. Rem bound approx. 0.2 mol of GTP[S]/mol of Rem, whereas GST–Rem2C bound approx. 0.11 mol of GTP[S]/mol of GST–Rem2.

Guanine nucleotide dissociation

Guanine nucleotide dissociation was measured by using the strategy described previously [19]. In brief, Rem (1 μg) was incubated with 5 μM [^{3}H]GDP (0.45 Ci/mmol) or 5 μM [α-^{32}P]GTP (0.9 Ci/mmol) in 1 mM EDTA for 5 min at 22 °C; $[Mg^{2+}]_{free}$ was adjusted to 1 mM and guanine nucleotide binding continued for 10 min at 22 °C. Non-radiolabelled nucleotide was added (100-fold molar excess) to initiate the dissociation assay. At the indicated times after the addition of unlabelled nucleotide, samples were withdrawn and diluted with ice-cold wash buffer, filtered and counted as described above. Binding measured before the addition of non-radiolabelled nucleotide was set at 100 % binding for comparison. Guanine nucleotide dissociation for Rem2 was measured by incubating GST–Rem2C (10 μg) with 20 μM [^{3}H]GDP (0.45 Ci/mmol) or 20 μM [^{35}S]GTP[S] (0.9 Ci/mmol) at 22 °C for 5 min in the presence of 1 mM EDTA, followed by a 15 min incubation at 22 °C after adjustment of $[Mg^{2+}]_{free}$ to 10 mM. Non-radiolabelled nucleotide was added

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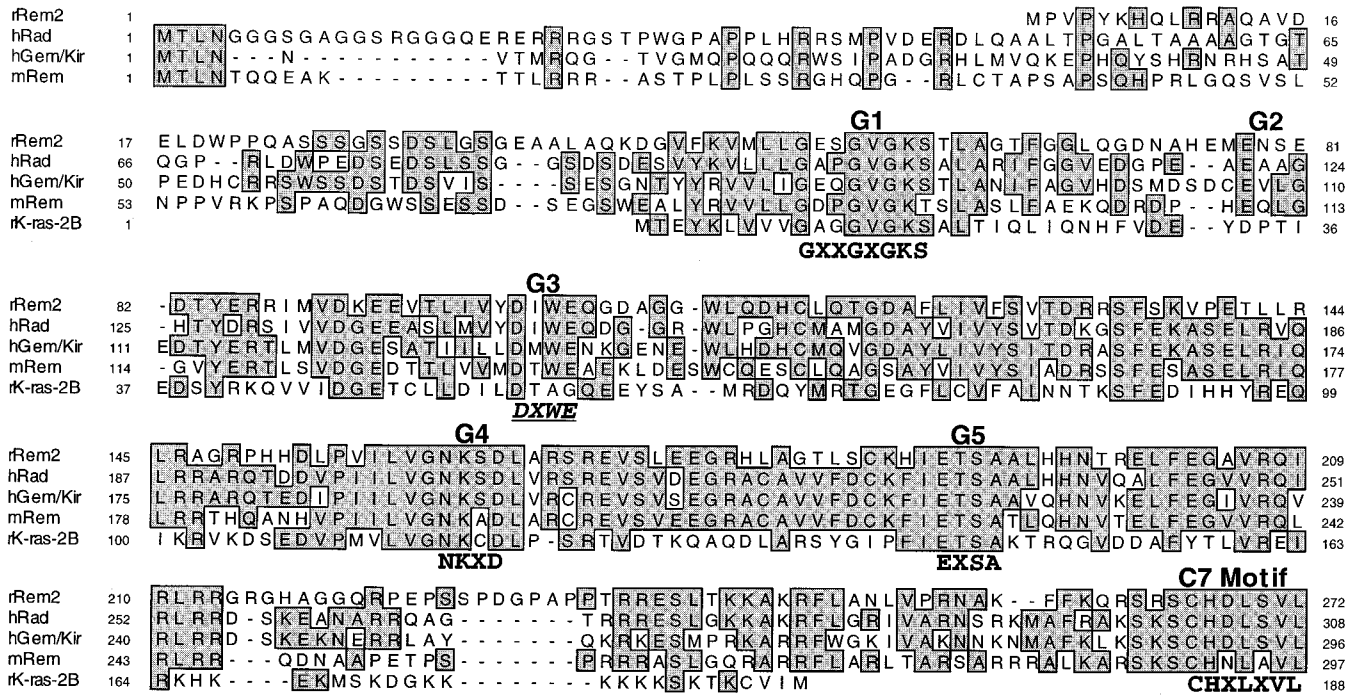

Figure 1 Comparison of the amino acid sequences of the rat (r) Rem2, mouse (m) Rem, human (h) Gem, hRad, hKir and rK-ras-2B proteins

The alignment was performed with the CLUSTAL W1.6 program [41]. Hyphens represent gaps introduced for optimal alignment. Numbers are residue numbers. Amino acid residues that were conserved in at least two of the five proteins in the alignment are placed in grey boxes. Consensus sequences for GTP-binding regions (G1–G5) and the conserved C7 sequence motif are labelled. The G3 consensus is unique to the RGK family and is underlined and in italics.

(50-fold molar excess) and bound radiolabelled guanine nucleotide was analysed as described above.

GTPase assay

Steady-state GTP hydrolysis was measured because the rapid release of GTP from both Rem and Rem2 proteins, even in the presence of high concentrations of Mg^{2+}, did not permit the isolation of the stable radiolabelled GTP complexes necessary for single turnover measurements of hydrolysis. Affinity-purified Rem/GST–Rem2C proteins were found to contain a small amount of contaminating phosphatase activity that resulted in a low background rate of [α-^{32}P]GTP hydrolysis. Unlabelled UTP (20 mM) was therefore added to each reaction to competitively inhibit this non-specific phosphatase activity. GTP hydrolysis assays were performed in buffer containing 20 mM Tris/HCl, pH 7.5, 100 mM NaCl, 1 mM dithiothreitol, 1 mM $MgCl_2$, Rem or GST–Rem2 protein (1 μg Rem, 10 μg GST–Rem2C), 10 μM [α-^{32}P]GTP, and either 30 mM UTP or 20 mM UTP and 10 mM GTP [20]. Reactions were incubated at 30 °C and 1 μl aliquots were removed at the indicated times and spotted directly on poly(ethyleneimine)-cellulose plates (EM Separations). Chromatograms were developed in 1 M LiCl/1 M formic acid and exposed to X-OMAT AR film (Kodak) for 15 h. The migration of authentic GTP and GDP standards was detected with UV (254 nm). The plate was quantified with a Molecular Dynamics PhosphorImager SF (model 455A). The percentage of GTP hydrolysis was calculated by dividing the amount of radioactivity in the GDP region by that in the sum of the GDP and GTP regions.

Expression of green fluorescent protein (GFP)–Rem and GFP–Rem2 in HT4 cells

The GFP expression vector pEGFP-C1 (Clontech) was used to produce expression constructs encoding wild-type and two distinct C-terminal deletions for Rem and Rem2. The deletion mutants were generated by PCR with specific 3′ oligonucleotide primers for either Rem or Rem2 that introduced stop codons at the indicated position within each cDNA [21]. The identity of the mutant cDNA species was confirmed by DNA sequence analysis.

HT4 cells were initially cultured on laminin-coated coverslips for at least 24 h before use. Cells were transfected by the calcium phosphate method as described previously [16]. Coverslips were harvested, washed in PBS with Ca^{2+} and Mg^{2+} (8 mM Na_2HPO_4/1.5 mM KH_2PO_4/137 mM NaCl/2.7 mM KCl/1.3 mM $CaCl_2$/0.9 mM $MgCl_2$), and then fixed in 3.7% formaldehyde in PBS (containing 1.3 mM $CaCl_2$ and 0.9 mM $MgCl_2$) for 15 min at room temperature. The coverslips were washed three times in PBS and mounted on microscope slides with Vectashield (Vector Laboratories, Burlingame, CA, U.S.A.). The samples were then examined under the appropriate illumination with a 40× objective lens on an E600 microscope (Nikon, Melville, NY, U.S.A.). No epifluorescence was observed in non-transfected cells (results not shown). Slides (35 mm) of the samples were taken with the Nikon U-III camera system. The slides were then scanned with a slide scanner (Nikon) at 600 dots per inch and the Figures were generated with Photoshop 5.0 (Adobe, San Jose, CA, U.S.A.).

Immunoblotting was performed on total cell lysates prepared from 10 cm dishes of cells transiently transfected with GFP

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fusion proteins with Rem or Rem2. The cells were harvested after 48 h, washed twice with 10 ml of PBS, then transferred in 1 ml of ice-cold PBS to a 1.5 ml microfuge tube and centrifuged (Microfuge; Eppendorf) at 12000 ***g*** for 1 min. The cell pellets were resuspended in 200 μl of PBS containing 1 % (v/v) Triton X-100 and lysed with a Kontes probe sonicator (six bursts, each of 10 s duration) on ice. Total lysates (50 μg) were resolved by SDS/PAGE [10 % (w/v) gel] and transferred to nitrocellulose. Immunoblotting was performed with a rabbit anti-GFP polyclonal antibody (Clontech) at 1:100 dilution by using the procedure described previously [22].

RESULTS

During the cloning of the cDNA for the Ras-related GTPase Rem [7], a human expressed sequence tag EST50903 (accession number H19231) was found to be similar to, but clearly different from, that of Rem and other members of the RGK subfamily. The DNA sequence of both strands of the approx. 1100 bp insert from this human cDNA clone was obtained and found to have an open reading frame of 217 residues, which was incomplete at the 5′ end. This partial cDNA clone, which we termed Rem2, was used to generate a radiolabelled probe that was used to screen a human brain cDNA library. However, repeated screening of this library failed to extend the 5′ end of the human cDNA. To identify a full-length cDNA clone, the same radiolabelled probe was used to screen a rat brain cDNA library, from which several cDNA clones were isolated. The largest of these cDNA clones was subjected to DNA sequence analysis and found to encode the entire putative open reading frame of rat Rem2.

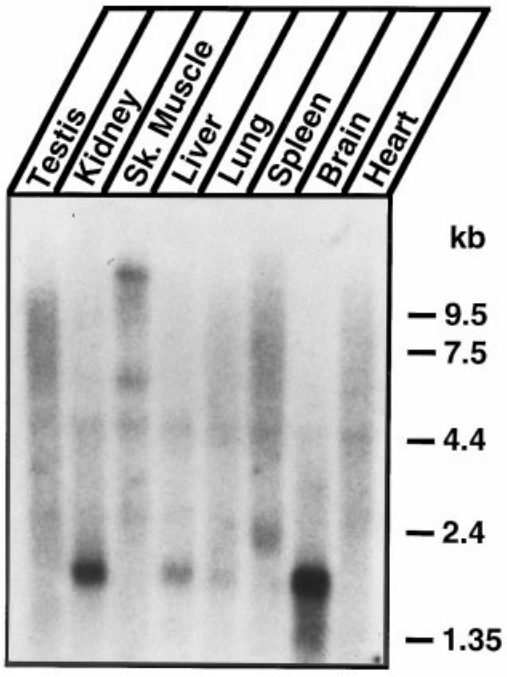

Figure 2 Tissue distribution of mRNA for rat Rem2

Northern blot analysis of poly(A)$^+$ RNA from the indicated rat tissues was performed as described in the Experimental section with a ^{32}P-labelled rat Rem2 probe. The Northern blot was exposed to Kodak X-OMAT-AR film for 42 h at −70 °C with two intensifying screens. The mobility of RNA mass standards is indicated at the right.

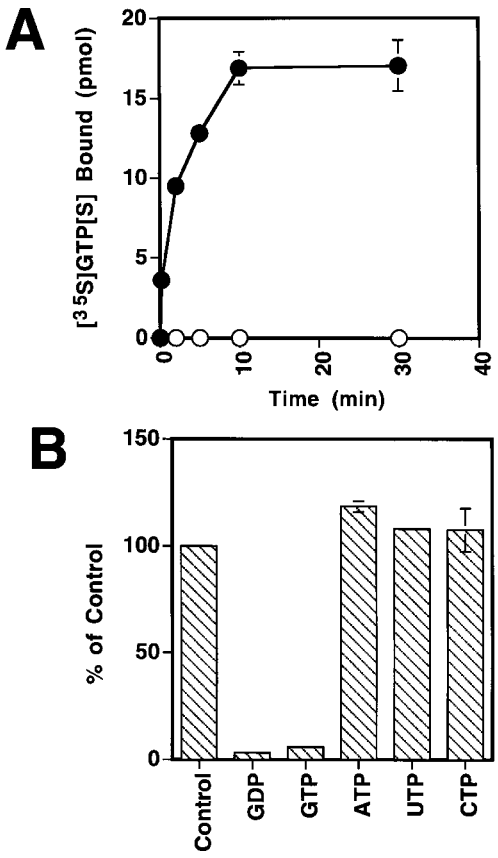

Figure 3 Time course and specificity of binding of [^{35}S]GTP[S] to Rem2

(**A**) GST–Rem2C (10 μg) and [^{35}S]GTP[S] (20 μM) were preincubated in the presence of 1 mM EDTA for 5 min at 22 °C. $[Mg^{2+}]_{free}$ was then adjusted to 10 mM $MgCl_2$ (●) or EDTA adjusted to 10 mM (○) and these reactions were incubated at 22 °C for the indicated durations. The amount of [^{35}S]GTP[S] binding was determined in a filter-binding assay as described in the Experimental section. (**B**) GST–Rem2C (10 μg) was incubated in the presence of 1 mM EDTA, [^{35}S]GTP[S] (20 μM) in the absence (control) or presence of the indicated ribonucleotides (1 mM) for 5 min at 22 °C. $[Mg^{2+}]_{free}$ was then adjusted to 10 mM $MgCl_2$ and the reactions proceeded for 1 h before being subjected to filter-binding assays to quantify bound radioactivity. Each value in (**A**) and (**B**) is the average of duplicate incubations and is representative of three separate experiments.

The deduced amino acid sequences of human and rat Rem2 are 95 % identical. This level of similarity is consistent with both proteins' being orthologues; therefore each is given the name Rem2 (for Rem, Rad and Gem/Kir related protein 2). The putative full-length rat Rem2 cDNA predicts a protein of 272 residues with a calculated molecular mass of 30032 Da. A search of the GenBank and SwissProt databases with the BLAST program revealed significant similarity to the known members of the RGK family. The greatest degree of similarity was with mouse Rad, which is over 54 % identical with human Rem2. However, over 47 % identity was observed with other members of the RGK family (Figure 1). For comparison, alignment with other members of the Ras superfamily revealed that Rem2 was 26–32 % identical with human Rab1A, Rac1, Rap1A or Ha-Ras proteins and 37–39 % identical when aligned with human Rit or Rin. The greatest similarity existed in regions corresponding to the guanine nucleotide-binding domains conserved in all Ras family members. The Rem2 protein exhibited all five of the domains (G1–G5) that have been shown to take part in both guanine nucleotide binding and the catalytic functions of

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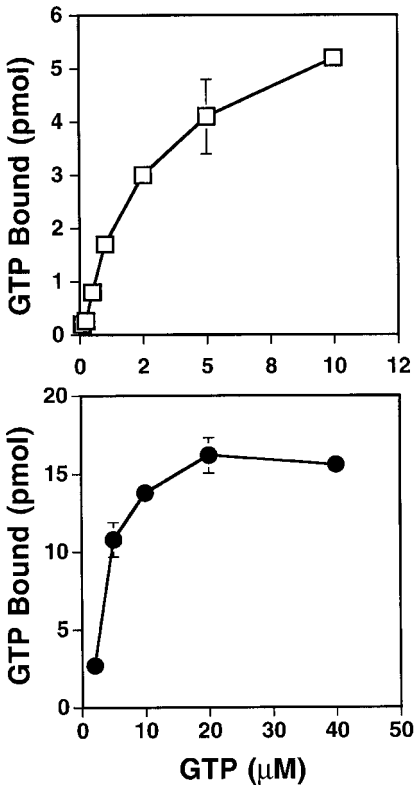

Figure 4 Dose-dependent binding of [^{35}S]GTP[S] to Rem (upper panel) and Rem2 (lower panel)

Rem (1 μg) or GST–Rem2C (10 μg) was incubated in the presence of the indicated amounts of [^{35}S]GTP[S] and 1 mM EDTA for 5 min at 22 °C; $[Mg^{2+}]_{free}$ was adjusted to 1 mM $MgCl_2$ (Rem) or 10 mM $MgCl_2$ (Rem2C) and the reaction proceeded for 10 min at 22 °C. The amount of [^{35}S]GTP[S] binding was determined in a filter-binding assay as described in the Experimental section. Each value is the average of duplicate incubations and is representative of two independent experiments.

the Ras protein superfamily (Figure 1) [1,23]. Although both the N- and C-terminal extensions past the Ras core region are divergent, the C-terminal 10 residues of Rem2 are highly conserved in the RGK family (Figure 1). This region does not contain a typical CaaX, XXCC or CXC prenylation site (where a represents an aliphatic amino acid) present in almost all Ras family members, although it does contain a conserved cysteine residue at position 7 from the C-terminus [11].

The tissue distribution of Rem2 mRNA was examined by Northern blot analysis of mRNA from various rat tissues. A probe derived from the entire coding region of the Rem2 cDNA hybridized with transcripts of approx. 1.9 kb in mRNA from several rat tissues (Figure 2). The highest basal levels of expression were detected in brain and kidney, with low levels of Rem2 mRNA in liver and barely detectable levels in lung, heart, skeletal muscle and kidney. The tissue distribution of Rem2 contrasted with that of Rem, Rad and Gem: Rem mRNA is expressed in cardiac and skeletal muscle, lung and kidney, Gem mRNA is most abundant in kidney, lung, and spleen, and Rad is expressed in cardiac and skeletal muscle and lung [7–10]. Thus Rem2 is the first member of the RGK family to be expressed at significant levels in neuronal tissues.

Although previous studies have demonstrated that Rem and other RGK proteins are GTP-binding proteins, the guanine nucleotide exchange rates for RGK proteins have not been determined and only Rad has been reported to hydrolyse GTP [10,24]. To begin to address these issues, recombinant Rem2 and Rem proteins were expressed in bacteria. The production of full-length Rem2 proved to be quite difficult, resulting in the expression of mostly insoluble recombinant protein (results not shown). However, the removal of 21 C-terminal amino acids from Rem2 and production as a GST fusion protein allowed high levels of expression (results not shown; see the Experimental section). Full-length Rem was produced by thrombin cleavage of a GST–Rem fusion protein as described previously [7]. GST–Rem2C was highly enriched after affinity purification (more than 90% pure) as revealed by SDS/PAGE and staining with Coomassie Blue (results not shown).

Binding of radiolabelled nucleotide to these proteins was assayed by rapid filtration on nitrocellulose filters [7]. As shown

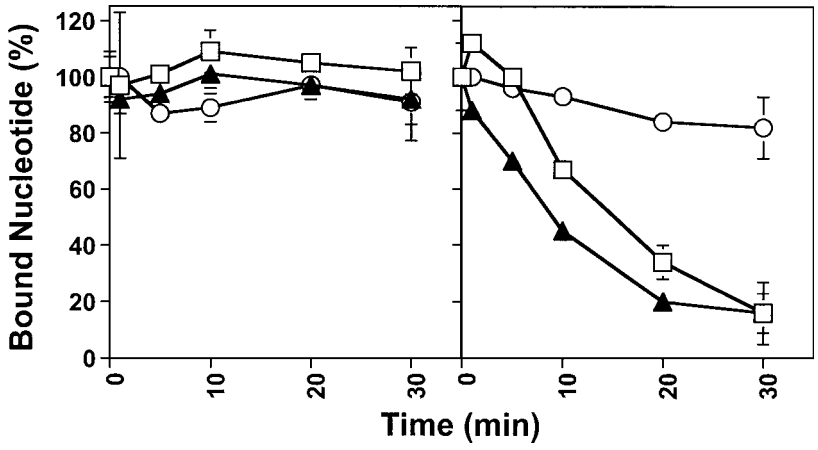

Figure 5 Dissociation of [^{3}H]GDP and [^{35}S]GTP[S] from Rem and Rem2

Rem (1 μg) (□) and either 5 μM [^{3}H]GDP (left panel) or 5 μM [^{35}S]GTP[S] (right panel) were incubated with 1 mM EDTA for 5 min at 22 °C; $[Mg^{2+}]_{free}$ was adjusted to 1 mM and guanine nucleotide binding continued for 10 min as described in the Experimental section. To initiate dissociation, a 100-fold excess of unlabelled GDP or GTP[S] was added and the exchange of radiolabelled nucleotide was measured as described in the Experimental section. The experiment was repeated as described above for GST–Rem2C (10 μg) (▲) and H-Ras (1 μg) (○) except that $[Mg^{2+}]_{free}$ was adjusted to 10 mM to initiate nucleotide binding. Each value is the average of duplicate incubations and is representative of two independent experiments.

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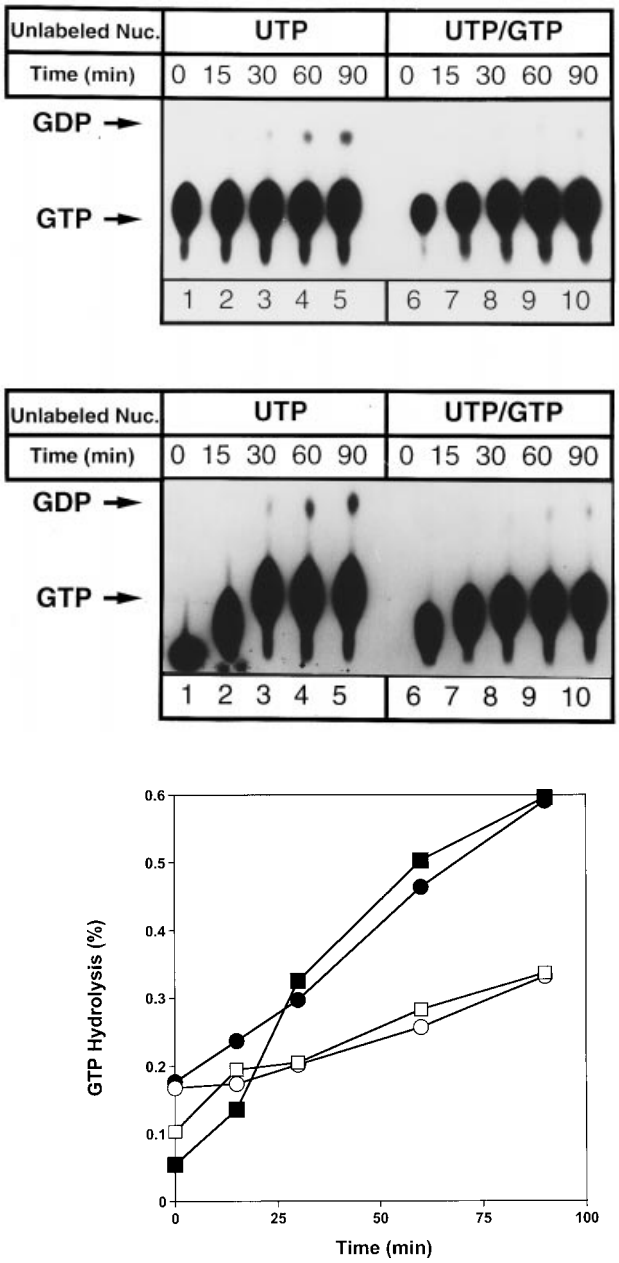

Figure 6 [α-^{32}P]GTP hydrolysis by Rem and Rem2

Rem (top panel) or GST–Rem2C proteins (middle panel) were incubated with [α-^{32}P]GTP (10 μM; 2 Ci/mmol) and 1 mM Mg^{2+} in the presence of either 30 mM unlabelled UTP or 20 mM unlabelled UTP and 10 mM unlabelled GTP. Aliquots were removed from the mixture at the indicated times and analysed by poly(ethyleneimine) TLC plates and autoradiography as described in the Experimental section. The migrations of authentic GTP and GDP standards are indicated. Bottom panel: the chromatography plates were quantified with a PhosphorImager and used to calculate the percentage GTP hydrolysis for Rem (○,●) and Rem2 (□,■) in the presence of either an excess of UTP (●,■) or UTP and GTP (○,□) as described in the Experimental section. The results are representative of three independent experiments.

in Figure 3, Rem2 exhibited a Mg^{2+}- and time-dependent [^{35}S]GTP[S] binding activity. As seen with other GTPases, the association of guanine nucleotides was greatly affected by the concentration of Mg^{2+} ions [19,25]. Replacement of the $MgCl_2$ in the reaction with EDTA completely abolished GTP[S] binding (Figure 3A). Binding of [^{35}S]GTP[S] to both Rem and Rem2 was also dose-dependent and saturable (Figure 4). The ability of various ribonucleotides to compete for [^{35}S]GTP[S] binding to Rem2 was also examined. Rem2 is a specific guanine nucleotide-binding protein because an excess (20-fold) of unlabelled GTP, GDP and GTP[S], but not of CTP, UTP or ATP, competed for [^{35}S]GTP[S] (Figure 3B). These GTP-binding properties closely resemble those of recombinant Rad [24] and Rem proteins [7].

The replacement of GDP by GTP is an essential step in the activation of all Ras-related GTPases, because the GDP-bound form of the enzyme constitutes the inactive conformational state and GDP dissociation is often the rate-limiting step in their activation [23]. The rates for the dissociation of guanine nucleotide from Rem and GST–Rem2C were obtained by prebinding radiolabelled GDP or GTP[S] to the recombinant proteins, adding a 100-fold excess of unlabelled nucleotide and then assaying for the loss of radioactivity at 22 °C as described in the Experimental section. As can be seen in Figure 5, the dissociation curves for both proteins are of single-exponential type, as expected for a single class of nucleotide-binding site. The half-time for [^{3}H]GDP release from both Rem and Rem2 was extremely long and was therefore difficult to assess accurately in this study (half-time more than 60 min). Surprisingly, both proteins released GTP[S] quite rapidly, with a half-time of approx. 15 min. This is in contrast with what is observed for most Ras-related proteins [26]. Under the same reaction conditions, less than 10% of prebound GTP[S] was released from recombinant Ha-Ras. In the absence of Mg^{2+}, the dissociation of both GDP and GTP from Rem and GST–Rem2C was greatly increased (results not shown). Indeed, structural studies indicate that the Mg^{2+} ion is involved in binding the β and γ phosphates of GTP to Ras proteins and that nucleotide exchange rates are often greatly increased in the absence of Mg^{2+} [19,27–29]. Our results suggest that, as with Ha-Ras, the Mg^{2+} ion is exposed to the solvent in the GTP-binding sites of Rem and Rem2.

Alignment of the sequences of the RGK family with other Ras proteins (Figure 1) revealed a number of non-conservative amino acid substitutions within the G1 and G3 regions. These primary-sequence motifs have been well conserved in Ras-related proteins; these amino acid differences within the RGK family include residues which are thought to be involved in GTP binding and hydrolysis, to be involved in stimulation by GAP proteins [30] and to have a role in the conformational change triggered by GTP hydrolysis [1,31]. It was therefore important to determine whether Rem and Rem2 were capable of GTP hydrolysis. The rapid release of GTP from both recombinant proteins precluded the use of single-turnover GTP hydrolysis experiments. We therefore measured steady-state hydrolysis by incubating Rem and GST–Rem2C proteins with [α-^{32}P]GTP and followed GTP hydrolysis by measuring the generation of [α-^{32}P]GDP. As shown in Figure 6, both recombinant proteins displayed a slow, time-dependent GTPase activity that could be inhibited by the addition of non-radiolabelled GTP but not UTP.

To determine the subcellular localizations of Rem and Rem2, we used transient expression of GFP-tagged Rem and Rem2 fusion proteins in HT4 cells. To initiate these studies, a series of GFP fusion proteins with Rem and Rem2 were generated and their expressions were characterized by immunoblot analysis. As shown in Figure 7 (left panel), transfection of wild-type Rem and Rem2 cDNA species led to the production of equivalent amounts of intact GFP fusion proteins as determined by anti-GFP immunoblotting. Truncated versions of both Rem and Rem2 produced amounts of protein similar to those produced by the cDNA species encoding the wild-type fusions. As expected, the size of these GFP-tagged proteins decreased progressively

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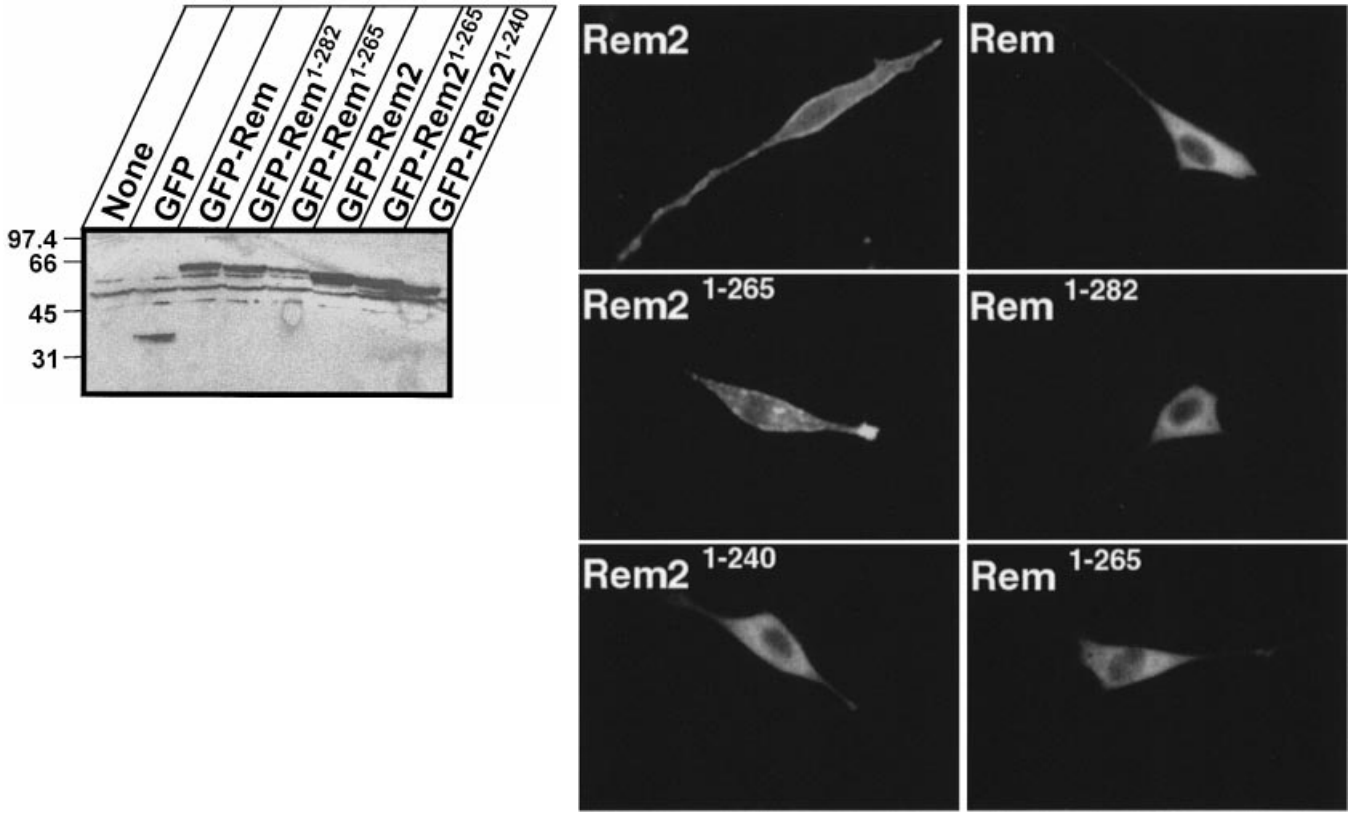

Figure 7 Cellular localization of wild-type and mutant Rem and Rem2 proteins

Left panel: HEK-293 cells transiently transfected with and expressing the indicated GFP fusion proteins were lysed and the total cellular homogenate was analysed by immunoblotting with an anti-GFP polyclonal antibody as described in the Experimental section. The positions of molecular mass standards are indicated (in kDa) at the left. Right panels: HT4 cells were initially cultured on laminin-coated glass coverslips for 24 h and transfected with DNA constructs encoding the indicated GFP-tagged versions of wild-type and mutant Rem and Rem2. At 18 h after transfection, the cells were fixed with 3.7% (v/v) formaldehyde and examined by epifluorescence microscopy as described in the Experimental section. The final images were generated with Adobe Photoshop 5.0.

as the proteins were subjected to C-terminal truncation. To determine the subcellular distribution of these GFP fusion proteins, HT4 cells were first plated on laminin-coated coverslips and transformed with calcium phosphate; 18 h after transfection, the cells were examined by epifluorescence microscopy (Figure 7, right panels). GFP–Rem was distributed uniformly throughout the cytosol. In contrast, the intracellular distribution of GFP–Rem2 was punctate, with the most intense signal at the cell perimeter, indicative of plasma membrane localization (Figure 7, right panels). These distributions were also seen for GFP–Rem and GFP–Rem2 expressed in HEK-293 and PC6 cells (results not shown). Epifluorescence microscopy of HT4 and HEK-293 cells expressing unfused GFP revealed a low but uniform labelling throughout the cell, including signal localized to the nucleus (results not shown). To eliminate the possibility that N-terminal fusion of the GFP protein might alter the subcellular distribution of either Rem or Rem2, the distribution of recombinant proteins bearing N-terminal HA (influenza haemagglutinin) epitopes were assessed by immunostaining with anti-HA antibody. The same intracellular distributions were seen with the HA-tagged versions of Rem and Rem2 expressed in HEK-293 cells (results not shown).

The C-terminal 11 residues of Rem2 are highly conserved in Rem, Rad and Gem/Kir, and are precisely conserved between human and rat Rem2, suggesting a possible role for this domain (termed the C-7 domain [11]) in the function or cellular localization of RGK family proteins. To determine the importance of the Rem and Rem2 C-termini to cellular localization, site-directed mutagenesis was used to delete either the C-7 motif or a larger deletion of 32 C-terminal residues from the C-terminus of each protein. The subcellular distribution of N-terminal GFP-tagged versions of these deletion mutants was then assessed by epifluorescence in HT4 cells. The deletion of either the C7 motif ($Rem^{1–290}$) or the larger C-terminal domain ($Rem^{1–265}$) failed to alter the cytosolic distribution of GFP–Rem mutants (Figure 7). Subcellular location was also not disrupted by the deletion of the C-7 domain from Rem2 (GFP–$Rem2^{1–265}$), because fluorescence remained punctate and seemed to be concentrated at the cell perimeter. However, the deletion of 32 residues from the Rem2 C-terminus resulted in its redistribution to the cytosol, as seen by the uniform distribution of GFP–$Rem2^{1–240}$ in HT4 cells. Deletion analysis therefore maps a critical cellular localization signal to residues 265–282 within the highly basic C-terminus of Rem2.

DISCUSSION

We report here the identification of the Rem2 Ras-related GTPase. Sequence analysis reveals that Rem2 has the highest degree of amino acid identity with RGK proteins, and by virtue of this similarity we include Rem2 as the newest member of this Ras subfamily. The highest degree of similarity between Rem2 and Ras proteins occurs within the core consensus motifs

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(G1–G5) involved in GTP binding and hydrolysis (Figure 1). However, Rem2 contains a unique G2 (effector) domain that diverges greatly from the same region in Rem, Rad, Gem and all other Ras-like GTPases. Effector domains are normally highly conserved within Ras subfamilies but divergence in the G2 (effector) domain is now seen in all RGK family members. This difference is significant because the effector domains of Ras-like GTPases have a critical role in defining effector protein interactions [1] and suggests that each member of the RGK family might interact with a distinct set of effectors and regulators.

Rem2 mRNA was found to have a unique tissue-specific distribution. Northern blot analysis found the message to be highly expressed in brain and kidney, with detectable expression in lung, heart, skeletal muscle and testis (Figure 2). Although this distribution does not suggest a function for Rem2, it is interesting that it has a complementary distribution to those of other RGK family members, being the first to be expressed at highest levels in the brain. All previously characterized members of the RGK family have been shown to be under transcriptional regulation. Studies to determine whether Rem2 is transcriptionally regulated in neuronal tissues are continuing.

Most Ras-related proteins are membrane-associated; membrane localization is both essential and central to their biological activity [32,33]. Membrane binding generally requires the post-translational addition of a C-terminal isoprenyl group, by a mechanism that involves the recognition of conserved cysteine-rich motifs (prenylation) [32]. For Ras proteins, high-affinity membrane binding requires an additional component of binding energy, provided by either a cluster of basic amino acids or the palmitoylation of internal cysteine residues [34,35]. The Arf GTPases contain a highly conserved glycine residue at position 2, which is the site of N-myristoylation and directs their membrane association [36]. One of the distinguishing structure features of RGK-family proteins is the absence of C-terminal prenylation consensus motifs or an N-terminal myristoylation motif. However, the RGK proteins do contain a conserved cysteine residue in the midst of a highly conserved C-terminal motif. This has led to the suggestion that this cysteine residue might lie within a novel lipid modification site (the C7 motif) [11].

The results of the present study, however, suggest that all RGK family proteins are not membrane-targeted and that although structural information important for directing their cellular distribution resides in the C-termini, the C-7 motif does not seem to have a role. Epifluorescence microscopy reveals that Rem is localized to the cytosol when expressed in a variety of cell types, whereas Rem2 has a punctate distribution that is concentrated at the cell perimeter, which is consistent with a plasma membrane localization (Figure 7). Deletion analysis was used to locate the subcellular targeting domain of Rem2. Thus, whereas the deletion of residues 265–272 from full-length Rem2 (deleting the C-7 motif) did not alter cellular distribution, the deletion of residues 240–272 resulted in a complete redistribution to the cytosol. On the basis of these mutants, the C-terminus of Rem2 has a critical role in directing cellular localization and the highly basic 25-residue region encompassing residues 240–265 might be particularly important in directing cellular distribution.

Interestingly, the reported cellular distributions for the highly related Gem and Rad proteins are also quite divergent [9,37]. Immunofluorescence studies suggest that Gem is localized to the cytoplasmic leaflet of the plasma membrane. Removal of the C-terminal 62 residues of Gem resulted in a relocalization of the protein to the nucleus, leading the authors to suggest that the C-7 motif might serve as a site for lipid modification that anchors Gem to the plasma membrane. In contrast, immunofluorescence and biochemical fractionation studies of Rad suggest that it has a largely cytosolic distribution with a portion also found in a Triton X-100-insoluble fraction, which the authors suggest might indicate cytoskeletal association [37]. Removal of the C-terminal 11 residues had no effect on partitioning with Triton X-100, leading to the conclusion that the C-7 motif was not involved in directing cellular localization. Because the final 32 residues of RGK proteins are highly conserved, the differential localization of Rem2/Gem and Rem/Rad is difficult to explain. In this regard our recent studies that have demonstrated phosphoserine-dependent association of Rem and Rad, but not Rem2, with 14-3-3 proteins is intriguing [22]. With the use of deletion mutants, the final 31 residues of Rem were shown to have a critical role in the binding of 14-3-3 proteins. Thus 14-3-3 binding might have a role in regulating the cellular distribution of these proteins. Studies to determine the functional consequences of 14-3-3 binding to Rem are continuing. Clearly, additional studies, including the immunolocalization of endogenous RGK proteins, will be necessary before the nature of the RGK family localization signal can be determined.

Rem2, like other RGK family members, contains unique differences in the G1 and G3 consensus domains relative to other Ras-like proteins. Structural studies and extensive mutagenesis have confirmed the important contributions of residues within these sequence motifs to GTP hydrolysis as well as GTP and Mg^{2+} binding. The most striking difference within the RGK family is the replacement of the G3 consensus DXXG with DXWE. In particular, the substituted glycine residue is thought to have a key role in the conformational change that occurs between the GDP- and GTP-bound forms of the Ras GTPases [29,31]. Thus RGK family members might use a different mechanism of GTP hydrolysis or might undergo a novel conformational change between their GDP- and GTP-bound states. Despite these differences, we have demonstrated that recombinant Rem and Rem2 bind guanine nucleotides in a specific and saturable manner (Figure 4) and that each exhibits a slow intrinsic GTP hydrolysis activity (Figure 6). This suggests that a cellular GAP would be necessary to regulate the nucleotide-state of both proteins. Indeed, recent studies have described a cellular GAP activity for Rad [24].

A surprising finding of the current study was the finding that uncatalysed GTP release was more rapid than GDP dissociation for both Rem and Rem2. The extremely slow rate of GDP release is similar to that seen for Ras and many other Ras-like GTPases [26]. This result suggests that, as for other Ras proteins, a cellular GEF would be necessary to allow the rapid activation of Rem and Rem2 *in vivo*. Although the rate of GTP release for both Rem and Rem2 is also similar to those determined for many Ras family members, it is surprising that it exceeds the rate of GDP release. This property, although uncommon among Ras-related GTPases, has been observed previously for Rit/Rin, R-Ras/TC21 [38] and Arf proteins [39]. For example, GDP release from Arf1 is undetectable in 1 mM Mg^{2+}, whereas 50 % GTP release occurs in 15–20 min under similar conditions [40]. We suggest that this rate might be modified in a cellular context by additional regulatory proteins [6] or lipids as seen with Arf [39]. Thus although Rem and Rem2 have unique biochemical properties, by analogy with previously characterized GTP-binding proteins, it is expected that they would behave as molecular switches in a cellular context whose state of activation would be regulated by putative GEFs, GAPs and guanine nucleotide dissociation inhibitors (GDIs). The future identification of these regulatory molecules as well as additional effector molecules for Rem and Rem2 will be important in clarifying the cellular role of this novel Ras subfamily. These biochemical studies provide important information needed to isolate these factors.

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