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Molecular structure of ras-related small GTP-binding protein genes of rice plants and GTPase activities of gene products in *Escherichia coli*

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We isolated two rice cDNA clones (ric1 and ric2) encoding proteins homologous to the ras-related small GTP-binding protein. The amino acid sequences of ric1 and ric2 are conserved in four regions involved in GTP binding and hydrolysis which are characteristic in the ras and ras-related small GTP-binding protein genes. In addition, two consecutive cysteine residues near the carboxyl-terminal end required for membrane anchoring are also present in ric1 and ric2. The ric1 and ric2 proteins synthesized in *Escherichia coli* possessed GTPase activity (i.e. hydrolysis of GTP to GDP).

cDNA; Rice; Small GTP-binding protein; GTPase; ypt/rab

1. INTRODUCTION

Small GTP-binding proteins identified in a large variety of evolutionary distinct organisms constitute at least three subfamilies (ras, rho and rab/ypt) [1]. They are believed to play a central role in signal transduction, cell differentiation and membrane vesicle transport [2–5], though the precise cellular function of most small GTP-binding proteins has not been defined. Although several reports have shown the presence of small GTP-binding proteins in plants [6–12], little is known about their function in higher plants.

We isolated putative plant genes by random sequencing of cDNA clones prepared from rice suspension cultured cells [13]. By comparing the partial sequences of cDNA clones to the GenBank database, we found two cDNA clones (ric1 and ric2) showing homology to small GTP-binding protein genes. In this paper, we report the complete nucleotide and deduced amino acid sequences of ric1 and ric2. Furthermore, we demonstrate here that these small GTP-binding proteins show GTPase activity.

2. MATERIALS AND METHODS

2.1. *Plant materials*

Suspension-cultured cells of rice (*Oryza sativa* L. var. Yamahoushi) were maintained in AA liquid medium [14]. The cultures were kept in a gyratory shaker (100 rpm) at 25°C and subcultured every 2 weeks. Seedlings (7 days old) were used for RNA extraction.

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2.2. *RNA preparation and cDNA libraries*

Total RNA was prepared from 5-day-old callus treated with 20% sucrose or 2% NaCl according to a modified method of Palmiter [15]. Poly(A)$^+$RNA was isolated from total RNA by oligotex-dT30 (Roche). cDNA was synthesized from poly(A)$^+$RNA using the cDNA synthesis system plus (Amersham) and ZAP-cDNA synthesis kit (Stratagene). A cDNA library of 20% sucrose-treated callus was constructed with pIBI31 and another one for 2% NaCl treated callus was constructed with pBluescript (Stratagene).

2.3. *Nucleotide sequencing*

Deletion mutants were generated by exonuclease III digestion using a Deletion Kit (Takara Co.). The nucleotide sequences of inserts were determined using the Sequenase Kit (United State Biochemical Co.) with the M13 universal and reverse primers.

2.4. *Southern blot analysis*

Total DNA was isolated from rice calli according to Shure et al. [16]. DNA (3 μg) digested with restriction enzymes (*Bam*HI, *Eco*RI and *Hin*dIII) was electrophoresed on a 1.0% agarose gel. The gel was then treated with 0.4 M NaOH, and denatured DNA was blotted onto a nylon membrane (Hybond-N Plus, Amersham). Hybridization was carried out with an α-^{32}P-labeled cDNA probe (the 680 bp *Hin*dIII fragment of ric1 and 430 bp *Xho*I fragment from the 3′ end of ric2) at 65°C. The nylon membrane was washed at 65°C with 2 × SSC, 0.1% SDS (2 × 30 min) and then exposed to an X-ray film with intensifying screen at −80°C.

2.5. *Northern blot analysis*

Total RNA (10 μg) isolated from rice callus and seedlings was electrophoresed in 1.2% formamide agarose gel, and transferred to a nylon membrane, which was then hybridized with a DNA probe (the 680 bp *Hin*dIII fragment of ric1 and 430 bp *Xho*I fragment from the 3′ end of ric2) as described in the Southern blot analysis.

2.6. *Expression of the ric proteins in* Escherichia coli

The coding region of ric1 was isolated by digestion with *Hin*dIII (position 131 and 811) was blunted with T4 DNA ligase and cloned

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into the *Sma*I site of the pGEX-1 expression vector (Pharmacia). The coding region of ric2 isolated by digestion with *Kpn*I (position 68 and vector) was blunted with T4 DNA ligase and cloned into the *Sma*I site of the pGEX-3x expression vector (Pharmacia). Clones were then transferred into *E. coli* DH5αF′. The expression of GST (glutathione *S*-transferase)-ric fusion proteins in *E. coli* was performed as described [17] and purified by glutathione Sapphires-4B column chromatography (Pharmacia).

2.7. *GTPase assay*

GTPase activity was determined according to the modified conditions of Wagner [18]. Briefly, the reaction was carried out in a final volume of 200 μl consisting of 65 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.5 mM DTT, 1 mM NaN_3, 4 μM GTP, 33 nM α-^{32}P-labeled GTP (3,000 Ci/mmol), 1 mM ATP and 1 μM of GST-ric1 and GST-ric2 fusion proteins at 30°C. Ten-microliter samples were collected at 1 h intervals followed by the addition of 10 μl 0.5 M EDTA to stop the reaction. Two microliter were spotted onto a PEI-cellulose TLC plate (Macherey-Nagel), which was then developed in 0.5 M KH_2PO_4 (pH 3.4). After drying, the plate was exposed to an X-ray film.

3. RESULTS AND DISCUSSION

3.1. *Two small GTP-binding protein genes (ric1 and ric2)*

cDNA libraries of rice callus were constructed using plasmid vectors pIBI31 and pBluescript. By random sequencing of these cDNA libraries and computer analysis using the GenBank database [13], we obtained two ras-related small GTP-binding protein genes (ric1 and ric2). Fig. 1 shows the entire nucleotide and deduced amino acid sequences of ric1 and ric2. ric1 consists of 955 bases with an ORF of 606 bases. This ORF starts at base 104 and ends with a TGA stop codon at base 710, capable of encoding for a 202 amino acid polypeptide. A consensus polyadenylation signal (AATAAA) was not found in the 3′-noncoding region. The ric2 consists of 965 bases with an ORF of 651 bases capable of encoding a polypeptide of 217 amino acids long. The first ATG codon is located at base 56, and the ORF ends with a TAA stop codon at base 707. We could not find any sequence homologous to the polyadenylation signal (AATAAA).

3.2. *Characterization of cDNA*

Fig. 2A shows a comparison of deduced amino acid sequences of ric1, yptm2 [10], ara5 [6] and ypt1 [19]. ric1 shares 83%, 81%, and 71% amino acid identity with yptm2, ara5 and ypt1, respectively. The amino acid sequences of ric1 are conserved in four regions (shaded boxes in Fig. 2A) which are now known to be important for binding and hydrolysis of GTP [1,20]. In addition, two consecutive cystein residues near the carboxyl-ter-

```
ric1      1                    TCGTGCTCCGCTTTCTCCCCCCAAATTCCTCGCCGCCCTCGGTCGTCGCCGCCGCCTCCGCCGCCTCGCCTCCCGGAGATTCGCCGCCTTCGCCGCCGCCGCC
ric2      1                                                                                  *TCGT***GATT*G**ACGAG*CTG*ACCTGCA*C***AGGAGAGGAGA*GA**A

ric1    104  ATGAATCCCGAG------------------TATGACTACCTCTTCAAGCTTCTGCTCATCGGAGACTCGGGCGTCGGGAAGTCTTGCCTGCTTCTGAGGTTTGCGGACGATTCATATCTG
             M  N  P  E  -  -  -  -  -  -  Y  D  Y  L  F  K  L  L  L  I  G  D  S  G  V  G  K  S  C  L  L  L  R  F  A  D  D  S  Y  L    34
ric2     56  ***GCGG*G*G*TACCGGGAGGAGGACGAC**C***************G*GG*C********C*****C********C*****CAA***C**CTCCC*C**CA*CCG*A*CGAG*TCAGC
             *  A  A  G  Y  R  E  E  D  D  *  *  *  *  *  *  V  V  *  *  *  *  *  *  *  *  *  *  N  *  *  S  *  *  T  R  N  E  F  S    40

ric1    206  GAGAGCTATATCAGTACCATCGGCGTTGATTTTAAAATCCGCACTGTTGAGCAAGATGGGAAGACAATAAAGCTGCAAATTTGGGATACTGCTGGCCAAGAGCGATTTAGGACCATTACA
             E  S  Y  I  S  T  I  G  V  D  F  K  I  R  T  V  E  Q  D  G  K  T  I  K  L  Q  I  W  D  T  A  G  Q  E  R  F  R  T  I  T    74
ric2    176  CTCGAG*CC*AGTCC**********C**G**CGCC*C****T*CC*CC**GTC**C**C***GTCG*C***GCC**G********C**C**C*****G*******ACC*TG*T*****T
             L  E  S  K  *  *  *  *  *  E  *  A  X  *  S  L  Q  V  *  *  *  V  V  *  A  *  *  *  *  *  *  *  *  *  *  Y  *  A  *  *    80

ric1    326  AGCAGCTACTACCGTGGTGCCCACGGGATCATTGTTGTTTATGATGTGACTGATCAGGAGAGCTTCAACAATGTCAAGCAGTGGCTGAATGAAATTGATAGGTATGCTAGTGAAAATGTG
             S  S  Y  Y  R  G  A  H  G  I  I  V  V  Y  D  V  T  D  Q  E  S  F  N  N  V  K  Q  W  L  N  E  I  D  R  Y  A  S  E  N  V   114
ric2    296  **TGCA**T*****A*****TGTT**AGCGT*GC****C********C**CCGG**CTCA*C***TG*G*****TG***GC***T****G***T*GAGGGACC**A*AGACCC***CA*A
             *  A  *  *  *  *  *  V  *  A  L  L  *  *  *  *  *  R  H  S  T  *  E  *  *  E  R  *  *  K  *  *  R  D  H  T  D  P  *  I   120

ric1    446  AACAAGCTCTTGGTGGGGAACAAGTGTGATCTAGCTGAGAACAGAGTGGTTTCTTATGAGGCTGGCAAGGCCCTTGCTGATGAGATTGGAATACCATTCCTGGAGACCAGTGCGAAGGAT
             N  K  L  L  V  G  N  K  C  D  L  A  E  N  R  V  V  S  Y  E  A  G  K  A  L  A  D  E  I  G  I  P  F  L  E  T  S  A  K  D   154
ric2    416  GTTGTCA*GC*A**T**C*******C******GCGCC*TCTTGT**C****CAAACC**T*AA**G*****AT*C**G**GAGAGAATCGC*CTAT***A********TC****CTC**G
             V  V  M  *  *  *  *  *  S  *  *  R  H  L  V  A  *  Q  T  D  E  *  *  *  F  *  E  R  E  S  L  Y  *  M  *  *  *  *  L  E   160

ric1    566  GCAACAAATGTGGAGAAGGCATTCATGACCATGGCAGGAGAGATAAAGAACAGGATGGCAAGCCAAGGCCGTACAAATGCAAGCAAGCCTGCAACCGTGCAAATGCCGAGGCAACCTGTT
             A  T  N  V  E  K  A  F  M  T  M  A  G  E  I  K  N  R  M  A  S  Q  G  R  T  N  A  S  K  P  A  T  V  Q  M  P  R  Q  P  V   194
ric2    536  T*C**C**C**T*****T*****TGCAGAGG*CTTGACCC****CT*CCG**TCG**AGC*AGAGGTCAGTCGA*GCA*GTGATG*TG*A*GTT*T*GT*CTGGCAA*G*TG*GAAGA*C
             S  *  *  *  *  N  *  *  A  E  V  L  T  Q  *  Y  R  I  V  S  K  R  S  V  E  A  G  D  D  A  G  S  G  P  G  K  G  E  K  I   200

ric1    686  GCCCAGCAAAGCAGCTGCTGCTCTTGATTATCAATCTAGATCACAATAGTGTGCTTGTTTGTGCACCAAGCAACACTTAATTGGTTGCTCTATCCCTGTAAATTAAGTCTATTAGTACTA
             A  Q  Q  S  S  C  C  S  *                                                                                                202
ric2    656  AATATAA*GGATGATGTT*CGG*GGTGAAGAAGGG*GGCTG*TGCTCG*GC*AAGCTC***CTGTATTTTTTTTGGGACC*GT**GAGCT*G*GTTATCGCCG*TCCAAGCGACTG*GA*
             N  I  K  D  D  V  S  A  V  K  K  G  G  C  C  S  G  *                                                                    217

ric1    806  TCAAAAAGCTTGATGTGTTCTGTGTCGGATATTATTGGAAAAAGTCTGTCCCCTAACTGTAACGAAGCGGCAGATGCTGTGGGCCTGTTTTAACTGCCTGTTCATATTTCTATGTTTCAA
ric2    776  ATTGTT**TAGT**TA*CAACT*C*AT*T**CGTAGA**GTTTA*TGC*ATAACTGAAAGGTTTGGC*A*ATC*GTT*AGACTT*CTA**GTTG*ATGGTC**CGCGGCAAC**GGG**T

ric1    926  GTGGCATCGATGTTCGACTTTAAAAAAAAA
ric2    896  *GT*TTGGTT*TCAT*TGAA*CC*TTGC**CCATGCTTGAACTGTTGAATCAAAAAAAAAAAAAAAAAAAA
```

Fig. 1. The nucleotide and deduced amino acid sequences of ric1 and ric2. Identical amino acid residues are indicated by asterisks. Gaps are introduced to obtain maximum similarity. The nucleotide sequence data of ric1 and ric2 appear in the DDBJ, EMBL and GenBank database under the accession numbers D10436 and D13758, respectively.

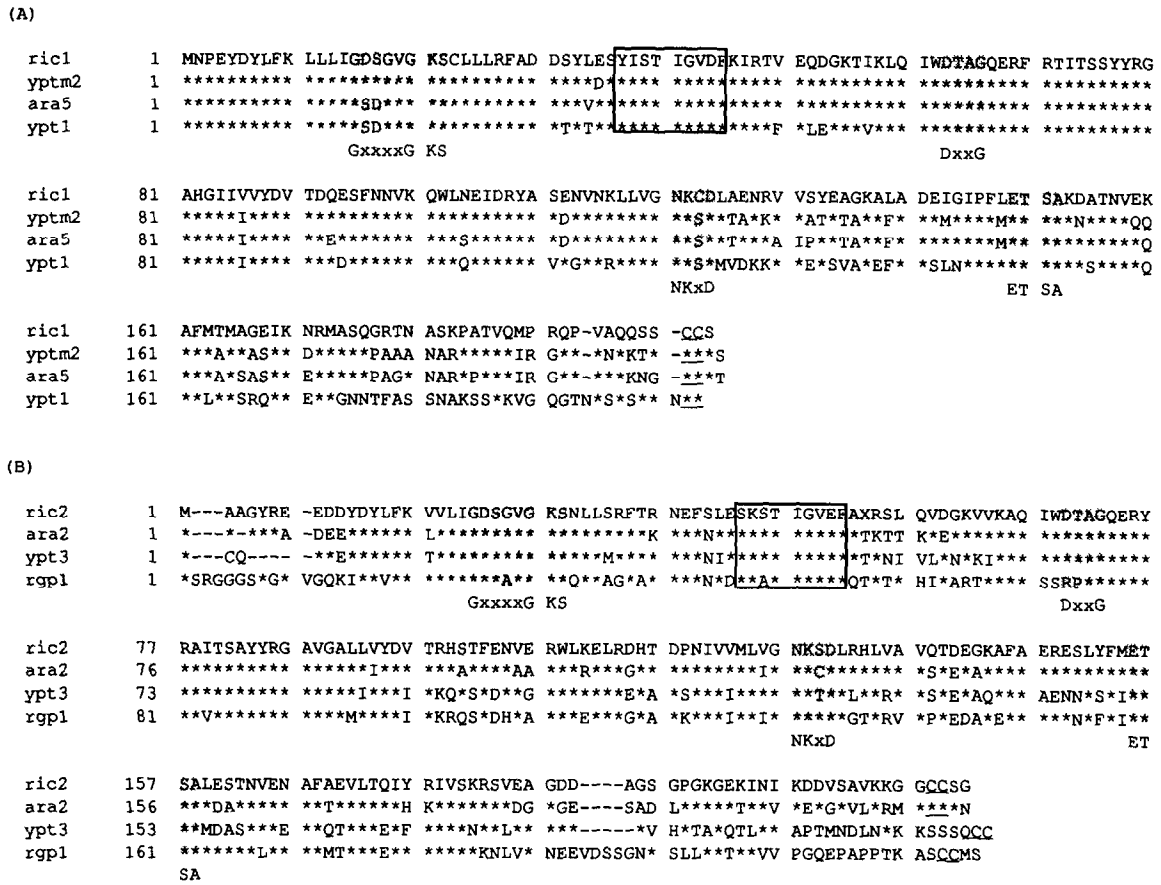

Fig. 2. Alignment of the amino acid sequences of ric1, yptm2, ara5 and ypt1 (A). Alignment of amino acid sequences of ric2, ara2, ypt3 and rgp1 (B). Each amino acid sequence is represented by the standard single letter code. Identical amino acid residues are indicated by asterisks. Gaps are introduced to obtain maximum similarity. The shaded boxes indicate highly conserved regions in all the ras and ras-related small GTP-binding protein genes characteristic for GTP binding and hydrolysis; the consensus amino acid sequences are shown at the bottom. Two consecutive cysteine residues near the carboxyl-terminal end required for membrane anchoring are underlined. The region corresponding to the effector domain of mammalian H-ras protein is boxed.

minal end are conserved in ric1. These cysteine residues required for geranylgeranylation and subsequent membrane anchoring are also seen in the rab/ypt subfamily of small GTP-binding protein genes [21]. The region of amino acid residues 37–45 (boxed in Fig. 2A) corresponds to amino acids 32–40 in the mammalian H-ras protein, known as the effector region interacting with GTPase-activating proteins (GAPs) [22]. The amino acid sequences of ric1, yptm2, ara5 and ypt1 are well conserved except for 40 amino acids at the C-terminus. Fig. 2B shows a comparison of deduced amino acid sequences of ric2, ara2 [6], ypt3 [19] and rgp1 [11]. ric2 shares 79%, 68% and 58% amino acid identity with ara2, ypt3 and rgp1, respectively. The amino acid sequences of ric2 are conserved in four regions (shaded boxes in Fig. 2B) required for binding and hydrolysis of GTP. In addition, two consecutive cysteine residues near the carboxyl-terminal end are conserved in ric2. Furthermore, the region interacting with GTPase-activating proteins is also seen in these four genes (boxed in Fig. 2B).

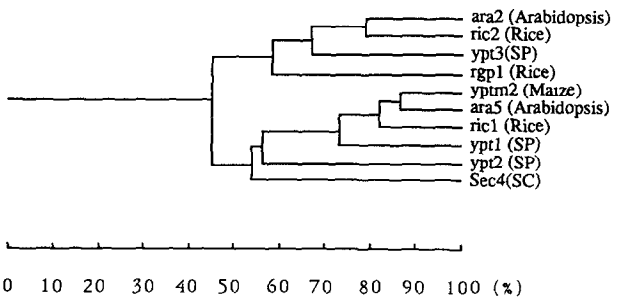

Fig. 3. The evolutional tree of ras-related small GTP-binding proteins. The scale on the bottom shows homology % of amino acid sequences. SP, *Schizosaccharomyces pombe*; SC, *Saccharomyces cerevisiae*.

3.3. *Evolutional comparison*

Recently, many ras-related small GTP-binding protein genes were identified in various organisms including plants [6–12]. To analyze an evolutional relationship between ric1 and other ras-related small GTP-binding protein genes [3,6,11,19,23,24] an evolutional tree on the basis of amino acid homology was made (Fig. 3). Apparently, ric1 belongs to a group of genes including *Zea mays* yptm2 [10], *Arabidopsis thaliana* ara5 [6] and

Schizosaccharomyces pombe ypt1 [19]. ric2 shows similarity to *A. thaliana* ara2 [6], *S. pombe* ypt3 [19] and *O. sativa* rgp1 [11].

3.4. *Southern and Northern blot analysis*

Both ric1 and ric2 hybridized to multiple bands of *Bam*HI, *Eco*RI and *Hin*dIII digested DNA (Fig. 4). This result shows that ras-related small GTP-binding protein genes of rice form a multiple gene family. We also isolated mRNA from callus and seedlings for Northern blot analysis. As seen in Fig. 5, both ric1 and ric2 showed the expected transcript size of 1.0 kb. Concerning the level of transcripts, ric2 was expressed at least five times higher than ric2 in both tissues. Interestingly enough, the level of ric mRNA expression was much higher in callus than in seedlings.

3.5. *GTPase activity*

Several instances demonstrating that plant ras-related small GTP- binding proteins are capable of GTP binding have been presented [7,8,11]. These experiments were carried out by incubating membranes containing proteins with [α-^{32}P]GTP. Here, we have tried to demonstrate GTPase activity of the RIC1 and RIC2 protein. For this purpose, we made a fusion protein of GST-RIC1 and GST-RIC2 (Fig. 6A). The free form of GDP was increased as the incubation progressed, whereas without proteins no such reaction was observed (Fig. 6B). The hydrolysis rate of GST-RIC1 and GST-RIC2 GTPase was about 0.002 min^{-1}, which is lower than the hydrolysis rate of 0.006 min^{-1} determined for the yeast YPT1 protein [18]. To our knowledge, this is the first report demonstrating that two plant small GTP-binding proteins show GTPase activity in vitro.

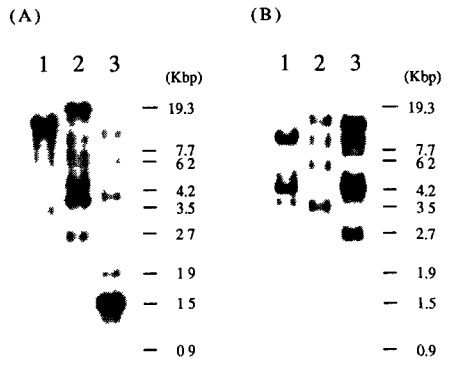

Fig. 4. Southern blot analysis of ric1 (A) and ric2 (B) in total rice DNA. Total DNA was digested with *Bam*HI (lane 1), *Eco*RI (lane 2) and *Hin*dIII (lane 3). α-^{32}P-labeled DNA (680 bp *Hin*dIII fragment of ric1 and 430 bp *Xho*I fragment from the 3′ end of ric2) was used as a probe. λDNA digested with *Eco*T14I was used as a size maker.

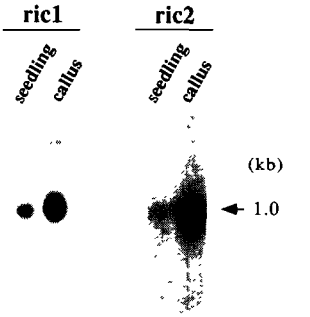

Fig. 5. Northern blot analysis of RNA from rice seedlings (7-day-old) and callus (5-day-old). α-^{32}P-labeled cDNA (the 680 bp *Hin*dIII fragment of ric1 and 430 bp *Xho*I fragment from the 3′ end of ric2) was used as a probe. The dark background in ric2 as a probe was due to the relatively long exposure time, since the ric2 mRNA level was much lower than that of ric1.

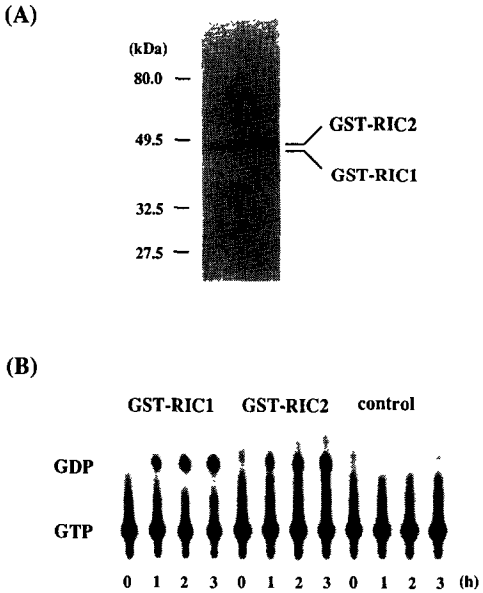

Fig. 6. GTP hydrolysis by GST-ric1 and GST-ric2 protein. SDS-PAGE (12.5%) of purified GST-RIC1 and GST-RIC2 proteins (A). Purified proteins were incubated with [α-^{32}P]GTP at 30°C. Reaction mixtures were separated into GTP and GDP on a PEI-cellulose TLC plate and visualized by autoradiography (B).

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