Characterization of the interaction between RhoA and the amino-terminal region of PKN

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Abstract The yeast two-hybrid system and in vitro binding assay were carried out to characterize the interaction between PKN and a small GTP-binding protein, RhoA. It was revealed that the region corresponding to the amino acid residues 33–111 in the amino-terminal region of PKN was sufficient to confer the ability to associate with RhoA. Each synthetic peptide fragment corresponding to the amino acid residues 74–93 and 94–113 of PKN inhibited the interaction between PKN and RhoA in the in vitro binding assay, suggesting that this region is important in the association with RhoA. The endogenous and the GAP-stimulated GTPase activity of RhoA was inhibited by the interaction with PKN, suggesting the presence of a regulatory mechanism that sustains the GTP-bound active form of RhoA.

Key words: Serine/threonine protein kinase; PKN; Small GTP-binding protein; RhoA; GTP hydrolysis

1. Introduction

PKN is a serine/threonine protein kinase with the catalytic domain highly homologous to that of protein kinase C, and its kinase activity is enhanced by unsaturated fatty acids such as arachidonic acid [1-3]. The N-terminal regulatory region of PKN contains repeats of a leucine zipper-like motif, suggesting protein-protein association through hydrophobic interaction [4], and a basic region located immediately N-terminal to the first leucine zipper-like motif, which are conserved through evolution in vertebrates [5]. We have recently reported that RhoA, a small GTP-binding protein implicated in multiple cell functions, specifically binds to the N-terminal regulatory region of PKN and activates PKN in a GTP-dependent manner, indicating that PKN functions as one of the target proteins of RhoA [6,7]. The precise binding region of PKN for RhoA and the effect of PKN on the GTPase activity of RhoA, however, are not known. In this study, we used the yeast two-hybrid system and in vitro binding assay to determine the essential region in the N-terminal regulatory region of PKN for binding with RhoA, and demonstrated that PKN inhibited the endogenous and the GAP-stimulated GTPase activity of RhoA.

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Abbreviations: Rho, the small GTP-binding protein of the *rho* gene product; GAP, GTPase activating protein; N-terminal, amino terminal; PCR, polymerase chain reaction; GST, glutathione *S*-transferase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; C-terminal, carboxyl terminal; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; MBP, maltose binding protein

2. Materials and methods

2.1. Materials and chemicals

[γ-^{32}P]GTP (1.11 TBq/mmol) was purchased from DuPont-New England Nuclear. Glutathione Sepharose 4B was purchased from Pharmacia Biotech Inc. Nitrocellulose filters (BA-85, 0.45 μm pore size) were obtained from Schleicher&Schuell. Synthetic peptide fragments for potential RhoA binding region of PKN were synthesized with an automated peptide synthesizer (Applied Biosystems, model 431). All other chemicals were from commercial sources.

2.2. Two-hybrid system

The scheme of the fusion constructs for the truncated PKN cDNA used in this study is shown in Fig. 1B. The cDNA fragments encoding various lengths of human PKN were inserted into the vector pBTM116 in frame with the upstream LexA DNA-binding domain sequences or the vector pVP16 in frame with the upstream VP16 transcription activation domain sequences. The cDNA fragments encoding human RhoA, RhoA^{val14} and RhoA^{ala37}, were obtained by digesting pGEX-RhoA, pGEX-RhoA^{val14}, and pGEX-RhoA^{ala37} [7], respectively, then cloned into pBTM116 and pVP16. The cDNA fragment for human RhoA lacking the C-terminal lipid modification site (amino acid residues 190–193), which was designated RhoA CLVL$^-$, was prepared by PCR and ligated into pBTM116 and pVP16.

The expression plasmids were transfected into yeast L40 cells and plated on semi-solid media lacking tryptophan and leucine. Interaction was examined using a filter lift assay for the β-galactosidase activity as described previously [8].

2.3. Preparation of recombinant proteins and in vitro binding assay

[^{35}S]Methionine-labeled in vitro translation product corresponding to amino acid residues 1–474 of human PKN was prepared as described previously [9]. Expression vector for the N-terminal region of human PKN fused to GST was made by subcloning the cDNA fragment encoding amino acid residues 1–540 of human PKN into pGEX vector (Pharmacia Biotech Inc.). GST fusion proteins were expressed in *E. coli* and affinity-purified on glutathione-Sepharose columns as described previously [7]. PKN (amino acid residues 7–155) fused to MBP was prepared as described previously [7]. 50 nM of the guanine nucleotide bound form of RhoA fused to GST was incubated with 1.5 μl of in vitro translated PKN in a total volume of 200 μl of binding buffer (20 mM Tris/HCl at pH 7.5, 1 mM EDTA, 0.5 mM DTT, 5 mM $MgCl_2$ and 1 μg/ml leupeptin) for 60 min at 4°C. Then 40 μl of glutathione-Sepharose beads preincubated in binding buffer containing 10 mg/ml *E. coli* extracts to block non-specific binding [10], was added and rotated for 30 min at 4°C. Unbound proteins were removed by four washes with binding buffer containing 0.2% Nonidet P-40 and 50 mM NaCl, and by two washes with binding buffer. Specifically bound proteins were eluted with binding buffer containing 10 mM reduced glutathione, subjected to SDS-PAGE, and to autoradiography as described previously [9]. Experiments with synthetic

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peptides were performed using the various concentrations of peptide in 200 μl of the binding mixture.

2.4. GTPase assay

The assay for endogenous GTPase activity was carried out as follows: 2 μM of purified RhoA fused to GST was incubated with 5 μM of [γ-^{32}P]GTP (1.11 TBq/mmol) in 50 mM Tris/HCl at pH 7.5, 10 mM EDTA, 1 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT, 0.3% CHAPS for 10 min at 30°C. The exchange reaction was stopped by placing the reaction mixture on ice, followed by addition of $MgCl_2$ to a final concentration of 10 mM. The [γ-^{32}P]GTP-loaded RhoA fused to GST or RhoA^{val14} fused to GST (0.2 μM) was incubated at 30°C either with or without PKN (amino acid residues 1–540) fused to GST in 60 μl of hydrolysis buffer (50 mM Tris/HCl at pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, 1 mM GTP and 0.1 mg/ml bovine serum albumin). The reaction was stopped by the addition of about 2 ml of ice-cold 20 mM Tris/HCl at pH 7.5 containing 100 mM NaCl and 25 mM $MgCl_2$, followed by rapid filtration on a nitrocellulose filter. The filter was washed three times with the same ice-cold buffer. The radioactivity collected on the filter was determined.

The GAP-stimulated GTPase activity of RhoA was assayed by measuring the decreased radioactivity of the [γ-^{32}P]GTP-bound form of RhoA fused to GST for 2 min at 25°C in the presence of 50 nM of p122 Rho GAP fused to GST [11] and the various concentration of PKN (amino acid residues 7–155) fused to MBP [7] in 100 μl of reaction mixture (20 mM Tris/HCl at pH 7.5, 10 mM EDTA, 1 mM DTT, 10 mM $MgCl_2$, 1 mM GTP, 0.075% CHAPS, 0.25 mM L-α-dimyristoylphosphatidylcholine, 100 nM [γ-^{32}P]GTP-bound form of RhoA fused to GST) [11].

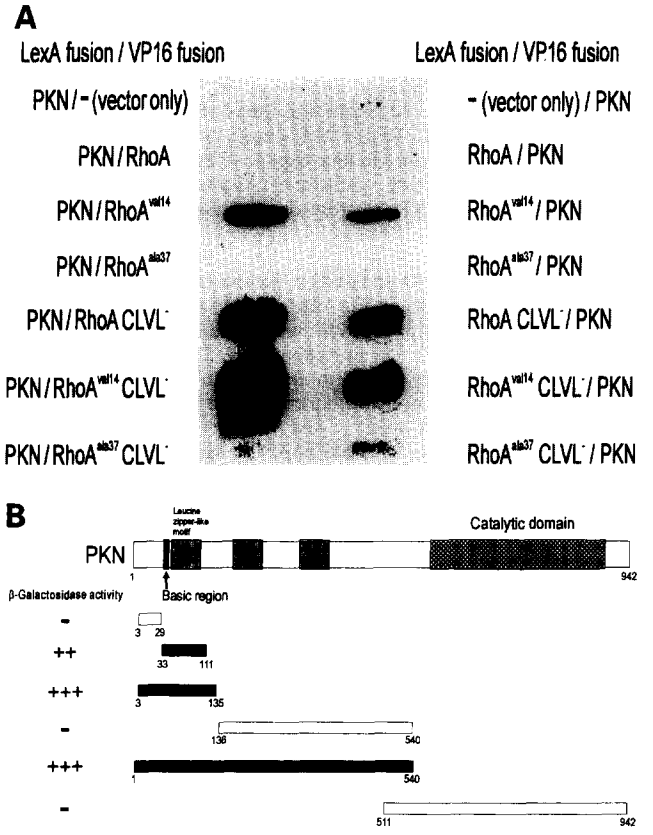

Fig. 1. Interaction of PKN with RhoA in the two-hybrid system. Yeast L40 cells were cotransfected with expression vectors encoding various LexA DNA binding domain and VP16 transcription activation domain fusion proteins. Interaction was examined using a filter lift assay for β-galactosidase activity. (A) Specificity of the interaction of the N-terminal regulatory region of PKN with wild type or mutants of RhoA. 'PKN' indicates the N-terminal regulatory region of PKN (amino acid residues 1–540). 'CLVL' indicates the deletion mutant which lacks the C-terminal lipid modification site. (B) Identification of the RhoA binding region of PKN. The schematic whole structure of PKN is represented at the top of the figure, and the deletion mutants are aligned below. 'LZ' indicates leucine zipper-like motif. '+++' and '++' indicate the development of strong color within 20 and 60 min from initiation of the assay, respectively. '—' indicates no development of color within 24 h. The results shown are representative of three independent experiments.

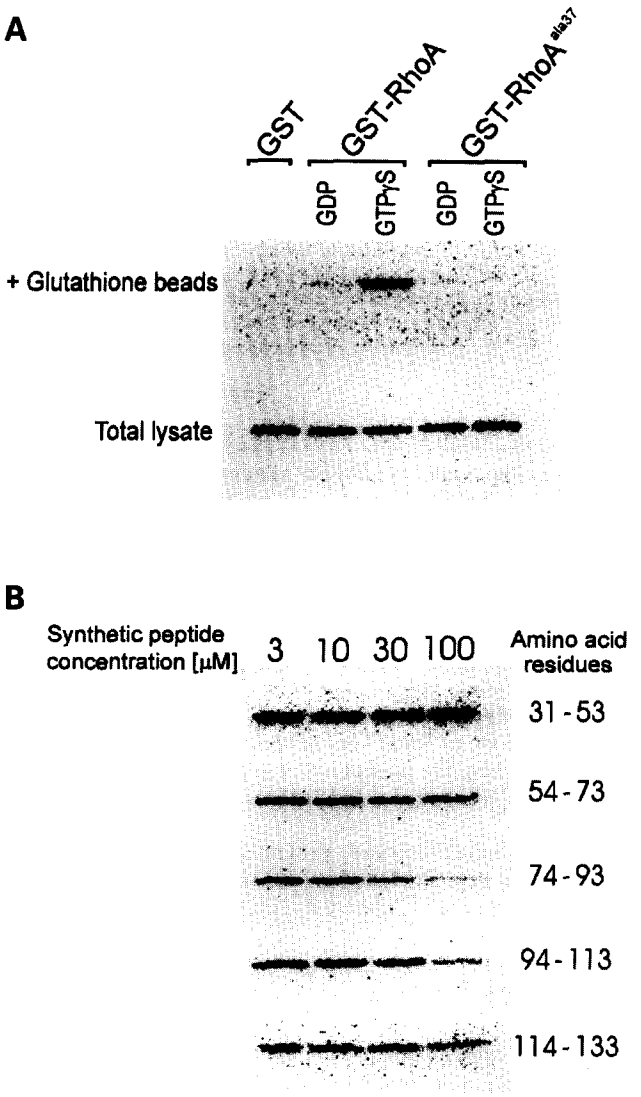

Fig. 2. In vitro interaction of the N-terminal regulatory region of PKN with RhoA. In vitro translated PKN and GST fusion proteins were incubated with glutathione-Sepharose beads, washed, and subjected to SDS-PAGE and to autoradiography, as described in section 2. (A) Specific interaction between the N-terminal regulatory region of PKN and the GTPγS-bound form of RhoA. (B) Effects of the various synthetic peptide fragments on the interaction between PKN and RhoA. The GTPγS-bound form of RhoA fused to GST was incubated with in vitro translated PKN in the presence of the indicated concentration of the peptides for the potential RhoA binding region of PKN. The results shown are representative of three independent experiments.

3. Results and discussions

We have recently shown that PKN forms a complex with the GTP-bound form of RhoA but not with the GDP-bound form [6,7]. To characterize the association between PKN and RhoA further, the yeast two-hybrid system was carried out. The N-terminal regulatory region of human PKN (amino acid

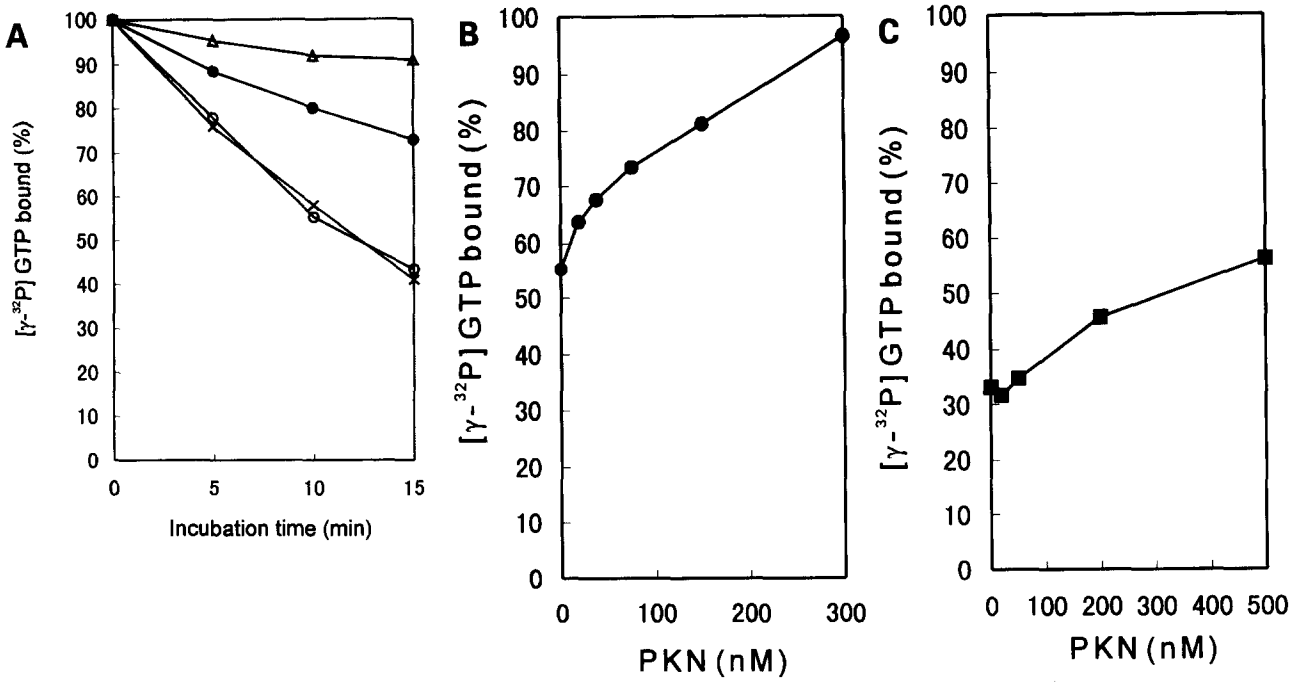

Fig. 3. Effects of PKN on the GTPase activity of RhoA. (A) Effects of added protein on the endogenous GTPase activity of RhoA. 200 nM of the [γ-^{32}P]GTP-bound form of RhoA (●, ○, ×) or RhoA^{val14} (△) were incubated with (●) or without (○) 150 nM of the N-terminal region of PKN fused to GST or incubated with GST alone (×) for the indicated times at 30°C, and the residual bound radioactivity was determined by a filter binding assay. The remaining [γ-^{32}P]GTP bound to each protein was expressed as a percentage of that measured at 0 min of incubation. (B) Dose-dependent effect of PKN. 200 nM of the [γ-^{32}P]GTP-bound form of RhoA fused to GST was incubated for 10 min with various concentrations of the N-terminal region of PKN fused to GST. The residual bound radioactivity of RhoA fused to GST preloaded with [γ-^{32}P]GTP was measured as described in the legend to (A). (C) Effects of PKN on GAP-stimulated GTPase activity of RhoA. 100 nM of the [γ-^{32}P]GTP-bound form of RhoA fused to GST was incubated for 2 min at 25°C in the presence of 50 nM of p122 Rho GAP fused to GST and various concentrations of the N-terminal region of PKN fused to MBP. The residual bound radioactivity of RhoA fused to GST preloaded with [γ-^{32}P]GTP was measured as described in the legend to (A). The results shown are representative of three independent experiments.

residues 1–540) fused to the LexA DNA binding domain was coexpressed with human RhoA or RhoA mutants fused to the VP16 activation domain in yeast strain L40. As shown in Fig. 1A, the β-galactosidase activity was obviously induced in the transformants expressing PKN and RhoA^{val14}, a GTPase-defective mutant [12], whereas it was detected in neither the transformants expressing PKN and wild-type RhoA nor the transformants expressing PKN and RhoA^{ala37}, an effector domain mutant [7,13]. The Rho family members have a CAAX motif (C, cysteine; A, aliphatic acid; X, any amino acid) in their C-terminal region, and undergo post-translational modifications including geranylgeranylation, proteolysis, and carboxyl-methylation [14,15]. To prevent association of RhoA fusion proteins with membranes, RhoA mutants designated CLVL$^-$, which lack the C-terminal lipid modification site, were tested by the two-hybrid system. PKN interacted with RhoA CLVL$^-$ and with RhoA^{val14} CLVL$^-$, though not with RhoA^{ala37} CLVL$^-$. Similar results were obtained from the combination of RhoA fused to LexA and PKN fused to VP16 (Fig. 1A). Thus, the specific interaction between activated RhoA and PKN was supported by the two-hybrid system, and these results suggest that the CAAX motif of RhoA is not required for the interaction with PKN.

To identify the binding region of PKN for RhoA, the various expression plasmids for the truncated PKN were transfected with RhoA^{val14} CLVL$^-$ into yeast cells. As shown in Fig. 1B, RhoA^{val14} CLVL$^-$ interacted with the region corresponding to amino acid residues 33–111 of PKN in the two-hybrid system. This result is consistent with recent observations [6,7]. Since this region of PKN contains the first leucine zipper-like motif [1,2], this motif may have some role in the interaction between PKN and RhoA.

Next, we measured the binding between in vitro translated PKN and the GTPγS-bound form of RhoA fused to GST in the presence of synthetic peptides corresponding to the various N-terminal regions of PKN. As shown in Fig. 2A, in the absence of peptide, in vitro translated PKN bound specifically to the GTPγS-bound form of RhoA fused to GST. Each synthetic peptide fragment corresponding to amino acid residues 74–93 and 94–113 of PKN inhibited the binding of PKN to the GTPγS-bound form of RhoA in a concentration-dependent manner (Fig. 2B). These results suggest that the region corresponding to amino acid residues 74–113 of PKN is critical for this interaction.

Several protein kinases, such as the activated Cdc42Hs-associated kinase p120^{ACK} and the p21 (Cdc42/Rac)-activated kinase p65^{PAK}, which bind to the active form of small GTPases, have been found to inhibit the endogenous and the GAP-stimulated GTPase activity of these small GTP binding proteins [16,17]. These effects of target protein kinases on GTPase activity are important for signal duration. To determine whether the GTPase activity of RhoA could be affected by the binding to PKN, the residual bound radioactivity of RhoA fused to GST or RhoA^{val14} fused to GST preloaded with [γ-^{32}P]GTP was measured in the presence or absence of PKN. As shown in Fig. 3A, the half-life of GTP bound to

RhoA fused to GST was 12 min, while that of RhoA^{val14} fused to GST was increased to greater than 100 min. The addition of the N-terminal region of PKN (amino acid residues 1–540) fused to GST increased the half-life of GTP bound to RhoA fused to GST from 12 to more than 20 min at 30°C, but the addition of GST alone did not affect the GTP hydrolysis rate. These results indicate that the N-terminal region of PKN has an activity to inhibit the endogenous GTPase activity of RhoA. This inhibition was dependent on the concentration of PKN (Fig. 3B). As shown in Fig. 3C, the N-terminal region of PKN fused to MBP (amino acids 7–155) inhibited p122 Rho GAP-stimulated GTPase activity of RhoA fused to GST in a concentration-dependent manner, suggesting that the N-terminal region of PKN inhibits the interaction of GAP with RhoA. Rhotekin, a novel putative target for Rho, bearing a homologous sequence to the N-terminal region of PKN, inhibits both the endogenous and the GAP-stimulated GTPase activity of RhoA [18], suggesting this conserved Rho binding motif is implicated in the regulation of the GTPase activity.

Recently, the GTP-bound form of RhoA was found to bind to and stimulate Rho kinase/ROKα and p160^{ROCK}, which are members of the serine/threonine protein kinases [19-21]. In *Saccharomyces cerevisiae*, the GTP-bound form of Rho1p, a yeast homologue of mammalian RhoA, interacts with Pkc1p, leading to the activation of the MAP kinase cascade [22]. However, there is no homology in primary amino acid sequences for the RhoA binding region between these protein kinases and PKN. ROKα was reported to have no effect on the GTPase activity of RhoA [20]. Taken together, there might be various modes of interaction of RhoA with the target. It is important to identify physiological targets for PKN and to examine the activation mechanism of PKN.

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References

[1] Mukai, H., Kitagawa, M., Shibata, H., Takanaga, H., Mori, K., Shimakawa, M., Miyahara, M., Hirao, K. and Ono, Y. (1994) Biochem. Biophys. Res. Commun. 204, 348–356.
[2] Mukai, H. and Ono, Y. (1994) Biochem. Biophys. Res. Commun. 199, 897–904.
[3] Kitagawa, M., Mukai, H., Shibata, H. and Ono, Y. (1995) Biochem. J. 310, 657–664.
[4] Landschulz, W.H., Jonson, P.F. and Mcknight, S.L. (1988) Science 240, 1759–1764.
[5] Mukai, H., Mori, K., Takanaga, H., Kitagawa, M., Shibata, H., Shimakawa, M., Miyahara, M. and Ono, Y. (1995) Biochim. Biophys. Acta 1261, 296–300.
[6] Watanabe, G., Saito, Y., Madaule, P., Ishizaki, T., Fujisawa, K., Morii, N., Mukai, H., Ono, Y., Kakizuka, A. and Narumiya, S. (1996) Science 271, 645–648.
[7] Amano, M., Mukai, H., Ono, Y., Chihara, K., Matui, T., Hamajima, Y., Okawa, K., Iwamatsu, A. and Kaibuchi, K. (1996) Science 271, 648–650.
[8] Vojtek, A.B., Hollenberg, S.M. and Cooper, J.A. (1993) Cell 74, 205–214.
[9] Mukai, H., Toshimori, M., Shibata, H., Kitagawa, M., Shimakawa, M., Miyahara, M., Sunakawa, H. and Ono, Y. (1996) J. Biol. Chem. (in press).
[10] Lee, J.W., Ryan, F., Swaffield, J.C., Johonston, S.A. and Moore, D.D. (1995) Nature 374, 91–94.
[11] Homma, Y. and Emori, Y. (1995) EMBO J. 14, 286–291.
[12] Ridley, A.J. and Hall, A. (1992) Cell 70, 389–399.
[13] Paterson, H.F., Self, A.J., Garrett, M.D., Just, I., Aktories, K. and Hall, A. (1990) J. Cell Biol. 111, 1001–1007.
[14] Adamson, P., Marshall, C.J., Hall, A. and Tilbrook, P.A. (1992) J. Biol. Chem. 267, 20033–20038.
[15] Katayama, M., Kawata, M., Yoshida, Y., Horiuchi, H., Yamamoto, T., Matsuura, Y. and Takai, Y. (1991) J. Biol. Chem. 266, 12639–12645.
[16] Manser, E., Leung, T., Salihuddin, H., Zhao, Z.S. and Lim, L. (1994) Nature 367, 40–46.
[17] Manser, E., Leung, T., Salihuddin, H., Tan, L. and Lim, L. (1993) Nature 363, 364–367.
[18] Reid, T., Furuyashiki, T., Ishizaki, T., Watanabe, G., Watanabe, N., Fujisawa, K., Morii, N., Madaule, P. and Narumiya, S. (1996) J. Biol. Chem. (in press).
[19] Matsui, T., Amano, M., Yamamoto, T., Chihara, K., Nakafuku, M., Ito, M., Nakano, T., Okawa, K., Iwamatsu, A. and Kaibuchi, K. (1996) EMBO J. (in press).
[20] Leung, T., Manser, E., Tan, L. and Lim, L. (1995) J. Biol. Chem. 270, 29051–29054.
[21] Ishizaki, T., Maekawa, M., Fujisawa, K., Okawa, K., Iwamatsu, A., Fujita, A., Watanabe, N., Saito, Y., Kakizuka, A., Morii, N. and Narumiya, S. (1996) EMBO J. (in press).
[22] Nonaka, H., Tanaka, K., Hirano, H., Fujiwara, T., Kohno, H., Umikawa, M., Mino, A. and Takai, Y. (1995) EMBO J. 14, 5931–5938.