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The GTP binding proteins Gem and Rad are negative regulators of the Rho–Rho kinase pathway

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The cytoskeletal changes that alter cellular morphogenesis and motility depend upon a complex interplay among molecules that regulate actin, myosin, and other cytoskeletal components. The Rho family of GTP binding proteins are important upstream mediators of cytoskeletal organization. Gem and Rad are members of another family of small GTP binding proteins (the Rad, Gem, and Kir family) for which biochemical functions have been mostly unknown. Here we show that Gem and Rad interface with the Rho pathway through association with the Rho effectors, Rho kinase (ROK) α and β. Gem binds ROKβ independently of RhoA in the ROKβ coiled-coil region adjacent to the Rho binding domain. Expression of Gem inhibited ROKβ-mediated phosphorylation of myosin light chain and myosin phosphatase, but not LIM kinase, suggesting that Gem acts by modifying the substrate specificity of ROKβ. Gem or Rad expression led to cell flattening and neurite extension in N1E-115 neuroblastoma cells. In interference assays, Gem opposed ROKβ- and Rad opposed ROKα-mediated cell rounding and neurite retraction. Gem did not oppose cell rounding initiated by ROKβ containing a deletion of the Gem binding region, demonstrating that Gem binding to ROKβ is required for the effects observed. In epithelial or fibroblastic cells, Gem or Rad expression resulted in stress fiber and focal adhesion disassembly. In addition, Gem reverted the anchorage-independent growth and invasiveness of Dbl-transformed fibroblasts. These results identify physiological roles for Gem and Rad in cytoskeletal regulation mediated by ROK.

Introduction

Gem (Cohen et al., 1994; Maguire et al., 1994), Rad (Reynet and Kahn, 1993), Rem1 (Finlin and Andres, 1997), and Rem2 (Finlin et al., 2000) are members of a small GTP binding family of proteins within the Ras superfamily, sometimes referred to as the RGK* (for Rad, Gem, and the mouse orthologue of Gem, Kir) family. The basic structure of RGK proteins consists of a Ras-related core, a non-CAAX–containing COOH-terminal extension, and NH_2-terminal extensions. The G3 motifs (DXXG) of RGK proteins are not conserved relative to other small GTPases, consistent with their low intrinsic GTPase activity (Cohen et al., 1994; Finlin et al., 2000). The function of RGK proteins may not be regulated via GTP hydrolysis or, alternatively, there is a unique molecular mechanism for GTPase activating protein–catalyzed GTP hydrolysis relative to other Ras superfamily members. Other potential mechanisms for modulating RGK protein activity have been described, including transcriptional regulation (Maguire et al., 1994; Finlin and Andres, 1997), phosphorylation of the NH_2- and COOH-terminal extensions (Maguire et al., 1994; Moyers et al., 1998; Finlin and Andres, 1999), 14-3-3 binding (Finlin and Andres, 1999), calmodulin binding (Fischer et al., 1996; Moyers et al., 1997), and signal-regulated protein degradation (Zhu et al., 1996).

Although structural features of the RGK family have been known for many years, the physiological role of individual proteins has not been readily forthcoming. Recently, a role for Gem has been proposed in cells expressing voltage-gated calcium channels, such as endocrine and neuronal cells. Gem was reported to down-regulate channel activity as a result of binding the β subunit and thereby inhibiting expression

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*Abbreviations used in this paper: aa, amino acid(s); GFP, green fluorescence protein; GST, glutathione-*S*-transferase; HA, hemagglutinin; LIMK, LIM kinase; MBS, myosin binding subunit; MLC, regulatory myosin light chain; PBD, p21 Rho binding domain; RGK, Rad, Gem, and Kir; ROKβ or ROKα, Rho kinase β or α; SC, synthetic complete.

Key words: Gem; Rad; Rho kinase; myosin light chain; neuroblastoma

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of the α subunit at the plasma membrane (Beguin et al., 2001). However, it is likely that Gem has additional roles. Gem is expressed in cells such as T lymphocytes that do not contain voltage-gated calcium channels, and furthermore, Gem has been indirectly implicated in playing a role in cytoskeletal reorganization. Overexpression of mouse Gem was found to induce invasive pseudohyphal growth in *Saccharomyces cerevisiae* (Dorin et al., 1995). Although there is no apparent Gem orthologue in yeast, this assay most likely reflects the interaction of Gem with a protein common to yeast and mammalian cells. Recently, immunofluorescence and cell fractionation studies have localized a portion of Gem to microfilaments and microtubules (Piddini et al., 2001). Also, Gem expression stimulates cell flattening and neurite extension in human and mouse neuroblastoma cells (Leone et al., 2001).

Other RGK family members are implicated in cytoskeletal interactions as well. Ges, the likely human orthologue of mouse Rem1, and Rem1 were recently described to induce endothelial cell sprouting (Pan et al., 2000). Rad binds β-tropomyosin in skeletal muscle and is associated partially with the cytoskeleton in C2C12 cells (Zhu et al., 1996; Bilan et al., 1998).

Rho family members regulate the dynamic organization of cytoskeletal proteins. As described herein, Gem and Rad bind Rho kinase (ROK), an effector of GTP-bound Rho that mediates a large proportion of the signals from Rho, leading to actinomyosin contractility. RhoA-dependent activation of ROK requires binding via the RhoA effector region and an additional activation function requiring the RhoA insert region (Zong et al., 2001). Two isoforms of ROK exist, referred to as either α and β or II and I, respectively, with an overall identity of 64% that is greatest in the kinase domain (90%) and least in the coiled-coil domain (55%) (Leung et al., 1995, 1996; Ishizaki et al., 1996; Nakagawa et al., 1996). Relatively few functional differences between the two isoforms are known presently. Both isoforms are ubiquitously expressed in tissues, although ROKα predominates in adult brain (Ishizaki et al., 1996; Leung et al., 1996; Matsui et al., 1996). In addition, ROKβ, but not ROKα, is a substrate for caspase-3 during apoptosis, leading to a constitutively active kinase that participates in bleb formation (Coleman et al., 2001; Sebbagh et al., 2001).

Several substrates for ROK are known. ROK controls actinomyosin filament assembly and myosin contractile activity by inducing the phosphorylation of the regulatory myosin light chain (MLC). Increased MLC phosphorylation results directly from ROK-mediated phosphorylation of MLC and indirectly by the inactivation of myosin phosphatase through ROK-mediated phosphorylation of myosin binding subunit (MBS) (Amano et al., 1996; Kimura et al., 1996). MLC phosphorylation is detected after ROK activation and associated with the formation of stress fibers and focal adhesions (Amano et al., 1997, 1998; Chihara et al., 1997; Ishizaki et al., 1997), smooth muscle contraction (Kureishi et al., 1997), and neurite retraction (Amano et al., 1998; Hirose et al., 1998). Other ROK substrates include members of the ezrin/radixin/moesin family, adducin, LIM kinase (LIMK), Na-H exchanger 1, and intermediate filaments, and the phosphorylation state of these proteins appears to be associated with specific cell functions (for review see Amano et al., 2000).

We show here that Gem binds ROKβ and inhibits ROK-mediated MLC phosphorylation. Ectopic Gem or Rad expression inhibits ROK-dependent functions such as formation of stress fibers and focal adhesions, neurite retraction, and Rho-dependent transformation. These data suggest that Gem and Rad perform regulatory functions in cytoskeletal remodeling, perhaps as spatially regulated inhibitors of ROK activity.

Results

Yeast two-hybrid analysis

To gain insight into its biochemical function, we used Gem as bait in a yeast two-hybrid analysis of a human B cell library. A clone encoding a fragment of ROKβ (also called ROCK I) was identified as interacting strongly with Gem and was of particular interest in light of various reports suggesting an association between RGK family members and the cytoskeleton (Dorin et al., 1995; Bilan et al., 1998; Pan et al., 2000). The ROKβ clone (amino acids [aa] 787–1027) spanned the COOH-terminal half of the coiled-coil domain

Table I. **A yeast two-hybrid assay**

	ROKβ regions bound	ROKα regions bound
Gem full-length (2–296)	a, c, e	–
Gem Δc (2–262)	a, c, e	–
Gem core (71–262)	a, c, e	–
Gem(S89N)	a, c, e	–
Rad (41–308)	–	c
Rad Δc (41–274)	a, c, e	–
Rem1 full-length (2–297)	–	–
Rem2 full-length (2–272)	–	–
Rem1 Δc (2–254)	–	–
Rho Q63L	a, c	a, c

Y190 yeast were transformed with combinations of plasmids expressing Gal4 DNA binding domain–GTP binding protein and Gal4 activation domain–ROKα or ROKβ fragments. The letters refer to the diagrammatic representation of the various ROK fragments shown in Fig. 1. At least three independent transformants were assayed in each case. Interactions were considered positive if growth occurred after 3 d at 30°C on selective medium lacking histidine and containing at least 2 mM aminotriazole, and if a blue color was observed within 5 min of initiating an in situ β-galactosidase assay.

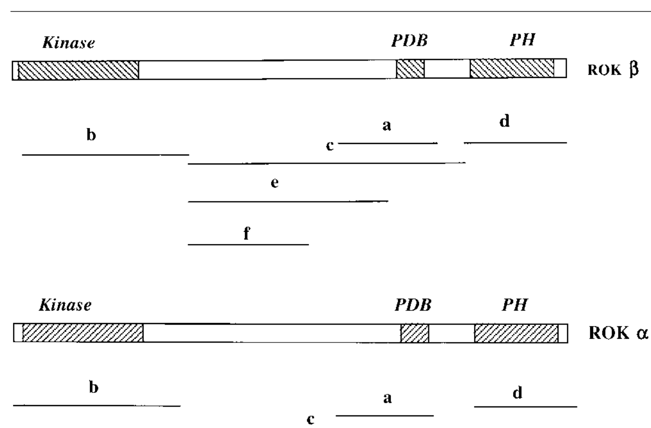


Figure 1. **ROK fragments used in two-hybrid assays and referred to in Table I.** ROKβ: a (787–1027), b (2–421), c (422–1097), d (1098–1354), e (422–933), f (422–727). ROKα: a (807–1056), b (2–438), c (439–1126), d (1127–1388).

Table II. **A yeast two-hybrid assay**

	Gem core	Rad Δc	Rho Q63L
ROKβ (787–1027)	+	+	+
ROKβ (787–1027) [N1004T, K1005T]	+	+	−
ROKβ (787–976)	+	+	−
ROKβ (907–1027)	−	−	+
ROKβ (726–1027, Δ787–906)	−	−	+

Y190 yeast were transformed with combinations of plasmids expressing Gal4 DNA binding domain–GTP binding protein and Gal4 activation domain–ROKβ fragments. ROKβ (787–976) is adjacent to but does not include the p21 binding domain. ROKβ (907–1027) contains the previously defined p21 binding domain. At least three independent transformants were assayed in each case. Interactions were considered positive if growth occurred after 3 d at 30°C on selective medium lacking histidine and containing at least 2 mM aminotriazole and if a blue color was observed within 5 min of initiating an in situ β-galactosidase assay.

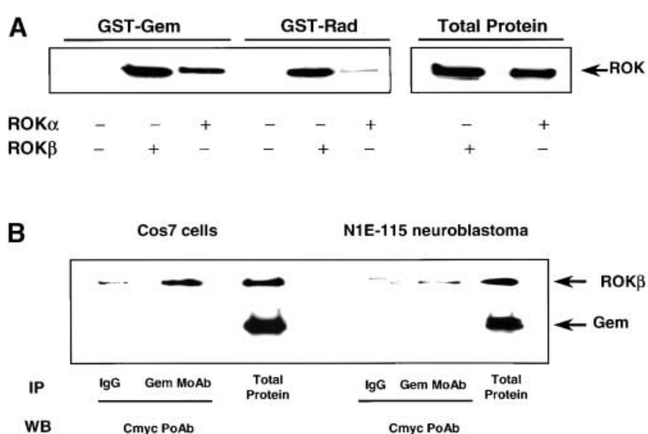


Figure 2. **Interaction of Gem and ROK**. (A) Interactions of Gem and Rad with ROK in vitro. Lysates from Cos cells transiently transfected with myc-tagged full-length ROKα or ROKβ were incubated with purified recombinant GST–Gem (GTPγS) or –Rad (GTPγS) bound to glutathione-Sepharose beads. ROK that cosedimented with Gem or Rad was shown by Western blot analysis with anti-myc antibody. (B) Interaction of Gem and ROKβ in vivo. Cos7 or N1E-115 cells were cotransfected with ROKβ and Gem. Cell lysates were subjected to immunoprecipitation with either anti-Gem monoclonal antibody or mouse IgG as a control for nonspecificity. Coprecipitated ROKβ was revealed by Western blot using anti-myc antibody.

and the p21 Rho binding domain (PBD) (Leung et al., 1996). The interaction of Gem with various regions of ROKβ was tested further in a two-hybrid assay (Table I and Fig. 1). Gem did not bind the PBD, PH/CRD, or NH_2-terminal kinase domains (Table I) but interacted with the coiled-coil domain exclusive of the PBD (Table II). In addition, point mutations introduced at aa 1004 and 1005 in the PBD were shown to inhibit interaction with Rho, whereas Gem binding remained intact (Table II). Therefore, Gem binds to a region of ROKβ adjacent to but distinct from Rho binding, and the interaction of Gem with ROKβ does not require Rho binding.

What are the structural features in Gem required for binding to ROKβ? Deletion of the Gem NH_2- and COOH-terminal extensions, leaving the Ras homology region core, did not prevent interaction with ROKβ (Table I). We constructed Gem(S89N) based upon the conservation of homologous serines at positions 17 in Ras and 89 in Gem. RasS17N is a widely used point mutant with dominant-negative activity. As shown in Table I, Gem(S89N) bound ROKβ in the two-hybrid assay.

An important question is the specificity of the Gem–ROK interaction. Using the yeast two-hybrid system, the interactions of ROKα and ROKβ with the different RGK family members (Gem, Rad, Rem1, and Rem2) were determined. Gem binding appeared to be specific for ROKβ, because various forms of Gem did not bind fragments spanning the length of ROKα. The length of the Rad construct that was assayed effected the binding specificity of Rad. Nearly full-length Rad (41–308) bound the coiled-coil domain of ROKα (439–1126) but did not bind various ROKβ fragments or other ROKα fragments. By contrast, Rad containing a COOH-terminal deletion (41–274) bound ROKβ similarly to Gem (Table I). Neither full-length nor COOH-terminal truncated Rem1 or Rem2 bound either ROK isoform, but full-length Rem1 bound 14-3-3β (unpublished data), as reported previously (Finlin and Andres, 1999).

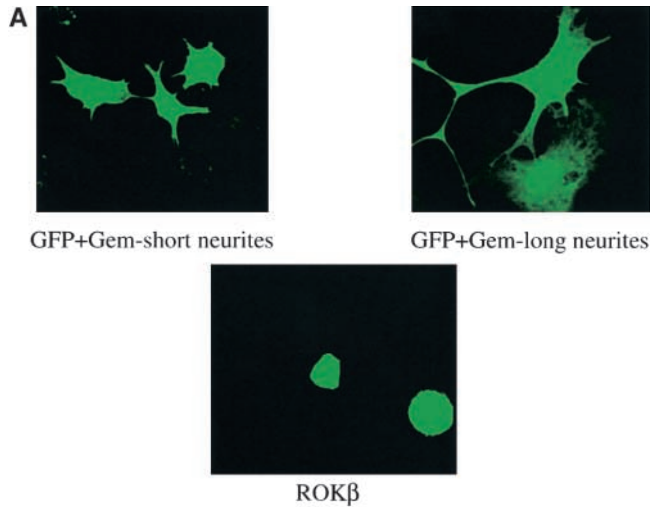
Gem–ROK interaction in mammalian cells

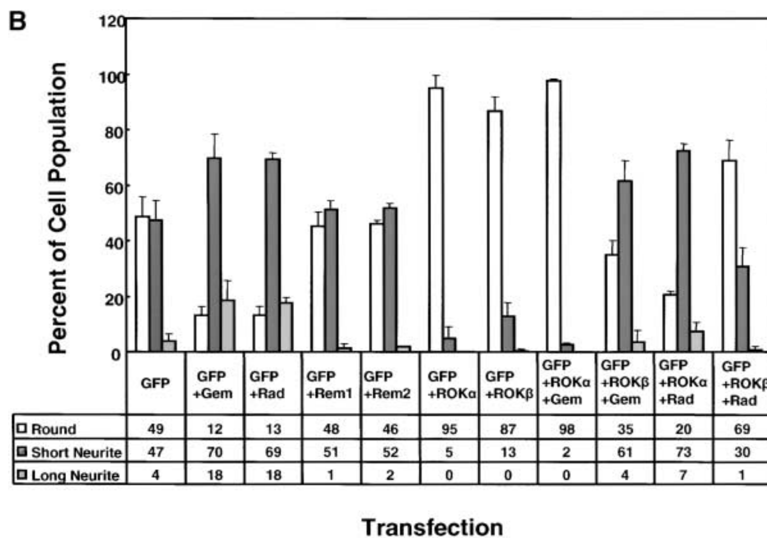
We also investigated the physical association of Gem and Rad with ROK (Fig. 2) and determined that binding can be observed. As shown in Fig. 2 A, recombinant glutathione-*S*-transferase (GST)–Gem and recombinant GST–Rad bound ROKβ and to a lesser extent ROKα in whole-cell extracts derived from transfected COS7 cells. The presence of GTP-γS did not effect the binding efficiency (unpublished data). In addition, coprecipitation of ROKβ with Gem was observed from extracts of either transfected COS7 or N1E-115 cells (Fig. 2 B), consistent with the interaction seen in the yeast two-hybrid analyses.

Neuroblastoma model

The roles of Rho and ROK have been explored extensively in a model of neuronal differentiation using mouse neuroblastoma N1E-115 cells. N1E-115 cells display a heterogeneous morphology with approximately half the cells rounded and the other half slightly flattened. Activation of Rho or ROK is necessary and sufficient to stimulate neurite retraction and extensive cell rounding in N1E-115, whereas, inhibition of ROK has been shown to lead to neurite extension (Hirose et al., 1998). To investigate the functional significance of the Gem–Rad–ROK interactions, we observed the effect of transiently expressing various RGK family members singly or together with ROK isoforms. ROKα and ROKβ as well as Gem and Rad are endogenously expressed in N1E-115 cells (unpublished data).

The functional effect observed after transient transfection of Gem or Rad suggested an inhibition of ROK activity (Fig. 3, A and B). That is, Gem or Rad individually stimulated flattening and neurite extension of N1E-115 cells, a phenotype that is observed after transfection of dominant-negative ROK or with the ROK inhibitor Y-27632 (see Fig. 8, A and B) (Hirose et al., 1998). Correlative with ROK binding in the yeast two-hybrid assay, Gem or Rad displayed functional





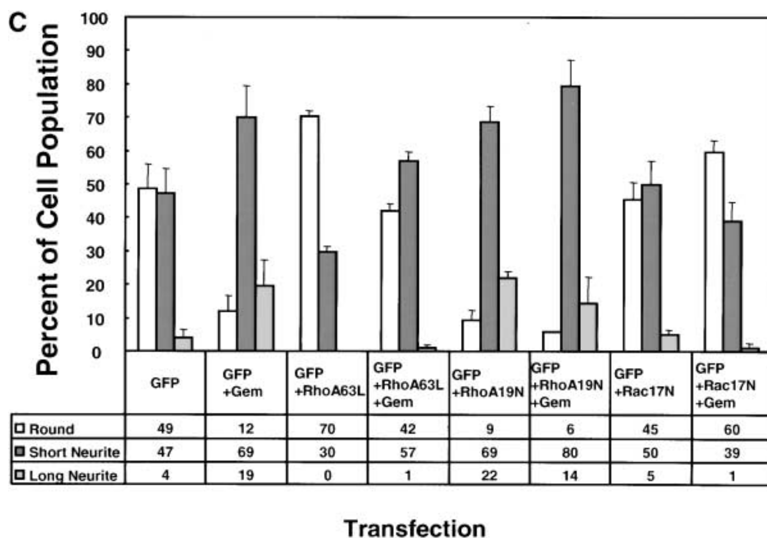


Figure 3. **Effect of RGK family members and ROK on N1E-115 mouse neuroblastoma phenotype.** N1E-115 mouse neuroblastoma cells were cotransfected with pEGFP-N1 and the DNA indicated. The total amount of DNA was normalized with vector DNA. Data presented in the bar graphs with standard deviations are the average of at least four independent experiments. (A) Representative round (ROKβ-transfected), flattened/short neurite (Gem-transfected), and long neurite (Gem-transfected) morphologies in N1E-115 cells. (B) Gem reverses ROKβ-, whereas Rad reverses ROKα-dependent neurite retraction. Rem1 and Rem2 are not involved in neurite induction. (C) Gem expression opposes neurite retraction stimulated by constitutively active RhoA(63L) but has little effect on cell flattening caused by the dominant-negative RhoA(19N). Dominant-negative Rac(17N) prevents Gem from stimulating cell flattening and neurite extension.

activity but Rem1 and Rem2 did not. In addition, an interference assay was used to investigate the specificity of Gem and Rad for the ROK isoforms. Transfection of either ROKα or ROKβ into N1E-115 cells caused enhanced cell rounding (Fig. 3, A and B). Previous investigations have shown that transfected ROK is active in the absence of Rho binding (Leung et al., 1996). Cotransfection of Gem opposed the effects of ROKβ but not ROKα. Surprisingly,

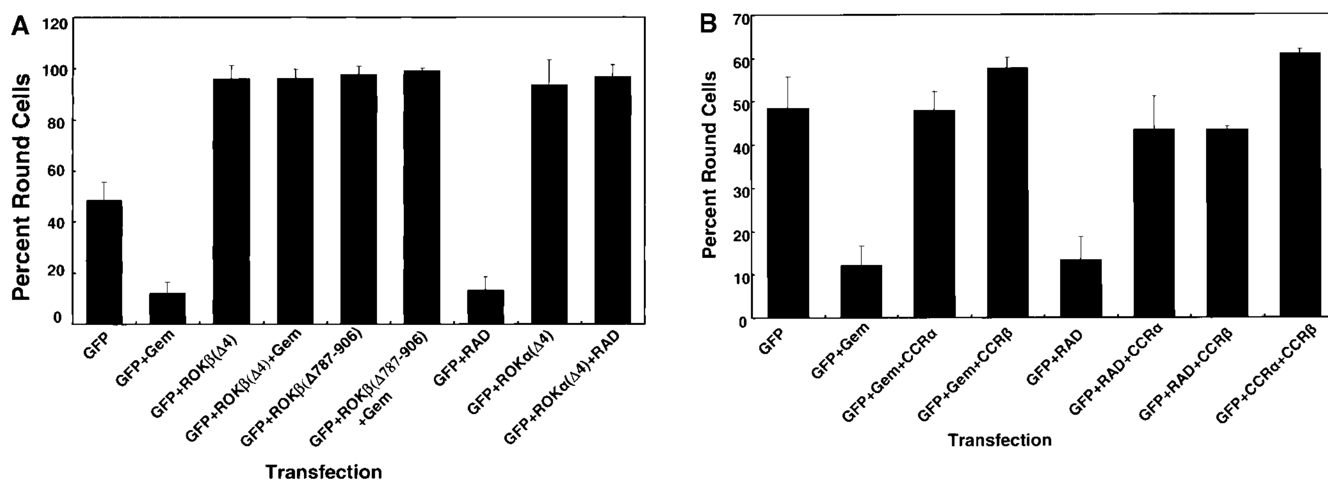


Figure 4. **Gem appears to inhibit ROKβ through a direct interaction.** N1E-115 mouse neuroblastoma cells were cotransfected with pEGFP-N1 and the DNA indicated above. The total amount of DNA was normalized with vector DNA. Data presented with standard deviations are the average of at least three independent experiments. (A) ROK isoforms missing the Gem/Rad binding domains are not inhibited by coexpression of Gem or Rad. (B) Gem- and Rad-induced cell flattening is inhibited by the ROKα (aa 807–1006) and ROKβ (aa 787–976) coiled-coil (CC) isolated domains.

cotransfection of Rad fully reversed the activity of ROKα and only weakly effected ROKβ. Therefore, in N1E-115 cells, Rad appears to have functional specificity for full-length ROKα, as compared with ROKβ. Western blots were used to verify equivalent expression levels for transfected Gem, ROKβ, and ROKα in the various experimental conditions depicted in Fig. 3 (unpublished data). The Gem(S89N) mutant stimulated cell flattening and neurite extension, consistent with its ability to bind ROKβ. Additionally, Gem and Rad expression in N1E-115 inhibited lysphosphatidic acid–induced cell rounding (unpublished data).

Consistent with acting downstream of Rho, Gem opposed the effect leading to cell rounding stimulated by constitutively active RhoA(63L) and had little effect on the cell flattening stimulated by dominant-negative RhoA(19N) (Fig. 3 C). In addition, the enhancement of cell flattening and neurite extension stimulated by Gem are not observed in the presence of dominant-negative Rac(17N), demonstrating a requirement for Rac in the neurite extension observed here.

An important question is whether the inhibitory effects of Gem and Rad on ROK-mediated functions require the interaction of Gem/Rad with ROK, or, alternatively, are an indirect effect. To address this question, we have assayed the ability of Gem or Rad to interfere with cell rounding initiated by ROK mutants missing Gem/Rad binding domains (Fig. 4 A). Constitutively active ROKβ or ROKα truncated shortly after the kinase domain robustly stimulated rounding of N1E-115 cells that was unaffected by Gem or Rad expression (Fig. 4 A), suggesting that Gem/Rad binding to ROK is required for inhibition. An additional ROKβ mutant, ROKβ(Δ787–906) was constructed by deleting the Gem binding domain but leaving other regulatory domains (including the Rho binding domain) intact. This mutant form of ROKβ was stably expressed in COS and N1E-115 cells (unpublished data). As shown in Fig. 4 A, ROKβ(Δ787−906) was effective at mediating neurite retraction that could not be reversed by Gem expression, further supporting the conclusion that Gem and ROKβ interact directly.

To further investigate in a functional assay the binding sites for Gem and Rad on ROKβ and ROKα, tagged coiled-coil domains (787–976 for ROKβ and 807–1006 for ROKα) were expressed alone or in combination with Gem or Rad in N1E-115 cells. As shown in Fig. 4 B, coexpression of the ROKβ or ROKα fragments with Gem or Rad reversed their neurite extension activity, consistent with binding between Gem or Rad and the coiled-coil fragments of ROKβ and ROKα. Because the interference assay showed specificity of Gem for ROKβ and Rad for ROKα, the determinants of specificity between Gem or Rad and the ROK isoforms appear to extend beyond the binding fragments.

We tested the possibility that these Gem and Rad binding domains in the coiled-coil regions of ROKβ and ROKα could act as dominant negatives for endogenous Gem and Rad. Expression of both protein fragments together in N1E-115 cells stimulated a small amount of cell rounding (Fig. 4 B), suggesting that Gem and Rad, in addition to other endogenous proteins, are responsible for maintaining the flattened morphology of these cells.

Recently, Gem was shown to bind the β subunit of L-type Ca^{2+} channels, resulting in reduced channel activity due to decreased α1 subunit expression at the plasma membrane (Beguin et al., 2001). Because N1E-115 cells express L-type channels, we investigated whether inhibition of channel activity using nitrendipine would lead to the morphological alterations induced by Gem. Green fluorescence protein (GFP)– or GFP–Gem-transfected N1E-115 cells were treated with nitrendipine (1, 5, 10, or 50 μM) for 24 h before being scored for neurite extension. Nitrendipine had no effect upon the distribution of morphological phenotypes in either GFP- or GFP–Gem-transfected cells (unpublished data), suggesting that inhibition of channel activity plays no role in the morphological differentiation described here.

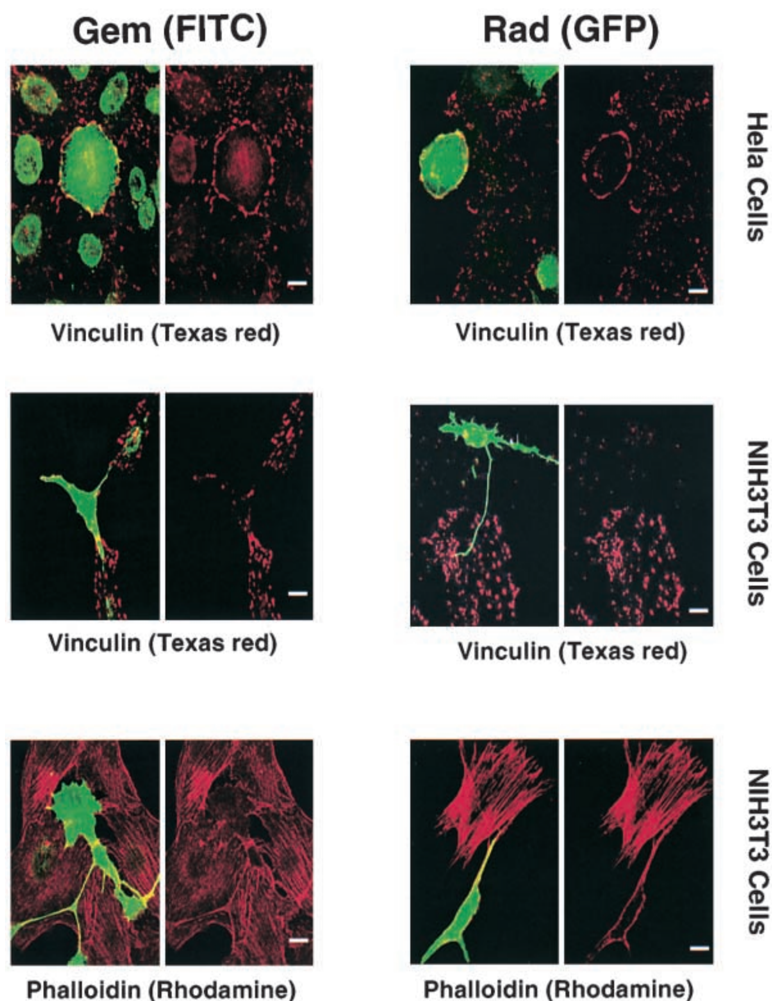


Figure 5. **Effect of Gem and Rad on the actin cytoskeleton.** HeLa and NIH-3T3 cells were transfected with Gem or cotransfected with pEGFP-N1 and Rad. The cells were analyzed via confocal microscopy. Gem was stained with polyclonal anti-Gem antibody and FITC-conjugated goat anti–rabbit. Focal adhesions were visualized by staining with antivinculin antibody and stress fibers were identified with rhodamine-conjugated phalloidin.

The effect of Gem upon the actin cytoskeleton

ROK has been shown to play a fundamental role in the regulation of the actinomyosin cytoskeleton, including the formation of stress fibers and focal adhesions. Therefore, we analyzed the effect of Gem and Rad expression in epithelial cells and fibroblasts on the cytoskeleton as judged by staining for F-actin and vinculin (Fig. 5). As shown for HeLa cells in Fig. 5, transient Gem or Rad overexpression inhibited the presence of focal adhesions in the main cell body while leaving peripheral focal complexes intact, in agreement with previous reports that ROK activity is required for the maintenance of central but not peripheral focal contacts (Totsukawa et al., 2000). Gem or Rad overexpression in fibroblasts often induced an unusual dendritic morphology (Fig. 5) characterized by abnormal cellular elongation or the presence of branching filopodial structures and rounding or retraction of the cell body. Gem or Rad expression was accompanied by loss of central but not peripheral focal contacts and loss of stress fibers. In addition, enhanced lamellipodia formation was evident in Gem-transfected cells (unpublished data). Interestingly, a dendritic morphology is induced in BALB/c 3T3 cells after prolonged inhibition of RhoA or ROK (Hirose et al., 1998). Low levels of Gem expression generally did not result in loss of focal adhesions or stress fibers or induction of a dendritic morphology, possibly as a result of residual ROK activity.

The effect of Gem upon Rho-dependent transformation and invasion

Rho-dependent transformation has been shown to require ROK signaling for its establishment and maintenance (Sahai et al., 1998). As one example, 3T3 cells transformed by Dbl (a Rho guanine nucleotide exchange factor) are inhibited in anchorage-independent growth by the ROK inhibitor Y-27632 (Sahai et al., 1999). Therefore, we investigated the effect of constitutively expressed wild-type and mutant (S89N) Gem on the ability of Dbl-transformed 3T3 cells to grow in soft agar. Polyclonal expression of Gem reduced the ability of Dbl-transformed cells to produce colonies in soft agar by ~60% (Fig. 6 A), consistent with the inhibition of ROK activity by Gem. Inhibition was observed for both wild-type and mutant (S89N) Gem, in accordance with their similar functional activities in neuroblastoma cells. Gem expression did not affect the growth rate of the Dbl-transformed cells (unpublished data).

ROK has been shown to play a role in Rho-dependent invasion of hepatocellular carcinoma (Genda et al., 1999; Itoh et al., 1999). We analyzed the Dbl-transformed 3T3 cells for their invasive capacity through a matrigel barrier and the effect of Gem coexpression on this activity. As shown in Fig. 6 B, after transformation by Dbl, 3T3 cells greatly increase their invasiveness through an extracellular matrix that is inhibited ~90% by coexpression of Gem or Gem(S89N).

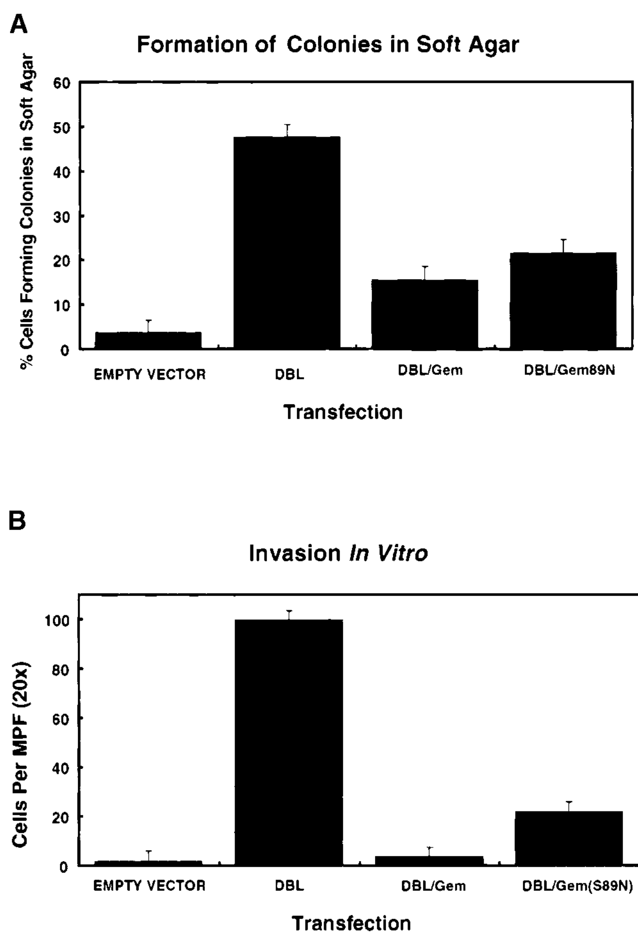


Figure 6. **Gem inhibits Rho-dependent transformation and invasion.** NIH-3T3 cells were permanently transfected with Dbl and further cotransfected with Gem or Gem(S89N). (A) The ability of the cells to form colonies in soft agar (average of three experiments shown) and (B) the invasiveness of the cells through matrigel (results are the average of five microscopic fields with background subtracted) were determined.

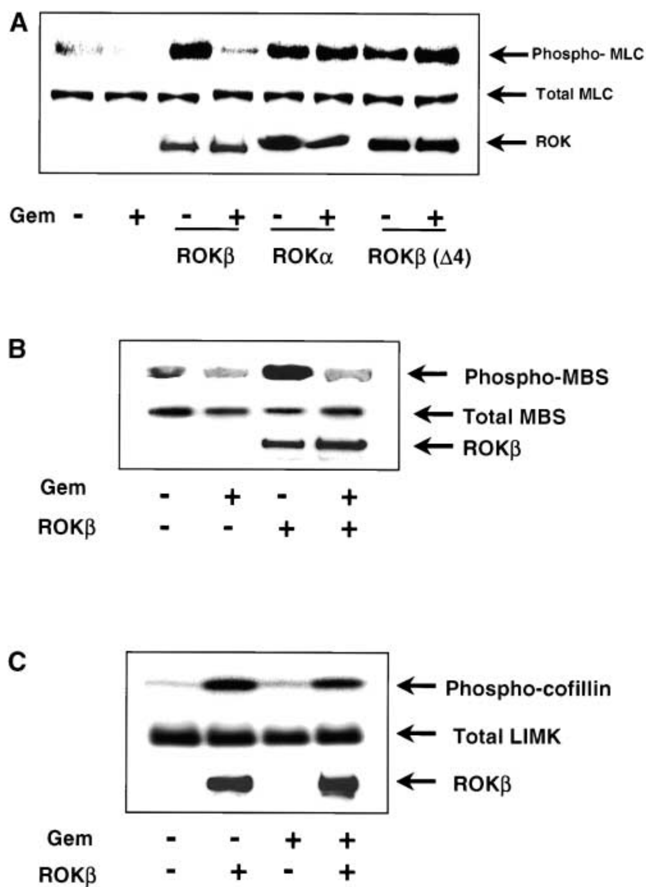


Figure 7. **Effect of Gem on ROK activity.** (A) Gem inhibits ROKβ- but not ROKα-dependent phosphorylation of MLC. Cos7 cells were transfected with flag-tagged MLC and myc-tagged ROKα, ROKβ, or the constitutively active kinase domain of ROKβ. The ability of Gem to inhibit MLC phosphorylation was demonstrated by cotransfection with full-length wild-type Gem. Western blots generated with antibodies specific for MLC phosphorylated on serine 19, total transfected MLC detected with antiflag antibodies, or ROK detected with anti-myc antibodies are shown. (B) Gem-mediated inhibition of MLC phosphatase phosphorylation by ROKβ was demonstrated by transfection of the MBS into Cos7 cells together with ROKβ and/or Gem. Western blots generated with antibodies specific for MBS phosphorylated on threonine 695 as well as total MBS and ROK are shown. (C) Gem has no effect on ROKβ-dependent LIMK activation. HA-tagged LIMK was cotransfected into Cos7 cells with ROKβ and/or Gem and then immunoprecipitated using anti-HA antibody. An in vitro kinase assay was performed using cofilin and [γ^{32}P]ATP as substrates for the phosphoryl transfer. Phosphorylated cofilin was revealed by autoradiography and ROK protein levels were assayed by Western blots.

Biochemical analyses

An important question is the mechanism of action whereby Gem functionally opposes ROKβ. We considered the possibilities that Gem (a) inhibits ROK kinase activity or (b) redirects ROK localization and/or substrate specificity. We have obtained no evidence suggesting a direct effect of Gem upon the kinase activity of ROKβ. For example, no change in the level of in vitro kinase activity was observed in immunoprecipitated ROKβ relative to the presence or absence of coexpressed Gem (unpublished data). Therefore, in order to test the second possibility, the effect of Gem expression upon the in vivo activity of ROKβ was investigated in COS cells for the substrates MLC, MBS, and LIMK. ROK-dependent phosphorylation of MLC and MBS was assayed with phosphospecific antibodies. LIMK phosphorylation was measured indirectly by an immune complex kinase activity assay using cofilin as the substrate. As shown in Fig. 7 A, ROKβ stimulated increased phosphorylation of MLC, which was reversed in the presence of coexpressed Gem. ROKα-mediated phosphorylation was unaffected by Gem as was phosphorylation mediated by the kinase domain of ROKβ in the absence of the Gem binding region. Similarly, ROKβ-dependent phosphorylation of MBS was inhibited by Gem coexpression (Fig. 7 B). By contrast, as shown in Fig. 7 C, immunoprecipitated LIMK demonstrated a ROK-dependent increase in cofilin-directed kinase activity, which was essentially unaffected by coexpressed Gem. Therefore, Gem had a selective effect on ROK-mediated phosphorylation, inhibiting MLC and myosin phosphatase phosphorylation, consistent with the opposition by Gem of ROK-activated acti-

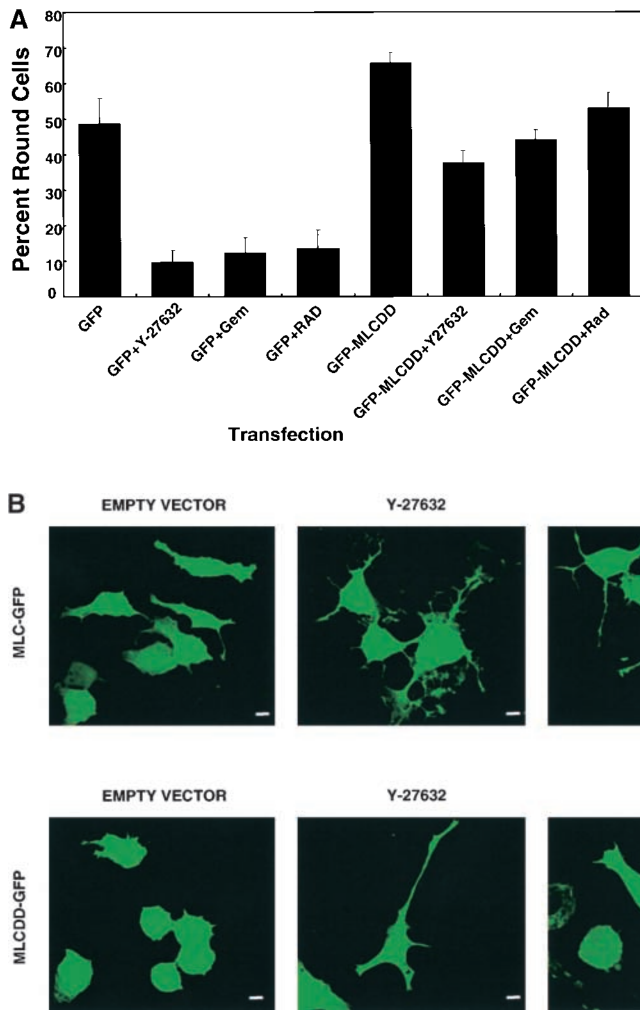


Figure 8. **Effect of constitutively active MLC on ROK inhibition in N1E-115 neuroblastoma.** NIE-115 cells were transfected with GFP-tagged MLC or activated MLC, MLC(18D,19D), along with Gem and/or Rad. Y-27632 was used as a control for ROK inhibition. The data presented with standard deviation are the average of at least four experiments. (A) Percent of cells with round phenotype was determined by microscopic examination of transfected cells. (B) Confocal scans of representative cells were generated using a ZEISS LSM 510.

nomyosin contractility. These data suggest that Gem most likely differentially modifies the access of ROK to its substrates.

Induction of neurites by Gem in N1E-115 cells can be partially reversed by an activated form of MLC

To determine whether the inhibition of MLC phosphorylation was principally responsible for the flattening and neurite extension observed in Gem- and Rad-transfected N1E-115 cells, we sought to reverse the Gem/Rad effect using a mutant MLC (T18D,S19D), a mimetic of phosphorylated MLC. MLC(T18D,S19D) has been shown to lead to the activation of myosin ATPase and a conformational change of myosin II when reconstituted with myosin heavy chains in vitro (Ikebe and Hartshorne, 1985). As shown in Fig. 8 A, MLC(18D,19D)–GFP expression enhanced cell rounding relative to GFP alone (66 as compared with 49%), consistent with previous findings (Amano et al., 1998). The number of rounded cells observed after Gem or Rad transfection alone (13 or 13%, respectively) or Y-27632 treatment alone (10%) was substantially increased (∼45%) upon MLC(18D,19D) expression. In addition, the morphology of the flattened cells induced by ROK inhibition was clearly different in the presence of wild-type MLC–GFP as compared with MLC(18D,19D)–GFP. As shown in Fig. 8 B, MLC–GFP cells cotransfected with Gem or treated with Y-27632 (Fig. 8 B) produced highly branched neurites, whereas MLC(18D,19D)–GFP cells coexpressing Gem (Fig. 8 B) or Rad (unpublished data) or treated with Y-27632 produced flattened bipolar cells. Therefore, cell flattening and neurite extension resulting from ROK inhibition by Gem/Rad or Y-27632 can be partially reversed with MLC(18D,19D) expression.

Discussion

In the present study, we have identified a functional interaction of Gem and Rad with ROK, leading to an inhibition of ROK-mediated actinomyosin-dependent contractility. Using the yeast two-hybrid approach, Gem was shown to interact with ROKβ in the coiled-coil region, adjacent and amino-terminal to the Rho binding domain. The interaction of Gem with ROKβ occurred independently of Rho (Tables I and II). Gem coexpression in cells inhibited ROKβ-dependent phosphorylation of MLC and the MBS of myosin phosphatase, events that would be predicted to work cooperatively in inhibiting contractility of the actinomyosin cytoskeleton. Consistent with this, ectopic Gem expression resulted in physiological changes, suggesting an inhibition of endogenous ROK function, including the stimulation of cell

flattening and neurite extension in N1E-115 cells, loss of stress fibers and focal adhesions in fibroblasts and epithelial cells, and inhibition in 3T3 cells of Rho-dependent anchorage-independent growth and invasion. In addition, interference assays demonstrated Gem inhibition of ROKβ- but not ROKα-mediated neurite retraction in N1E-115 cells (Fig. 3 B) and actin filament bundling in HeLa cells (unpublished data). Furthermore, the ability of Gem to interfere with ROKβ-mediated neurite retraction was dependent upon the presence of the Gem binding domain in ROKβ, demonstrating that the functional effects of Gem are a result of its binding to ROK (Fig. 4 A).

There are many parallel functional effects of Gem and Rad expression, such as the stimulation of neurite extension and loss of stress fibers and focal adhesions, that suggest inhibition of endogenous ROK-dependent actinomyosin contractility. Although a COOH-terminal truncated Rad was found to bind ROKβ in the yeast two-hybrid assay, interference assays with full-length Rad and ROKα or ROKβ indicated a specificity of Rad for ROKα in the neurite retraction assay (Fig. 3 B) or in an actin fiber bundling assay in HeLa cells (unpublished data). The interference data suggest that the interaction of Rad with ROK isoforms in mammalian cells may be tightly regulated.

The specificity of Gem for ROKβ was observed with regard to binding in the yeast two-hybrid system, biochemical assays, and functional assays. The significance of this specificity is currently unknown. To date, relatively few distinctions between potential ROKα and ROKβ functions have been described. One exception is that ROKβ is sensitive to apoptosis-induced caspase-3 cleavage and subsequent constitutive activation, leading to membrane blebbing (Coleman et al., 2001; Sebbagh et al., 2001). The caspase cleavage site in ROKβ is located at positions 1110–1113, COOH-terminal to the Gem binding region, suggesting that Gem could influence the outcome of caspase-3 regulation of ROKβ.

After the introduction of exogenous ROK, Gem inhibits ROK-dependent phosphorylation of MLC and myosin phosphatase in situ, but does not appear to inhibit ROK-dependent activation of LIMK, implying substrate specificity to the inhibitory function of Gem. Such specificity suggests that Gem is not affecting a generalized regulatory function for ROK, such as Rho binding. Thus, the effects of Gem on transfected ROK appear independent of Rho binding. Although it seems unlikely, we have not formally demonstrated that the loss of actinomyosin contractility, which occurs in the presence of Gem after activation of the Rho pathway, could not involve an effect of Gem upon Rho binding to endogenous ROK.

The in vitro kinase activity of immunoprecipitated ROK directed against purified MLC or meromyosin is unaffected by Gem coexpression (unpublished data), suggesting that Gem does not induce an inhibitory covalent modification of subsequently purified ROK. Gem may selectively effect the substrate specificity of ROK as a result of being localized in the cytoskeletal fraction (Piddini et al., 2001), a possibility supported by the association of ROK and myosin phosphatase with isolated stress fibers containing phosphorylation-competent MLC (Kawano et al., 1999; Katoh et al., 2001). Alternatively, Gem binding may obscure a region in ROK that plays a role in substrate-specific binding. Inhibition of ROK activity toward selective substrates by Gem provides a means for fine tuning the response of cytoskeletal components to ROK and by extension, Rho activation.

Although Rad expression mimics the functional effects of Gem with regard to inhibiting actinomyosin contractility, it has not been possible to assign a biochemical mechanism similar to that of Gem. Cotransfection of ROK and MLC with Rad resulted in the rapid turnover of MLC protein (unpublished data), the physiological significance of which merits further investigation.

Ectopic expression of Gem or Rad in fibroblasts or epithelial cells resulted in a loss of stress fibers and focal adhesions but not peripheral focal complexes, consistent with previous reports of the cytoskeletal organization observed after treatment with the ROK inhibitor Y27632 (Rottner et al., 1999). Peripheral focal complex formation has been shown to be regulated by Rac activation (Nobes and Hall, 1995) and dependent upon myosin 2 contractility, whereas focal complex maturation into focal adhesions is ROK dependent (Rottner et al., 1999). MLC phosphorylation at the cell periphery appears to be regulated by MLC kinase (Totsukawa et al., 2000) but not ROK. Thus, it has been suggested that ROK plays an important role in maintaining cytoplasmic or tonic tension within both smooth muscle (Katoh et al., 2001) and nonmuscle cells (Totsukawa et al., 2000). The dendritic morphology of fibroblasts induced by Gem or Rad probably results in part from a loss of cytoplasmic tension and rounding within the cell body while maintaining adhesion along the cell periphery.

We have observed increased lamellipodia formation in Gem-expressing cells, indicating that Rac activity may be increased. A mutual antagonism between Rac and Rho pathways has been previously proposed in studies on the regulation of neurite extension in neuronal cells (Leeuwen et al., 1997; Hirose et al., 1998) and actin filament reorganization in fibroblasts (Moorman et al., 1999). It will be interesting to determine whether the inhibition of ROK by Gem is accompanied by an increase in Rac activity.

What is the expected biological role of Gem and/or Rad with regard to regulating actinomyosin contractility? The actin cytoskeleton is central to such cellular processes as neurite extension, substrate adhesion, motility, secretion, cellular polarization, and cell cleavage (Carpenter, 2000). Dynamic processes such as motility and secretion cycle through periods of assembly and disassembly of the actin cytoskeleton. For example, in some cells, movement and positioning of exocytotic granules requires an intact cytoskeleton, whereas cortical F-actin disassembly appears to be a prerequisite for juxtamembrane apposition of granules and exocytosis (Muallem et al., 1995; Lang et al., 2000).

The data presented here demonstrating that Gem(S89N) is functional in ROK inhibition suggest that the effect of Gem upon ROKβ function is regulated by a mechanism other than differential GTP or GDP binding. Gem expression is highly responsive to various signaling pathways (Leone et al., 2001), and both Gem and Rad proteins are potentially regulated by not only GTP binding but phosphorylation (Maguire et al., 1994; Moyers et al., 1998; Finlin and Andres, 1999) and binding to other proteins such

as 14-3-3 (Finlin and Andres, 1999) and calmodulin (Fischer et al., 1996; Moyers et al., 1997). In summary, Gem and Rad provide a mechanism for localized signal-responsive regulation of the Rho–ROK-mediated assembly and contraction of the actin cytoskeleton.

Increased Gem protein levels have been shown to be associated with ganglionic differentiation of neuroblastoma in vivo (Leone et al., 2001), and ectopic Gem expression stimulates neurite extension in vitro (Fig. 3 A and Fig. 7 B), consistent with a potential role for Gem in morphological regulation of neurites/dendrites. Also, recently, binding of the β subunits of L-, P/Q-, and N-type voltage-gated calcium channels to Gem was shown to inhibit their transport to the plasma membrane (Beguin et al., 2001). Interestingly, Rho and ROK have been reported to control the intracellular localization of the water channel aquaporin-2 via regulation of the F-actin cytoskeleton (Klussmann et al., 2001). Inhibition of Rho or ROK induces translocation of aquaporin-2 to the plasma membrane, a process that is normally stimulated by vasopressin and cAMP production. It will be interesting to determine whether actin filament dynamics and/or regulation of ROK play a role in Gem-regulated transport of the β subunit.

Materials and methods

Plasmids and antibodies

Affinity-purified antiphosphoserine 19 MLC polyclonal antibody (Matsumura et al., 1998) was from Fumio Matsumura (Rutgers University), and affinity-purified antiphosphothreonine 695 MYPT1 polyclonal antibody ($pM1333^{T695}$) (Feng et al., 1999) was supplied by Masaaki Ito (Mie University School of Medicine). pEFBos-myc–ROKα was obtained from Kozo Kaibuchi (Nagoya University Graduate School of Medicine, Aichi, Japan) and pCAG-myc–ROKβ and pCAG-myc–ROKβ(Δ4) were from Shuh Narumiya (Kyoto University, Kyoto, Japan). pCAG-myc–ROKβ(Δ787–906) was made as follows. PCR was used to generate fragments with complementary ends encoding ROKβ (aa 683–786) and ROKβ (aa 907–1027). Recombinant PCR was used to generate 683(Δ787–906)–1027, which was subsequently cut with XhoI and SphI and used to replace the wild-type fragment between the 5′ XhoI and SphI sites of pCAG-myc–ROKβ. The construct was sequenced and determined to be unchanged outside the deleted region.

The pEGFPN1-MLC and pCMV-flag–MLC constructs were made using T7-7-MLC from Kathy Trybus (University of Vermont, Burlington, VT) as a template. Quik Change site-directed mutagenesis (Stratagene) was used to generate the pEGFPN1-MLC(18D,19D) mutant. PGex2T-Rad was a gift from Ron Kahn (Joslin Diabetes Center, Boston, MA) and pEFBos-Rad was from James Lenhard (Glaxo Wellcome Inc., Research Triangle Park, NC). PMT2T-Rem1 and -Rem2 were generated by PCR cloning using pGexKG-Rem1 and -Rem2 (Douglas Andres, University of Kentucky, Lexington, KY) as templates. pLEGFPN1-M133 was a gift from David Hartshorne (University of Arizona, Phoenix, AZ), and pUCD2-3xHA–LIMK1 and $pQE60Amp^r$-His–cofilin were from Kensaku Mizuno (Tohoku University, Sendai, Japan). pCEV-RhoA63L, -RhoA19N, and -Rac17N were obtained from Silvio Gutkind (National Cancer Institute, Bethesda, MD) and CTV-Dbl was from Geoff Clark (National Cancer Institute).

Yeast two-hybrid analysis

All Gal4 DNA binding domain fusions were generated by cloning into pGBT9 (CLONTECH Laboratories, Inc.). *S. cerevisiae* strain Y190 (obtained from Stephen Elledge, Baylor College of Medicine, Houston, TX) was sequentially transformed with the pGBT9 full-length Gem bait vector and a human Raji cDNA library in the GAL4 activation domain vector pACT1 (CLONTECH Laboratories, Inc.) according to the protocols described for the MATCHMAKER yeast two-hybrid system (CLONTECH Laboratories, Inc.). Transformants were plated on synthetic complete (SC) plates lacking Trp, Leu, and His for ~3 d. Colonies were rescreened for expression of the lacZ marker after lifting onto nitrocellulose filters. 5 million colonies were plated, and 3/27 clones that specifically interacted with Gem were identical to ROKβ. For further two-hybrid analyses, DNA fragments were inserted into derivatives of pGBT9 and pGAD424. Yeast were cotransformed by pairs of binding and activation domain plasmids, selected on Trp-, Leu-deficient SC plates, and subsequently patched to Trp-, Leu-, His-deficient SC plates containing 2, 25, or 50 mM 3-aminotriazole. Interactions were assessed by growth after 48 h, and β-galactosidase activity was measured after transfer to nitrocellulose filters.

Immunofluorescent staining and confocal microscopy

Exponentially growing cells were plated on glass coverslips (A. Daigger & Co.) in 24-well cell culture dishes and incubated overnight at 37°C and 5% CO_2. The next day, cells on each coverslip were transfected with 0.05 μg pEGFP-N1 and 0.5 μg pEFBos-Rad or 0.5 μg PMT2T-Gem using Lipofectamine Plus (Invitrogen). 24 h later, cells were fixed with 4% paraformaldehyde for 10 min at room temperature, rinsed three times with PBS, and permeabilized with 1% Triton X-100 in 0.02% BSA–PBS for 2 min at room temperature. Cells were blocked in 20% goat serum containing 2% BSA–PBS for 20 min at 37°C. Cells transfected with Gem were incubated for 1 h at room temperature with polyclonal anti-Gem antibody. One half of the coverslips transfected with Gem or Rad were then incubated with monoclonal antivinculin antibody (Sigma-Aldrich) for 1 h at room temperature, rinsed three times with PBS, and incubated at room temperature for 30 min with Texas red-X–conjugated goat anti–mouse antibody (Molecular Probes). The other half of the coverslips were incubated with rhodamine–phalloidin (Molecular Probes) for 30 min at room temperature. All antibodies were diluted in 2% goat serum in 2% BSA–PBS. After three more rinses with PBS, coverslips were inverted into 7 μl of mounting medium containing antifade agents (Biomeda Corp.) and were allowed to dry at room temperature in the dark. Stained cells were examined on a ZEISS Axioplan microscope equipped with a 100×/1.4 oil immersion objective. Confocal images were generated using an LSM 510 scanning laser microscope (ZEISS).

N1E-115 neuroblastoma morphology assay

Neurite remodeling was assayed as previously described (Leone et al., 2001). ROK and Gem expression were assayed by Western blots to determine relative levels in samples compared for morphology. Data presented are the average of at least four independent experiments. ROK inhibitor Y-27632 was obtained from Hiroyuki Sueoka (Welfide Corp, Osaka, Japan). Cells were treated with 10 μM of Y-27632 for 30 min to inhibit ROK. Transfections were done using Lipofectamine Plus.

Cosedimentation of recombinant Gem and Rad with ROK

Cos7 cells were plated on 10-cm cell culture dishes (2.0×10^6 cells/plate) and the next day were transfected with pCAG-myc–ROKβ (4 μg) or pEF-Bos-myc–ROKα (4 μg) using Lipofectamine Plus. Cell lysates were prepared as described below and precleared at 4°C on glutathione-Sepharose 4B (Amersham Pharmacia Biotech) for 30 min. Precleared Cos cell extracts were incubated at 4°C for 1 h with glutathione-Sepharose beads that were prebound to 50 μg GST–Gem or –Rad and blocked with 500 μl Cos cell lysate from nontransfected cells. The presence of ROK that had formed a physical complex with Rad or Gem on glutathione-Sepharose was revealed by Western blot analysis as outlined below.

Coimmunoprecipitation and Western blot analysis

Cos7 or N1E-115 cells (2.0×10^6 cells/10-cm cell culture plate) were transfected with PMT2T-Gem (2 μg) and pCAG-myc–ROKβ (2 μg) using Lipofectamine Plus. Soluble protein extracts were prepared as previously described (Leone et al., 2001) and precleared on 50 μl of recombinant protein G agarose beads (Invitrogen) for 30 min at 4°C. Gem was immunoprecipitated by incubating 1 ml of precleared lysate (one plate of cells) with 25 μl of packed recombinant protein G agarose beads and 25 μg of anti-Gem monoclonal antibody P7G4 for 2 h at 4°C. Beads were then washed three times in 40 volumes of lysis buffer. ROKβ that cosedimented with Gem was visualized by Western blot analysis using anti-myc polyclonal antibody (Upstate Biotechnology) and chemiluminescence (Pierce Chemical Co.).

Soft agar colony forming assay

NIH 3T3 cells were permanently transfected with CTV vector or CTV-hemagglutinin (HA)–Dbl and polyclonal populations were selected with hygromycin (300 μg/ml). Selected cells were checked for Dbl expression using Western blot analysis with monoclonal anti-HA antibody (Roche Molecular Biochemicals) and were then transfected with pRCCMV vector, pRCCMV-Gem, or pRCCMV-Gem(S89N). These cells were selected with geneticin (400 μg/ml) and expression of Gem and/or Dbl was visualized

by Western blot. Dbl expression was the same in cells with empty pRC-CMV vector and those transfected with pRCCMV-Gem or -Gem(S89N). Cells were assayed for their ability to form colonies in soft agar using the method of Cox and Der (1994).

In vitro invasion assay

Invasion capability of cells was determined as described previously (Ward et al., 2001). 10% FBS was used as the chemoattractant.

Phosphorylation of MLC and MLC phosphatase (MBS)

Cos7 cells were cotransfected with 2 μg pCEV-flag–MLC and empty vector or 2 μg PMT2T-Gem and/or 1 μg pEFBos-ROKα, pCAG-ROKβ, or pCAG-ROKβ(Δ4) using Lipofectamine Plus. Transfected cells were TCA precipitated with 5% TCA (2 mM DTT) and MLC was extracted with urea sample buffer (20 mM Tris, 22 mM glycine, 10 mM DTT, 8.3 mM urea, 0.1% bromophenol blue). Extract was filtered through a 0.45-μm centrifugal filter (Millipore), and proteins were resolved on a 15% SDS-polyacrylamide gel. Phosphorylated and total MLC were detected by Western blot analysis using antiphosphoserine 19 MLC polyclonal antibody and anti–flag M5 (Sigma-Aldrich) respectively. The effect of Gem on MBS phosphorylation was determined using the same procedure except that cells were transfected with pLEGFPN1-M133 instead of MLC, and pM1333^{T695} polyclonal and antimyosin phosphatase polyclonal (Berkeley Antibody Company) antibodies were used to detect phosphorylated and total MBS, respectively.

LIMK activity assay

Cos7 cells were cotransfected with 1 μg pUCD2-3xHA–LIMK1 and empty vector or 3 μg PMT2T-Gem and/or 1 μg pCAG-myc–ROKβ. The effect of Gem on ROK-dependent phosphorylation of LIMK1 was determined using the in vitro kinase assay of Ohashi et al. (2000).

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