Ras-interacting domain of RGL blocks Ras-dependent signal transduction in *Xenopus* oocytes

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Abstract RalGDS family members (RalGDS and RGL) interact with the GTP-bound form of Ras through its effector loop. The C-terminal region (amino acids 602–768) of RGL is responsible for binding to Ras. In this paper we characterized a Ras-interacting domain of RGL using deletion mutants of RGL(602–768). RGL(602–768), RGL(632–768), and RGL(602–734) bound to the GTP-bound form of Ras and inhibited the GAP activity of NF-1. RGL(646–768) showed a low binding activity to Ras and inhibited GAP activity of NF-1 weakly. None of RGL(659–768), RGL(685–768), RGL(602–709), and RGL(602–686) bound to Ras or inhibited GAP activity of NF-1. These results indicate that amino acids 632–734 of RGL constitute a nearly minimal domain that contains the binding element for Ras. RGL(632–734) inhibited v-Ras- but not progesterone-induced *Xenopus* oocyte maturation. Furthermore, RGL(632–734) inhibited v-Ras- but not v-Raf-dependent extracellular signal-regulated kinase activation in *Xenopus* oocytes. These results clearly demonstrate that the Ras-interacting domain of RGL is important for Ras-dependent signal transduction in vivo.

Key words: RID; RGL; Ras; Extracellular signal-regulated kinase; *Xenopus* oocyte

1. Introduction

Ras is a member of small GTP-binding protein and is important for cell growth and differentiation [1–4]. Ras has the GDP-bound inactive and the GTP-bound active forms, and the GTP-bound form interacts with the effector proteins [1,3]. The interaction of Ras with the effector proteins occurs through the effector loop of Ras (amino acids 32–40) [1,3]. The effector proteins of Ras have some activities that are regulated by its interaction with Ras, and the activities are responsible for some of biological effects of Ras in the cells. Therefore, identification of the effector proteins which directly interact with the active form of Ras and exert biological effects has been a major goal of research of Ras to clarify the mode of action of Ras.

Evidence has been accumulated that Ras has multiple effector proteins such as Raf-1, p120-GAP, PI3-kinase, and MEKK [5–16]. Raf-1 is a serine/threonine kinase and activates ERK pathway [5–13]. The N-terminal domain of Raf-1 interacts with Ras through its effector loop. It has been found that about 80 residues of Raf-1 constitute a binding domain for Ras [10,17–20]. p120-GAP interacts with the GTP-bound form of Ras and acts downstream of Ras in several signal transduction systems [14,21,22]. The catalytic domain of p120-GAP is 343 residues of its C-terminus (amino acids 702–1044) and this domain is required for the interaction with the effector loop of the GTP-bound form of Ras [14]. However, it has been also suggested that p120-GAP functions as a negative regulator of Ras [14]. p110 subunit of PI3-kinase associates with the GTP-bound form of Ras through its effector loop and nerve growth factor-dependent PI3-kinase activation is inhibited by a dominant negative mutant of Ras in PC12 cells, although the Ras-binding domain of PI3-kinase has not yet been determined [15]. However, we have recently demonstrated that a constitutively active mutant of PI3-kinase stimulates the GDP/GTP exchange of Ras [23]. These results suggest that PI3-kinase functions as a positive regulator of Ras. Whether GAP and PI3-kinase are the effector proteins of Ras might be dependent on cell types. MEKK binds to and is activated by the GTP-bound form of Ras [16,24]. The C-terminal kinase domain of MEKK interacts with Ras and the peptide corresponding to the effector loop of Ras blocks this interaction [16].

Three groups including us have found that RalGDS family members, RalGDS and RGL (RalGDS like), are new putative effector proteins of Ras [25–27]. RGL shares 70% amino acid homology with RalGDS [25,28]. RalGDS stimulates GDP/GTP exchange of Ral [28]. Ral has been originally isolated by probing with an oligonucleotide corresponding to one of the GTP-binding domains of Ras [29]. Although the function of Ral has not yet been understood, RalGDS and RGL have been implicated in the regulation of the GTP state of Ral [25,28]. Therefore, it is possible that RalGDS and RGL mediate the signal from Ras to Ral. We have demonstrated that a 166-amino acid fragment (amino acids 603–768) of RGL binds to the GTP-bound form of Ras and designated this fragment as RID, *R*as-*i*nteracting *d*omain [25,30]. Although no homology in the primary structures of RGL(RalGDS), Raf-1, p120-GAP, p110 of PI3-kinase, and MEKK has been found, it is speculated

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Abbreviations: GAP, GTPase activating protein; PI3-kinase, phosphatidylinositol 3-kinase; MEKK, MEK kinase; ERK, extracellular signal-regulated kinase; RGL, RalGDS like; RID, Ras-interacting domain; NF-1, neurofibromatosis 1; HA, hemaggulutinin; PCR, polymerase chain reaction; GST, glutathione-*S*-transferase; Sf9, *Spodoptera frugiperda*; *E. coli*, *Escherichia coli*; DTT, dithiothreitol; BSA, bovine serum albumin; GVBD, germinal vesicle breakdown; MEK, mitogen-activated protein kinase and ERK kinase; SAPK, stress-activated protein kinase.

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that these molecules may share some structural similarities to bind to Ras. To determine how closely the Ras-interacting domains of RGL, Raf-1, p120-GAP, p110 of PI3-kinase, and MEKK are situated in three dimension, and to identify the Ras-interacting residues of RGL by X-ray crystallography and NMR analysis, it is necessary to know the smallest RID of RGL. In this paper, we show that a 103-amino acid fragment (amino acids 632–734) of RGL is a nearly minimal domain that is containing the binding element for Ras. Furthermore, we demonstrate that RID of RGL inhibits the Ras-dependent signal transduction in *Xenopus* oocytes.

2. Materials and methods

2.1. Materials and chemicals

The NF-1 cDNA was provided by Dr. G. Xu (University of Utah, UT) [31]. pSP64T and pSP64T/v-Raf were provided by Dr. A. MacNicol (University of Chicago, IL). pCGN, pGEX1R, pGEX2TR, pGIKS, and pGEX2T were provided by Drs. Q. Hu, D. Pot, and D. Milda (University of California, San Francisco, CA). [α-^{32}P]GTP and [γ-^{32}P]GTP were purchased from DuPont NEN Research Product (Wilmington, DE). The anti-ERK antibody was from Santa Cruz Biotechnology (Santa Crutz, CA). v-Ras cDNA was synthesized by PCR as described [13]. c-Ras was purified to near homogeneity from the cytosol fraction of Sf9 cells expressing c-Ras [25,32]. To express the GST-fused to proteins in *E. coli*, transformed *E. coli* were initially grown at 37°C to an absorbance of 0.8 (600 nm) and subsequently transferred to 25°C. Then, isopropylthio-β-D-galactoside was added at a final concentration of 0.1 mM and further incubation was carried out for 10 h at 25°C. The GST-fused to proteins were purified as described [25,33].

2.2. Plasmid constructions

Deletion mutants of RGL(602–768) were constructed as follows. The oligonucleotide corresponding to the Myc epitope was synthesized by PCR. This oligonucleotide was designed to have the *Eco*RI site at the 5′ end and the *Bam*HI site at the 3′ end. pBSKS containing RID [25] was digested with *Xho*I and *Eco*RI. RID and the Myc epitope were ligated with pGEM7z which was digested with *Xho*I and *Bam*HI to generate pGEM7z/RGL(602–768). To construct pGEX1R/RGL(602–768), pGEM7z/RGL(602–768) was digested with *Xho*I and *Bam*HI and was inserted into the *Xho*I- and *Bam*HI-cut pGEX1R. To construct pGEX2TR/RGL(632–768), about 0.5-kb fragment fused to the Myc epitope containing the *Kpn*I and *Bam*HI sites was synthesized by PCR. This fragment was digested with *Kpn*I and *Bam*HI and inserted into the *Kpn*I- and *Bam*HI-cut pGEX2TR. To construct pGIKS/RGL(602–734), about 0.5-kb fragment containing the *Kpn*I and *Sac*I sites was synthesized by PCR. This fragment was digested with *Kpn*I and *Sac*I and inserted into the *Kpn*I- and *Sac*I-cut pGIKS. To construct pGEX2TR/RGL(646–768), pGEX2TR/RGL(659–768), and pGEX2TR/RGL(685–768), the fragments fused to the Myc tag containing the *Kpn*I and *Bam*HI sites were synthesized by PCR. These fragments were digested with *Kpn*I and *Bam*HI and inserted into the *Kpn*I- and *Bam*HI-cut pGEX2TR. To construct pGIKS/RGL(602 709) and pGIKS/RGL(602–686), the fragments containing the *Kpn*I and *Sac*I sites were synthesized by PCR. These fragments were digested with *Kpn*I and *Sac*I and inserted into the *Kpn*I- and *Sac*I-cut pGIKS. To construct pGEX2T/RGL(632–734), the fragment containing the *Bam*HI and *Eco*RI sites was synthesized by PCR. This fragment was digested with *Bam*HI and *Eco*RI and inserted into the *Bam*HI- and *Eco*RI-cut pGEX2T. To construct pSP64T/RGL(632–734), pGEM7z/RGL(632–734) was digested with *Bam*HI and *Eco*RI and both sites were blunted with *Klenow* fragment. This fragment was inserted into pSP64T which was digested with *Nco*I and *Sma*I (*Nco*I site was blunted with *Klenow* fragment). pSP64T/v-Ras and pSP64T/v-Raf were constructed as described [23,34].

2.3. Binding assay of RID and Ras in vitro

To make the GTP-bound form of Ras, Ras (2.5 pmol) was incubated for 5 min at 30°C in 5 μl of preincubation mixture (50 mM sodium phosphate [pH 6.8], 5 mM EDTA, 2.5 mM $MgCl_2$, 0.5 mg/ml BSA, 0.5 mM DTT, and 0.5 μM [α-^{32}P]GTP [20,000–30,000 cpm/pmol]). To this preincubation mixture, 45 μl of reaction mixture (22 mM Hepes/NaOH [pH 7.5], 2.2 mM Tris/HCl [pH 7.5], 5.5 mM $MgCl_2$, 1.1 mg/ml BSA, 0.11 mM DTT, and 5.5 μM GTP) containing the indicated concentrations of RGL fragments was added, and a second incubation was performed for 30 min at 4°C. RGL fragments were precipitated with glutathione Sepharose 4B, the precipitates were washed, and the remaining radioactivities were counted.

2.4. GAP assay of NF-1

Ras (2.5 pmol) was preincubated for 5 min at 30°C in 5 μl of the same preincubation mixture described above except that [γ-^{32}P]GTP was used instead of [α-^{32}P]GTP. To this preincubation mixture, 45 μl of the same reaction mixture described above containing RGL fragments (50 pmol) was added, and a second incubation was performed for 30 min at 4°C. Then, GST-NF-1 (0.5 pmol) was added and a third incubation was carried out for 15 min at 24°C. Assays were quantified by rapid filtration on nitrocellulose filters. GAP activity was calculated from the decrease of the radioactivity of [γ-^{32}P]GTP compared with a reaction performed in the absence of GST-NF-1, and GAP inhibition activity was expressed as percentage decrease of GAP activity of GST-NF-1.

2.5. Maturation and ERK assays in Xenopus oocytes

RNAs containing a 5′-GpppG cap were made by linearized plasmids of pSP64T/v-Ras, pSP64T/v-Raf, or pSP64T/RGL(632–734) with the SP6 RNA polymerase as described [34–36] and resuspended in RNAase free water. Large oocytes (Dumount stage VI) were removed from adult female frogs. Oocytes were manually dissected and treated with collagenase. Oocytes were maintained in 1×-modified Barth's saline (10 mM Hepes/NaOH [pH 7.4], 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, and 0.41 mM $CaCl_2$), 1 mg/ml BSA, and 1 mg/ml Ficoll. Each oocyte was injected with 5 ng of RNA. Oocytes were incubated for 16 h at 18°C, and then some were treated with 2 μg/ml of progesterone. Oocytes were scored for the appearance of a white spot on the animal pole as an index of GVBD. Immunoprecipitation of ERK from oocyte lysates was carried out as described [36]. ERK activity was determined by incubating the ERK immunoprecipitates with myelin basic protein (2 μg) in 30 μl of reaction mixture (50 mM Hepes/NaOH [pH 7.5], 1 mM DTT, 10 mM $MgCl_2$, and 50 μM [γ-^{32}P]ATP [2000–4000 cpm/pmol]) for 30 min at 24°C [36]. After the incubation, the samples were subjected to SDS-polyacrylamide gel electrophoresis, and the phosphoproteins were visualized by autoradiography.

3. Results

3.1. Ras-interacting domain of RGL

In order to investigate the domain of RGL which might critically affect the interaction of RGL with Ras, we constructed eight overlapping fragments of RGL; RGL(602–768), RGL(632–768), RGL(602–734), RGL(646–768), RGL(659–768), RGL(685–768), RGL(602–709), and RGL(602–686) (Fig. 1). They were expressed as GST-fused to proteins in and purified from *E. coli*. RGL(602–768), RGL(632–768), RGL(646–768), RGL(659–768), and RGL(685–768) were tagged with the Myc epitope at the C-terminal end. RGL(602–734), RGL(602–709), and RGL(602–686) were tagged with the HA epitope between GST and RGL fragments. To quantitate the Ras-binding activity of these RGL fragments, the [α-^{32}P]GTP-bound form of Ras was incubated with RGL fragments. RGL(602–768), RGL(632–768), and RGL(602–734) showed a Kd value of 100 nM for the GTP-bound form of Ras (Fig. 2). Under these conditions, about 0.5 pmol of Ras bound to 20 pmol of RID. RGL(646–768) displayed weak interaction (Fig. 2). None of RGL(659–768), RGL(685–768), RGL(602–709), and RGL(602–686) bound to Ras (Fig. 2). These results suggest that amino acids 632–734 of RGL constitute a minimal domain to interact with Ras (Fig. 1). Indeed, RGL(632–734) bound to

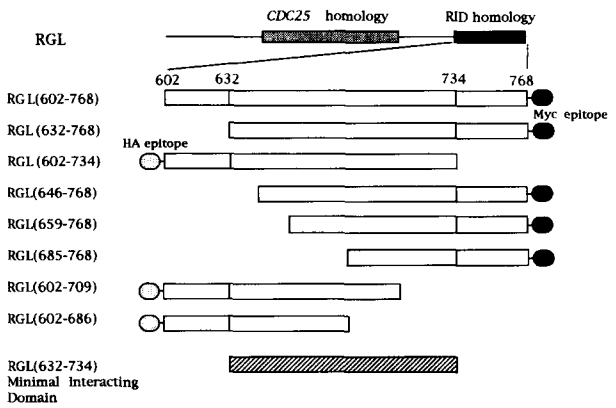

Fig. 1. Schematic structures of RGL fragments. The names of each RGL fragment, its first and last residues, are indicated. The hatched box indicates a possible minimal interacting domain with Ras. Light halftone symbol, the HA epitope; dark halftone symbol, the Myc epitope.

the GTP-bound form of Ras with the affinity similar to RGL(602–768) (Fig. 2).

3.2. GAP inhibition activity of RID of RGL

It is known that GAP stimulates the GTPase activity of Ras by interacting with the effector loop of the GTP-bound form of Ras [14]. Therefore, we examined whether RGL fragments inhibited the GAP activity of NF-1. None of RGL fragments had the GAP activity or the GDP/GTP exchange activity (data not shown). As expected, RGL(602–768), RGL(632–768), RGL(602–734), and RGL(632–734) inhibited strongly the GAP activity of NF-1, while RGL(646–768) inhibited weakly (Fig. 3). None of RGL(659–768), RGL(685–768), RGL(602–709), and RGL(602–686) affected the GAP activity of NF-1 (Fig. 3). These results are consistent with those obtained in Fig. 2 and indicate that RID of RGL interacts with the effector loop of Ras.

3.3. Inhibition of v-Ras-induced oocyte maturation and ERK activation by RID of RGL

Finally, we examined the biological effect of a minimal Ras-interacting domain of RGL. *Xenopus* oocytes provide a convenient system to analyse signal transduction. Ras initiates maturation which associates with GVBD [37–39]. It is known that Raf-MEK-ERK pathway is involved in the Ras-induced GVBD and that progesterone initiates GVBD through Raf-MEK-ERK pathway, but does not require Ras [36–39]. Progesterone is found to activate Raf-1 without Ras activation [36]. When 5 ng of RNA encoding v-Ras was injected into oocytes, GVBD was observed in 70% of oocytes (Fig. 4A). Injection of RGL(632–734) mRNA markedly reduced v-Ras-induced oocyte maturation. GVBD was observed in only 20% of oocytes injected with both of v-Ras and RGL(632–734) mRNAs (Fig. 4A). On the other hand, injection of RGL(632–734) mRNA did not affect progesterone-induced maturation (Fig. 4A). These results indicate that RGL(632–734) specifically binds to the active form of Ras and that it blocks Ras-dependent signal transduction. Since ERK activation is required for oocyte maturation [38,39], we investigated whether RGL(632–734) affected ERK activity. Coinjection of RGL(632–734) mRNA with v-Ras mRNA suppressed v-Ras-dependent ERK activity (Fig. 4B). However, coinjection of RGL(632–734) mRNA with v-Raf mRNA did not affect v-Raf-dependent ERK activity (Fig. 4B).

4. Discussion

We have determined a domain in the C-terminal region of RGL to bind to Ras with a high affinity. Our results indicate that the 103-amino acid residues of RGL(632–734) constitute a nearly minimal domain that contains the binding elements for Ras (Fig. 1), since RGL fragments which do not include whole of this domain neither interact with Ras nor inhibit the GAP activity of NF-1. RGL(632–734) shares about 80% amino acid homology with RalGDSa(721–821) and RalGDSb(764–864). In the overall sequences of RGL and RalGDS, these regions share the highest homology with each other. It has been found that RalGDSa(726–852) and RalGDSb(767–864) interact with Ras [26,27]. Therefore, these regions could be critical for the binding of RalGDS family to Ras.

Among the effector proteins of Ras, Raf-1 has been extensively studied [5–13]. Biochemical analysis has shown that the 80-amino acid residues of Raf(51–130) constitute a minimal binding domain to Ras [10,17–20]. The X-ray crystal structure of the complex between the Ras-binding domain of Raf-1 and Rap, a Ras-related small GTP-binding protein, has been solved [40]. It shows that an antiparallel β-sheet is formed by the β-sheet (amino acids 66–71) from Ras-binding domain of Raf-1 and the β-sheet (amino acids 36–45) from Ras. Since RID of RGL does not show any homology with Ras-binding domain of Raf-1, it is not known which amino acid residues of RID are critical for its interaction with Ras. By analogy with the interaction of Ras and Raf-1, the β-sheet structure in RID might be important. Other putative effector proteins of Ras such as GAP, PI3-kinase, and MEKK do not share any homology with Raf-1 in their primary structures, either. These results also suggest that not primary but secondary or tertiary structure of

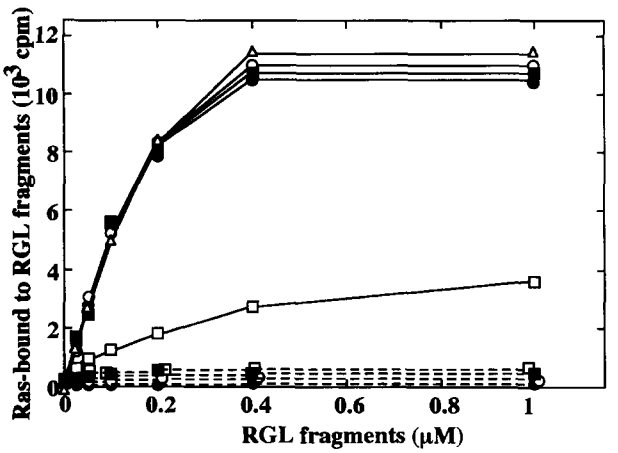

Fig. 2. Binding activity of RGL fragments to Ras. The [α-^{32}P]GTP-bound form of Ras was incubated with the indicated concentrations of the RGL fragments for 30 min at 4°C. The mixtures were precipitated with glutathione Sepharose 4B and the radioactivities of the precipitates were counted. (●—●), RGL(602–768); (■—■), RGL(632–768); (○—○), RGL(602–734); (□—□), RGL(646–768); (●--●), RGL(659–768); (■--■), RGL(685–768); (○--○), RGL(602–709); (□--□), RGL(602–686); (△—△), RGL(632–734). The results shown are representative of three independent experiments.

Ras-binding domain of Ras-effector proteins is important to interact with Ras. It is necessary to clarify the X-ray crystal structure of these effector proteins of Ras to understand the interaction of Ras with its effector proteins.

Our results have shown that RGL(632–734) inhibits v-Ras- but not progesterone-induced oocyte maturation. It is known that the anti-Ras antibody, Y13–259 (the neutralizing antibody), blocks Ras- but not progesterone-induced oocyte maturation and that progesterone does not require Ras to induce oocyte maturation [37]. Therefore, our results indicate that a direct interaction between Ras and RID is sufficient to inhibit the Ras action. Since RalGDS has been found to inhibit the interaction of Ras with Raf-1 in vitro [25–27], it is possible that RID of RGL interferes their association in vivo, resulting in inhibiting Ras-dependent Raf-1 activation. Consistent with this, our results have demonstrated that RID inhibits v-Ras- but not v-Raf-dependent ERK activation. Taken together, these results indicate that RID blocks at least Ras-dependent signal transduction through Raf-1, suggesting that RID can be utilized as a tool to analyse the function of Ras. The effect of RID on Ras-dependent signal pathway through the effector proteins other than Raf-1 remains to be clarified.

It is conceivable that RalGDS and RGL mediate the signal from Ras to Ral [25–27]. Although the function of Ral is not clear, RalBP1 has been found to be a putative effector protein of Ral [41,42]. RalBP1 shows the GAP activity for CDC42 and Rac1, members of small G proteins which regulate the cytoskeleton and SAPK activation [43–45]. Taken together with the observations that a dominant negative mutant of Rac reverses the v-Ras-dependent transformation [46,47], it is intriguing to speculate that there is a new signal pathway consisting of Ras-RalGDS(RGL)-Ral-RalBP1-CDC42/Rac. Furthermore, recently it has been reported that Ral interacts with phospholipase D and that Ras-dependent phospholipase D activity is inhibited by a dominant negative mutant of Ral [48]. Therefore, the new signal pathway from Ras to Ral could be important. It is necessary to develop the specific inhibitor for this new signal pathway to clarify the role of this pathway. One possible approach is to produce the anti-RID antibody which inhibits the interaction of Ras with RID. Screening to find this antibody is now under way.

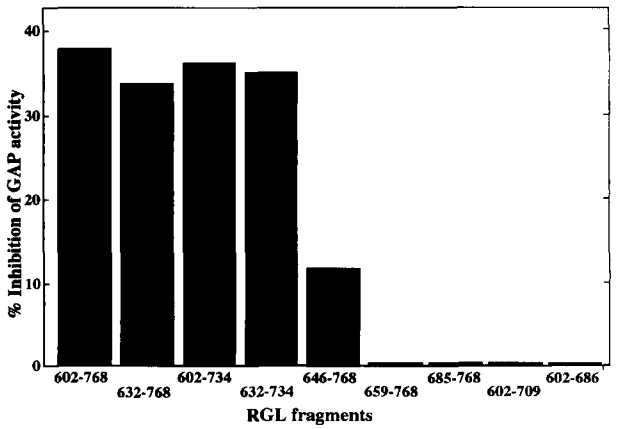

Fig. 3. Inhibition of GAP activity of NF-1 by RGL fragments. After the [γ-^{32}P]GTP-bound form of Ras was incubated with 1 μM RGL fragments for 30 min at 4°C, the mixture was incubated with 10 nM GST-NF-1 for 15 min at 24°C. The mixtures were then collected on filters, and the radioactivities were counted. GAP inhibition activity was expressed as percentage decrease of GAP activity of GST-NF-1. The results shown are representative of three independent experiments.

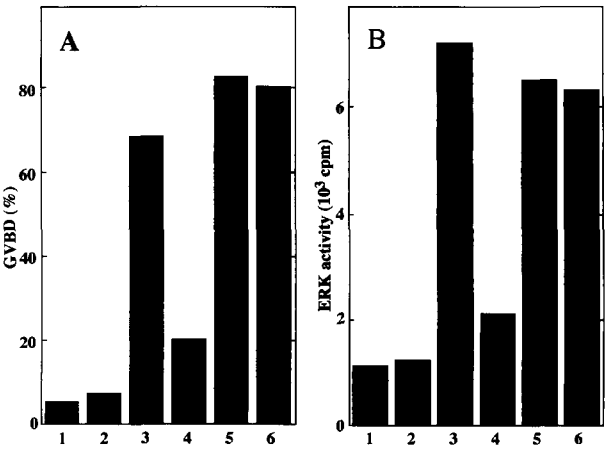

Fig. 4. Inhibition of v-Ras-dependent signal transduction in *Xenopus* oocytes by RGL(632–734). (A) Oocyte maturation. At least 50 oocytes were injected with water (lanes 1 and 5) or RNAs of RGL(632–734) (lanes 2 and 6), v-Ras (lane 3), or both v-Ras and RGL(632–734) (lane 4). Some oocytes (lanes 5 and 6) were treated with progesterone. The numbers of oocytes on which a white spot appeared were scored and the percentage of GVBD was expressed as an index of maturation. (B) ERK activity. At least 50 oocytes were injected with water (lane 1) or RNAs of RGL(632–734) (lane 2), v-Ras (lane 3), both v-Ras and RGL(632–734) (lane 4), v-Raf (lane 5), or both v-Raf and RGL(632–734) (lane 6). ERK activity was measured using myelin basic protein as a substrate. The results shown are representative of three independent experiments.

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