The EMBO Journal vol.14 no.2 pp.286–291, 1995

A dual functional signal mediator showing RhoGAP and phospholipase C-δ stimulating activities

Yoshimi Homma[1] and Yasufumi Emori[2]

Department of Biosignal Research, Tokyo Metropolitan Institute of Gerontology, Itabashi-ku, Tokyo 173 and [2]Department of Biophysics and Biochemistry, Faculty of Science, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

[1]Corresponding author

Communicated by W.Moolenaar

We have cloned a novel regulator protein, p122, in the PLC-δ signalling pathway by screening a rat brain expression library with antiserum raised against purified phospholipase C-δ1 (PLC-δ1). This novel p122-RhoGAP binds to PLC-δ1 and activates the phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolyzing activity of PLC-δ1. As suggested by the deduced amino acid sequence, this regulator protein shows a similarity to the GTPase activating protein (GAP) homology region of Bcr and possesses GAP activity for RhoA, but not for Rac1; no guanine nucleotide exchange activity for RhoA and Rac1 was detected. These findings suggest that this novel RhoGAP is involved in the Rho signalling pathway, probably downstream of Rho activation, and mediates the stimulation of PLC-δ, which leads to actin-related cytoskeletal changes through the hydrolysis of PIP_2, which binds to actin binding proteins such as gelsolin and profilin.

Key words: GTPase activating protein/phospholipase C/PIP_2/Rho/signal transduction

Introduction

Phosphatidylinositol 4,5-bisphosphate (PIP_2) is a minor component of membrane phospholipids and functions as a precurcer of intracellular second messengers. PIP_2 is hydrolyzed by activated phospholipase C (PLC) to generate diacylglycerol and inositol 1,4,5-triphosphate (IP_3). Diacylglycerol is the physiological activator of protein kinase C and IP_3 activates Ca^{2+}-dependent systems through the release of Ca^{2+} from internal stores (Berridge, 1993). In addition to these roles, another function of PIP_2 has been proposed, since a number of PIP_2 binding proteins, such as gelsolin, cofilin, profilin and α-actinin, have been found (Lassing and Lindberg, 1985; Goldschumidt-Clermont *et al.*, 1990; Yonezawa *et al.*, 1990; Yu *et al.*, 1990; Banno *et al.*, 1992; Fukami *et al.*, 1992; Janmey *et al.*, 1992). These PIP_2 binding proteins are known to bind to actin and regulate actin assembly. This indicates that PIP_2 is involved in the regulation of various cell functions in which cytoskeletal organization is involved, such as cytokinesis and cell motility.

Multiple members of the PLC superfamily have been identified in mammalian cells. These can be divided into three main subfamilies, PLC-β, PLC-γ and PLC-δ, which are activated by different mechanisms (Rhee and Choi, 1992). PLC-β subfamily members have been identified as primary targets of heterotrimeric G proteins. Specifically, PLC-β1−β3 have been shown to be activated by α subunits of the Gq subfamily of G proteins and PLC-β2 and PLC-β3 are also activated by βγ subunits of some G protein species. On the other hand, it has been demonstrated that tyrosine kinases are involved in the activation of PLC-γ. Both PLC-γ1 and PLC-γ2 bind to growth factor receptors via their Src homology 2 (SH2) domains in a ligand-dependent manner and are then phosphorylated by their receptors, leading to activation. Recent reports indicate that non-receptor type tyrosine kinases also phosphorylate and potentiate PLC-γ. In contrast to the PLC-β and PLC-γ subfamilies, the activation mechanism of PLC-δ remains unclear. Neither receptors nor transducers that couple to PLC-δ members have been identified so far.

The *rho* and *rac* gene products (Rho and Rac) are members of the Ras superfamily of small G proteins (Hall, 1990). Rho and Rac have been shown to regulate various cell functions, such as cell morphology (Rubin *et al.*, 1988; Chardin *et al.*, 1989; Paterson *et al.*, 1990; Ridley and Hall, 1992; Miura *et al.*, 1993), cell motility (Takaishi *et al.*, 1993), cytokinesis (Kishi *et al.*, 1993) and platelet aggregation (Morii *et al.*, 1992). These processes are triggered by a variety of extracellular stimuli, such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) (Ellis *et al.*, 1990). Rho/Rac proteins, activated by GDP/GTP exchange reactions that follow ligand binding signals to the receptors, transfer the signals to their targets, although the molecular natures of the target proteins have not yet been elucidated. Subsequently, the activated Rho/Rac are down-regulated by GTPase activating proteins (GAPs) (Bourne *et al.*, 1990). In the case of the Ras subfamily, it is suggested that some GAPs may function as targets for Ras, as well as negative regulators (Bollag and McCormick, 1991; Hall, 1992). Several proteins have been identified as candidates for RhoGAP and RacGAP (Hall *et al.*, 1990, 1993; Garrett *et al.*, 1991; Hart *et al.*, 1991; Morii *et al.*, 1991; Agnel *et al.*, 1992; Settleman *et al.*, 1992; Leung *et al.*, 1993), which contain a region homologous with the C-terminal region of Bcr (the gene product of the break-point cluster region) (Lifshitz *et al.*, 1988) which functions as a RacGAP.

In the course of this study, we unexpectedly cloned a novel RhoGAP containing the Bcr-related GAP region by screening a rat brain expression library with antiserum raised against purified PLC-δ1. This novel RhoGAP was shown to associate with PLC-δ1 and potentiate its catalytic activity. Therefore, this protein may be a key to understanding not only the activation mechanisms of PLC-δ, but

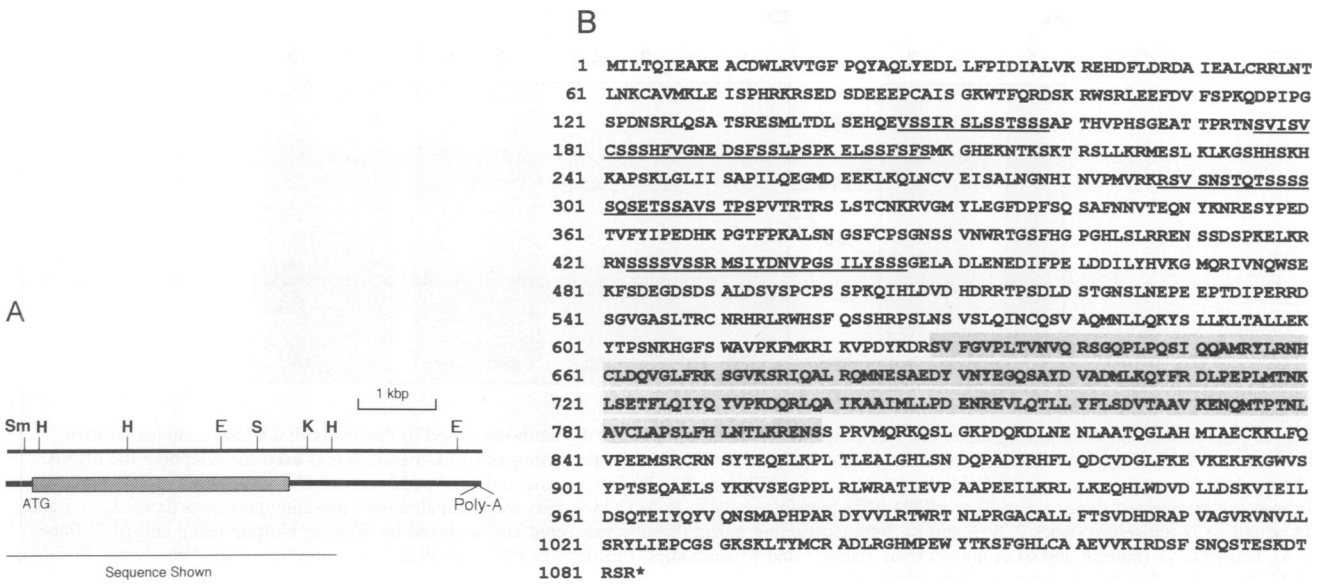

Fig. 1. The p122-RhoGAP cDNA clone and the deduced amino acid sequence. (**A**) The schematic structure of the p122-RhoGAP mRNA is shown in the middle (shaded portion). Digestion sites for relevant restriction enzymes are noted at the top: Sm, *Sma*I; H, *Hin*dIII; E, *Eco*RI; S, *Sal*I; K, *Kpn*I. The sequence shown in (B) is indicated at the bottom. (**B**) Amino acid residues deduced from p122 cDNA are numbered beginning with the initiation methionine and the residue numbers are shown to the left of each line. A region similar to the GTPase activating region of Bcr and Bcr-related proteins is indicated with shading. Four serine-rich regions are underlined. The nucleotide sequence has been submitted to the GenBankTM/EMBL Data Bank with accession number D31962.

also the involvement of PLC-δ in the regulation of cytoskeletal organization. Consequently, we propose that in growth factor-mediated cytoskeletal re-organization, Rho signalling is transferred via a novel regulator protein, designated p122-RhoGAP, to PLC-δ, which hydrolyzes PIP_2 bound to actin binding proteins.

Results

Cloning of a novel RhoGAP

A rat brain expression library of ~1×10^6 clones was screened with antisera raised against PLC-δ1 purified from rat brain (Homma *et al.*, 1988). Several clones were shown to react with the antisera, among which one clone gave significant and reproducible signals to different lots of sera. However, sequencing analysis indicated that the clone encodes a novel protein not related to any of the PLC isoforms, including the δ types. We therefore hypothesized that the positive signals originated from an unknown protein tightly associated with PLC-δ1, which exists in small quantities below the detection level of SDS−PAGE and recognizes our polyclonal antibodies as antigens. Thus a full-length cDNA clone was obtained from a rat brain λgt10 library in order to characterize this putative PLC-δ binding protein.

As shown in Figure 1, the cDNA clone contained an insert of ~6 kb, consistent with the length of the mRNA shown in Northern blot analysis (data not shown). The sequence contained a single open reading frame consisting of 1083 amino acids with a predicted molecular weight of 122.442 kDa; thus the encoded protein was termed p122.

p122 associates with PLC-δ

In the total sequence of p122, no regions showing similarity to PLC sequences were detected, although the encoded protein clearly reacted with the antisera used as probes to a lesser extent than PLC-δ when expressed in *Escherichia coli* (Figure 2A). This observation supports the idea that the clone might encode a PLC-δ binding protein. To examine this possibility, we obtained immunoprecipitates using anti-p122 antibody from COS-7 cells, which over-express p122, and the immunoreacted materials were analyzed using antibodies specific for each PLC isoform. These antibodies were raised independently in rabbits using recombinant PLC isoforms produced in *E.coli* as antigens. Thus, the anti-PLC-δ1 antibody used here was completely different from that used for screening the expression library and did not recognize p122 at all (Figure 2A).

When COS-7 cells over-expressing p122 were used, PLC-δ1, but neither PLC-β1 (data not shown) nor PLC-γ1, was immunoprecipitated together with p122 by the anti-p122 antibody (Figure 2B), suggesting a specific association of p122 with PLC-δ *in vivo*. On the other hand, since the expression level of p122 in control, mock-transfected COS-7 cells was quite low, only trace amounts of p122 were detected (lane 2 in Figure 2B).

p122-RhoGAP functions as a PLC-δ activator

We further studied the effect of p122 on the regulation of PLC-δ. PLC-δ1 was obtained as a glutathione S-transferase (GST) fusion protein and purified from bacterial extracts. The PIP_2 hydrolyzing activity of recombinant PLC-δ1 was determined at various concentrations of Ca^{2+} in the presence or absence of p122. Figure 3A shows that recombinant PLC-δ1 in the absence of p122 catalyzes the hydrolysis of PIP_2 in a Ca^{2+}-dependent manner, while no significant difference in Ca^{2+} dependency between recombinant and cellular PLC-δ1 was seen (data not shown). In the presence of p122, the PIP_2 hydrolyzing

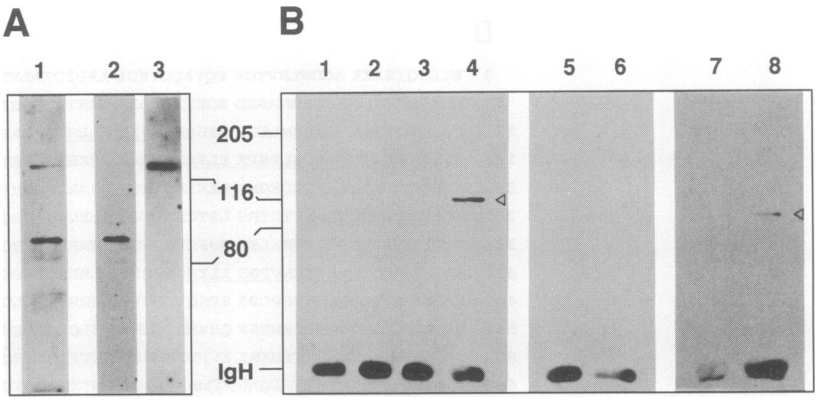

Fig. 2. Analyses of immunoreacting materials. (**A**) Detection of p122 and PLC-δ1 by antibodies used in this study. Each lane contains purified recombinant p122 (1 μg) and PLC-δ (1 μg). They were analyzed by Western blotting using anti-PLC-δ1 antiserum used for screening the cDNA library (lane 1), anti-recombinant PLC-δ1 (lane 2) and anti-p122 (lane 3). (**B**) *In vivo* association of p122 with PLC-δ. Cell lysates were prepared from mock-transfected (lanes 1 and 2) or pSRD-p122-transfected (lanes 3–8) COS-7 cells and incubated with pre-immune serum (lanes 1, 3, 5 and 7) or anti-p122 antiserum (lanes 2, 4, 6 and 8). Immunoreacting materials were recovered and analyzed by Western blotting using anti-p122 (lanes 1–4), anti-PLC-γ1 (lanes 5 and 6) or anti-PLC-δ1 (lanes 7 and 8) antibodies. Positions of p122 and PLC-δ are shown by open arrowheads. Positions of M_r markers and Ig heavy chain are also indicated.

activity of PLC-δ1 was greatly enhanced, without any effect on the Ca^{2+}-dependency of the enzyme (Figure 3B). p122 also stimulated the enzyme activity of PLC-δ1 purified from bovine brain. Neither PLC-β1 nor PLC-γ1, which were purified from bovine brain and thymus, were significantly activated by p122 (Figure 3B). Although it is of interest to know whether or not enzyme activation by p122 is further regulated by the GTP-bound form of RhoA, no significant effect of recombinant RhoA on PLC-δ activity has been detected so far.

p122 has intrinsic RhoGAP activity, but not RacGAP activity

To investigate the structural and functional features of p122, the deduced amino acid sequence was subjected to a database search using a protein databank (SWISS-PROT, release 25). The search yielded two proteins, *n*-chimearin (Hall *et al.*, 1990) and Bcr (Lifshitz *et al.*, 1988), which were initially identified as gene products of an mRNA present in abundance in brain and the break-point cluster region of chromosome translocation, respectively. The homologous regions consisted of ~170 residues and were located in the C-terminal regions of all three proteins. The most intriguing characteristic was that the homologous region corresponds precisely to the GAP domains of the Rho/Rac subfamily of small G proteins (Diekmann *et al.*, 1991). In addition, similar GAP domain sequences have recently been identified in other proteins exhibiting Rho/Rac GAP activity, as listed in Figure 4. Other regions of this novel protein provided no apparent information related to known functions, except for the presence of serine-rich motifs in the N-terminal region.

The sequence similarity to the GTPase activating domain of Bcr suggests that p122 acts as a GAP for Rho or Rac. To address this possibility, we measured the GTPase activating potency of p122 using recombinant p122 obtained from *E.coli* as a GST fusion protein and RhoA and Rac1 prepared independently from Sf9 cells and preloaded with [γ-^{32}P]GTP. As predicted, p122 caused a significant stimulation of the intrinsic GTPase activity of RhoA in a dose-dependent manner (Figure 5A). However,

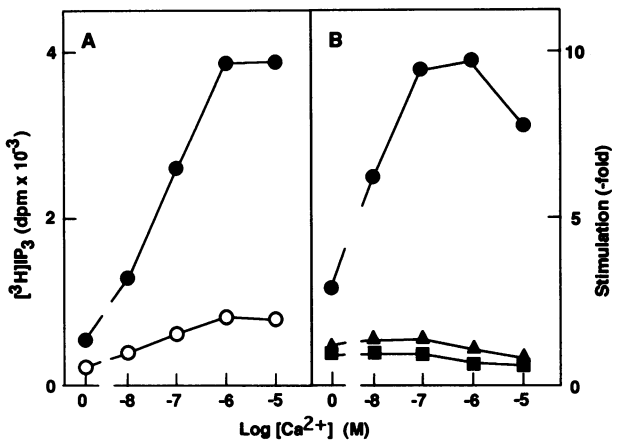

Fig. 3. Effect of p122 on PIP_2 hydrolyzing activity of PLC isoforms. (**A**) The PIP_2 hydrolyzing activity of 1 pmol reconbinant PLC-δ1 was determined at the indicated Ca^{2+} concentrations in the presence of ~1 pmol p122 (closed circles) or ~1 pmol GST (open circles). Assays were performed for 10 min at 30°C. Released [^{3}H]IP_3 was extracted and radioactivity was measured. (**B**) The PIP_2 hydrolyzing activity of 0.1 μg PLC-β1 (squares), -γ1 (triangles) or -δ1 (circles) was determined at the indicated Ca^{2+} concentrations. Data were expressed as ratios betweeen the enzyme activity measured in the presence of p122 and that measured in the presence of GST.

there was no effect of p122 on GTPase activity of Rac1 (Figure 5A and B). We also examined the effect of p122 on the rate of GDP dissociation. RhoA and Rac1 were preloaded independently with [^{3}H]GDP and then incubated with p122. p122 did not affect GDP dissociation from these two small G proteins (Figure 5C). These results suggest that p122 is a GAP for Rho rather than Rac, without any effect on GDP/GTP exchange.

Discussion

We have cloned a novel p122-RhoGAP that associates with PLC-δ1. In addition, this novel protein was revealed to activate the PIP_2 hydrolyzing activity of PLC-δ1. Based on its ability to interact with both RhoGAP and PLC-δ, we designate this novel protein ARP (adaptor for Rho and

```
p122 RhoGAP      630:VFGVPLTVNVQ-RSGQPLPQSIQQAMRYLRNHCLDQVGLFRKSGVKSRIQALRQ------MNESAEDYVNYEGQSAYDV---ADM
Bcr             1061:VFGVKIAVVTK-RERSKVPYIVRQCVEEIERRGMEEVGIYRVSGVATDIQALKA------AFDVN-NKDVSVMMSEMDVNAIAGT
n-Chimaerin      105:VYSCDLTTLVK-AHTTKRPMVVDMCIREIKSRGLNSEGLY-VSGFSDLIEDVKM------AFDRDGEKADISVNMYEDINIITGA
p190 RhoGAP     1246:VFGVPLTTVVT--PEKPIPIFIERCIEYIEATGLSTEGIYRVSGNKSEMESLQR------QFDQDHNLDLAEKDF--TVNTVAGA
Rotund           143:KRGCLSDYAPR--VAPMVPALIVHCVTEIEARGLQQEGLYRVVSSTREKCKRLR--------RKLLRGKSTPHLGNKDTHTLCCC
C.elegans GAP    778:VLGVRIADCPTGSCEDHVPMIVQACVCVIETYGMDTVGIYRIPGNTAAVNALKESLSNRGFDSVDLSKVESLDPRWRDVNVVSSL

LKQYFRDLPEPLMTNKLSETFLQIYQYVPKDQRLQAIKAAIMLLPDENREVLQTLLYFLSDVTAAVKENQMTPTNLAVCLAPSLFHLNTLKRENSSPRVMQRK: 807
LKLYFRELPEPLFTDEFYPNFAEGIALSDPVAKESCMLNLLLSLPEANLLTFLFLLDHLKRVAEKEAVNKMSLHNLATVFGPTLLRPSEKESKLPANPSQPIT:1230
LKLYFRDLPIPLITYDAYPKFIESAKIMDPDEQLETLHEALKLLPPAHCETLRYLMAHLKRVTLHEKENLMNAENLGIVFGPTLKRSPELDAMAALNDIRYQR: 285
MKSFFSELPDPLVPYSMQIDLVEAHKINDREQKLHALKEVLKKFPKENHEVFKYVISHLNRVSHNNKVNLMTSENLSICFWPTLMRPDFSSMDALTATRSYQT:1423
VKDFLRQLVHPLIPIYHRRDFEEATRHEDRLAVEMAVYLAVLELHQAHRDTLAYLMLHWQKIAES-PAVRMTVNNLAVIFAPTLFGDLDLTLENVVTWQRVLK: 320
LKMFLRKLPEPLLTDKLYPFFIDANRISTHHNRLHKLRNLLRKLPRPHYDTLRFLIVHLSEITKHSDVNKMECRNLALMFGPSIVRPSDDNMATMVTHMSDQC: 965
```

Fig. 4. Sequence comparison of the GTPase activating region of p122. Aligment with Bcr (Diekmann *et al.*, 1991), *n*-chimaerin (Hall *et al.*, 1990), p190 (Settleman *et al.*, 1992), Rotund (Agnel *et al.*, 1992) and *C.elegans* RhoGAP (Chen *et al.*, 1994) is based on the analysis by Diekmann (Diekmann *et al.*, 1991). Regions showing sequence identity are marked with shading.

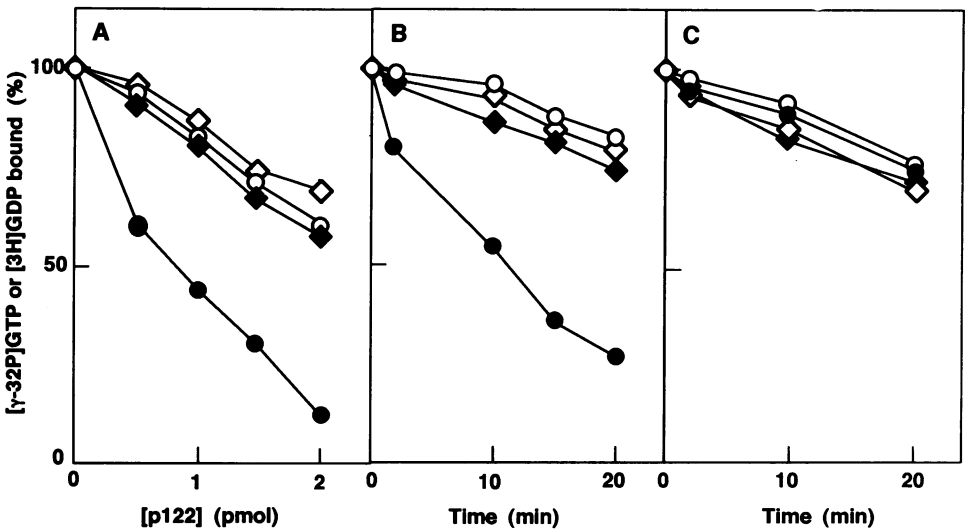

Fig. 5. Effect of p122 on the GDP/GTP conversion of RhoA and Rac1. (**A**) The concentration-dependent GTPase stimulating activity. The GTPase activities of RhoA (circles) and Rac1 (squares) were assayed by measuring the radioactivity of [γ-^{32}P]GTP bound to RhoA or Rac1 after incubation for 10 min with the indicated doses of GST (open symbols) or p122 (closed symbols) by the filtration method using a nitrocellulose filter. (**B**) The time-dependent GTPase stimulating activity. The GTPase activities of RhoA (circles) and Rac1 (squares) were assayed by measuring the radioactivity of [γ-^{32}P]GTP bound to RhoA or Rac1 after incubation with 0.5 pmol GST (open symbols) or 0.5 pmol p122 (closed symbols) by the filtration method using a nitrocellulose filter. (**C**) The dissociation of [^{3}H]GDP from RhoA (circles) or Rac1 (squares) was assayed by measuring the radioactivity of [^{3}H]GDP bound to RhoA or Rac1 after incubation with 0.5 pmol GST (open symbols) or 0.5 pmol p122 (closed symbols) by the filtration method.

PLC-δ). It is known that Rho regulates various cell functions through the cytoskeletal organization and that PIP_2 also binds to a number of actin binding proteins and regulates the actomyosin system. These findings imply a functional association between the Rho and PIP_2 systems and this novel p122^{ARP} GAP could be a key molecule for regulation of PIP_2 hydrolysis by Rho.

Rho has two interconvertible forms, GDP-bound inactive and GTP-bound active forms (Hall, 1990). The GDP-bound form is converted to the GTP-bound form by the GDP/GTP exchange proteins (GEPs) and the GTP-bound form is converted to the GDP-bound form by GAPs. We demonstrate in this study that p122 preferentially activates the intrinsic GTPase of Rho, but not that of Rac, without any effect on the rate of GDP/GTP exchange. In addition, preliminary results indicate that p122 is inactive on the GTPase of k-Ras, Rab3 and Cdc42Hs proteins. Therefore, we conclude that p122 is a GAP specific for Rho, but not a GEP for Rho or Rac. A number of GAPs, including at least Bcr, *n*- and α2-chimaerin, p190, p29, p150 and p145, have been reported for the Rho and Rac subfamilies (Hall *et al.*, 1990, 1993; Diekmann *et al.*, 1991; Garrett *et al.*, 1991; Hart *et al.*, 1991; Morii *et al.*, 1991; Agnel *et al.*, 1992; Manser *et al.*, 1992; Settleman *et al.*, 1992; Leung *et al.*, 1993; Chen *et al.*, 1994). Most of these proteins possess GAP activity for both Rho and Rac. Moreover, recent findings by Hall and colleagues have demonstrated that Rho and Rac regulate the assembly of focal adhesions and actin stress fibers (Ridley and Hall, 1992) and membrane ruffling (Ridley *et al.*, 1992) respectively in response to growth factors, indicating different physiological roles for these small G proteins. It is, therefore, possible that Rho and Rac are activated and down-regulated by independent mechanisms and transduce different intracellular signals. In this context, p122 should be a unique regulator and/or signal transducer specific for Rho rather than Rac.

Nothing is known about the activation mechanism of PLC-δ, whereas PLC-β and PLC-γ have been shown to be regulated by heterotrimeric G proteins and tyrosine kinases respectively. Here we demonstrate that p122 interacts with and potentiates PLC-δ1, suggesting that p122 functions as a specific activator for the PLC-δ subfamily. In addition, it is possible that Rho is also

involved in the regulation of PLC-δ. Namely, the exchange of GDP for GTP on Rho may induce a functional association between p122 and PLC-δ, resulting in activation of PLC-δ through changes in the conformation and/or location (cellular compartmentation).

Analyses of the enzyme kinetics of PLC-δ1 demonstrate that p122 potentiates the catalytic activity of PLC-δ1 by altering its K_m or V_{max} value rather than its Ca^{2+}-dependency. The regions of p122 required for activation of PLC-δ are not yet precisely identified, but the preliminary results so far obtained indicate that a region homologous to the GTPase activating domain of Bcr and Bcr-related proteins is also important for the activation of PLC-δ1. This region may contain two independent domains that activate the intrinsic GTPase of Rho and the PIP_2 hydrolysis of PLC-δ. It is also possible that one domain regulates both activities. In this case, it is of interest whether or not Bcr and Bcr-related proteins activate PLC-δ subfamily members.

Finally, we refer to the significance of PLC-δ activation in cytoskeletal changes involving Rho signalling. A number of PIP_2 binding proteins, including gelsolin, cofilin and profilin, regulate the formation of actin stress fibers. However, none of the PLC isoforms are known to catalyze the hydrolysis of protein-bound forms of PIP_2 except for phosphorylated PLC-γ1, which is phosphorylated and activated by growth factor receptors and is able to attack protein-bound PIP_2 (Goldschumidt-Clermont *et al.*, 1991). The finding that gelsolin co-purifies with PLC-δ as well as PLC-γ suggests that, like phosphorylated PLC-γ, $p122^{ARP}$-activated PLC-δ may be involved in the hydrolysis of protein-bound PIP_2, resulting in a significant effect on actin assembly. Further biochemical and cell biological studies on this novel dual function protein are required to evaluate the above hypothesis.

Materials and methods

Materials

PIP_2, phosphatidylethanolamine (PE) and glutathione were purchased from Sigma. Glutathione–Sepharose was from Pharmacia Biotech. [γ-^{32}P]GTP (1.11 TBq/mmol), [^{3}H]GDP (518 Gbq/mmol), [α-^{32}P]CTP (111 TBq/mmol) and [^{3}H]PIP_2 (370 GBq/mmol) were obtained from Du Pont-New England Nuclear. Nitrocellulose filters (BA-85, 0.45 mm pore size) were from Schleicher & Schuell. RhoA and Rac1, both of which were purified from Sf9 cells as described, were generous gifts from Y.Takai (University of Osaka, Osaka, Japan). Antisera used for screening the expression library were raised against PLC-δ1 by immunizing rabbits with highly purified PLC-δ1 (designated PLC-III in the original manuscript by Homma *et al.*, 1988). These antisera were shown to recognize PLC-δ1 and a few cellular proteins quite weakly, including p122, when these proteins were dissolved and concentrated by column chromatography. Polyclonal antibodies against PLC-β1, -γ1 and -δ1 were independently raised in rabbits using recombinant PLC isoforms produced in *E.coli* as antigens (Homma *et al.*, 1990). The anti-PLC-δ1 antibody produced by this method was completely different from that used to screen the expression library and did not recognize p122 at all. PLC-β1 was purified from bovine brain and PLC-γ1 and -δ1 were from bovine thymus (Homma and Takenawa, 1992). p122 was not detected in these purified samples.

Molecular cloning of p122-RhoGAP

A rat brain cDNA library constructed in λgt11 was screened with the rabbit anti-PLC-δ1 antisera described above. A positive clone was isolated and subjected to sequencing and immunoblotting analyses. To obtain full-length cDNA, another cDNA library constructed in λgt10 was screened with a cDNA insert of the above positive clone as a probe and the clones were subjected to sequencing analysis.

Expression and purification of p122 and PLC-δ1

cDNA fragments encoding PLC-δ1 and p122 were amplified by PCR using primers that corresponded to sequences containing initiation and termination codons consisting of 20 nucleotides. The amplified fragments were each inserted in frame with glutathione S-transferase (GST) at the *Sma*I site of the pGEX-3X vector. *Escherichia coli* harboring each of the expression plasmids constructed as above were cultured in L-broth and expression of the fusion proteins of PLC-δ1 or p122 with GST was induced by isopropyl-β-D-thiogalactoside (IPTG). Over-expressed fusion proteins were purified on glutathione–Sepharose CL-4B (Pharmacia). The GST carrier was cleaved from the fusion proteins by digestion with factor Xa and the released proteins were further purified by column chromatography using HiTrap–heparin (1 ml; Pharmacia).

Production of anti-p122 antibody

A *Hin*dIII fragment of 2.6 kb encoding residues 379 to the C-terminus (Figure 1) was inserted into the pUC18 vector downstream of and in-frame with *lacZ'* and the fusion protein was expressed by induction with IPTG. An *E.coli* extract was subjected to SDS–PAGE and the protein band visualized by Coomassie brilliant blue staining was excised and crushed to a paste. The paste was sonicated in a buffer consisting of 10 mM Tris–HCl, pH 7.5, 1 mM EDTA and used as antigen for immunization of guinea pigs.

Assays

The GTPase activities of RhoA and Rac1 were assayed by measuring the radioactivity of [γ-^{32}P]GTP bound to RhoA or Rac1 after incubation with recombinant p122 by the filtration method using a nitrocellulose filter as described previously (Hori *et al.*, 1991; Hart *et al.*, 1992). The dissociation of [^{3}H]GDP from RhoA or Rac1 was assayed by measuring the radioactivity of [^{3}H]GDP bound to RhoA or Rac1 after incubation with recombinant p122 by the filtration method as described (Ueda *et al.*, 1990). PIP_2 hydrolysis was determined in a reaction mixture (50 μl) consisting of 50 mM Mes–NaOH, pH 6.5, 50 μM PIP_2 (25 000 d.p.m. [^{3}H]PIP_2), 400 μM PE, 3 mM EGTA, 1 pmol recombinant PLC-δ1 and an aliquot of purified p122. $CaCl_2$ was added to the assay mixture to give the indicated Ca^{2+} concentrations, which were calculated as described elsewhere (Fabiato, 1988). Assays were performed for 10 min at 30°C. Released [^{3}H]IP_3 was extracted and radioactivity was measured. Under these conditions, [^{3}H]IP_3 formation was linear with respect to time and enzyme concentration when less than 20% of substrate was consumed. Thus, the amount of enzyme was reduced so as to produce <5000 d.p.m. of product during the incubation.

Immunoprecipitation

An expression plasmid in COS-7 cells was constructed by inserting a 3.6 kb *Sma*I–*Kpn*I fragment containing the total coding sequence into the SRD-α vector (Ohno *et al.*, 1988). COS-7 cells transfected with pSRD-p122 and over-expressing p122 were harvested and cell lysates were prepared by solubilization with 0.5 % (w/v) Nonidet P-40 in buffer consisting of 20 mM Tris–HCl, pH 7.4, 2 mM sodium vanadate, 2 mM EDTA, 1 mM EGTA, 10 mM sodium pyrophosphate, 100 mM NaF, 1 μM leupeptin, 10 μM phenylmethanesulfonyl fluoride and 10 μM diisopropyl fluorophosphate. After centrifugation (200 000 *g*, 15 min), the lysates were incubated at 4°C for 2 h with anti-p122 antiserum. Immunocomplexes recovered by protein G–Shepharose were analyzed by SDS–PAGE (6.5 % acrylamide).

Ackowledgements

We thank Y.Takai for generous gifts of RhoA and Rac1 purified from Sf9 cells and K.Kaibuchi and T.Sasaki for valuable discussions.

References

Agnel,M., Roder,L., Vola,C. and Griffin-Shea,R. (1992) *Mol. Cell. Biol.*, **12**, 5111–5122.

Banno,Y., Nakashima,T., Kumada,T., Ebisawa,K., Nonomura,Y. and Nozawa,Y. (1992) *J. Biol. Chem.*, **267**, 6488–6494.

Berridge,M.J. (1993) *Nature*, **361**, 315–325.

Bollag,G. and McCormick,F. (1991) *Nature*, **348**, 125–132.

Bourne,H.R., Sanders,D.A. and McCormick,F. (1990) *Nature*, **348**, 125–132.

Chardin,P., Boquet,P., Madaule,P.,Popoff,M.R., Rubin,E.J. and Gill,D.M. (1989) *EMBO J.*, **8**, 1087–1092.

Chen,W., Blanc,J. and Lim,L. (1994) *J. Biol. Chem.*, **269**, 820–823.
Diekmann,D., Brill,S., Garrett,M.D., Totty,N., Hsuan,J., Monfries,C., Hall,C., Lim,L. and Hall,A. (1991) *Nature*, **351**, 400–402.
Ellis,C.M., Moran,M., MaCormick,F. and Pawson,T. (1990) *Nature*, **343**, 377–381.
Fabiato,A. (1988) *Methods Enzymol.*, **157**, 378–417.
Fukami,K., Furuhashi,K., Inagaki,M., Endo,T., Hatano,S. and Takenawa,T. (1992) *Nature*, **359**, 150–152.
Garrett,M.D., Major,G.N., Totty,N. and Hall,A. (1991) *Biochem. J.*, **276**, 833–836.
Goldschumidt-Clermont,P.J., Machesky,L.M., Baldassare,J.J. and Pollard,T.D. (1990) *Science*, **247**, 1575–1578.
Goldschumidt-Clermont,P.J., Kim,J.W., Machesky,L.M., Rhee,S.G. and Pollard,T.D. (1991) *Science*, **251**, 1231–1233.
Hall,A. (1990) *Science*, **249**, 635–640.
Hall,A. (1992) *Cell*, **69**, 389–391.
Hall,C., Monfries,C., Smith,P., Lim,H.H., Kozma,R., Ahmed,S., Vanniasingham,V., Leung,L. and Lim,L. (1990) *J. Mol. Biol.*, **211**, 11–16.
Hall,C., Sin,W.C., Teo,M., Michael,G., Smith,P., Dong,J.M., Lim,H.H., Manser,E., Spurr,N.K., Jones,T.A. and Lim,L. (1993) *Mol. Cell. Biol.*, **13**, 4986–4998.
Hart,M.J., Shinjo,K., Hall,A., Evans,T. and Cerione,R.A. (1991) *J. Biol. Chem.*, **266**, 20840–20848.
Hart,M.J., Maru,Y., Leonard,D., Witte,O.N., Evans,T. and Cerione,R.A. (1992) *Science*, **258**, 812–815.
Homma,Y. and Takenawa,T. (1992) *J. Biol. Chem.*, **267**, 21844–21849.
Homma,Y., Imaki,J., Nakanishi,O. and Takenawa,T. (1988) *J. Biol. Chem.*, **263**, 6592–6598.
Homma,Y., Emori,Y., Shibasaki,F., Suzuki,K. and Takenawa,T. (1990) *Biochem. J.*, **269**, 13–18.
Hori,Y., Kikuchi,A., Isomura,M., Katayama,M., Miura,Y., Fujioka,H., Kaibuchi,K. and Takai,Y. (1991) *Oncogene*, **6**, 515–522.
Janmey,P.A., Lamb,J., Allen,P.J. and Matudaira,P.T. (1992) *J. Biol. Chem.*, **267**, 11818–11823.
Kishi,K., Sasaki,T., Kuroda,S., Itoh,T. and Takai,Y. (1993) *J. Cell. Biol.*, **120**, 1187–1195.
Lassing,I. and Lindberg,U. (1985) *Nature*, **314**, 472–474.
Leung,T., How,B.E., Manser,E. and Lim,L. (1993) *J. Biol. Chem.*, **268**, 3813–3816.
Lifshitz,B., Fainstein,E., Marcelle,C., Shtivelman,E., Amson,R., Gale,R.P. and Canaani,E. (1988) *Oncogene*, **2**, 113–117.
Manser,E., Leung,T., Monfries,C., Teo,M., Hall,C. and Lim,L. (1992) *J. Biol. Chem.*, **267**, 16025–16028.
Miura,Y., Kikuchi,A., Musha,T., Kuroda,S., Yaku,H., Sasaki,T. and Takai,Y. (1993) *J. Biol. Chem.*, **268**, 510–515.
Morii,N., Kawano,K., Sekine,A., Yamada,T. and Narumiya,S. (1991) *J. Biol. Chem.*, **266**, 7646–7650.
Morii,N., Teru-uchi,T., Tominaga,T., Kumagai,N., Kozaki,S., Ushikubi,F. and Narumiya,S. (1992) *J. Biol. Chem.*, **267**, 20921–20926.
Ohno,S., Akita,Y., Konno,Y., Imajoh,S. and Suzuki,K. (1988) *Cell*, **53**, 731–741.
Paterson,H.F., Self,A.J., Garrett,M.D., Just,I., Aktories,K. and Hall,A. (1990) *J. Cell. Biol.*, **111**, 1001–1007.
Rhee,S.G. and Choi,K.D. (1992) *Adv. Second Messenger Phosphoprotein Res.*, **26**, 35–61.
Ridley,A.J. and Hall,A. (1992) *Cell*, **70**, 389–399.
Ridley,A.J., Paterson,H.F., Johnston,C.L., Diekmann,D. and Hall,A. (1992) *Cell*, **70**, 401–410.
Rubin,E.J., Gill,D.M., Boquet,P. and Popoff,M.R. (1988) *Mol. Cell. Biol.*, **8**, 418–426.
Settleman,J., Narasimhan,V., Foster,L.C. and Weinberg,R.A. (1992) *Cell*, **69**, 539–549.
Takaishi,K., Kikuchi,A., Kuroda,S., Kotani,K., Sasaki,T. and Takai,Y. (1993) *Mol. Cell. Biol.*, **13**, 72–79.
Ueda,T., Kikuchi,A., Ohga,N., Yamamoto,J. and Takai,T. (1990) *J. Biol. Chem.*, **265**, 9373–9380.
Yonezawa,N., Nishida,E., Iida,K., Yahara,I. and Sakai,H. (1990) *J. Biol. Chem.*, **265**, 8382–8386.
Yu,Y.-X., Johnston,P.A., Sudhof,T.C. and Yin,H.L. (1990) *Science*, **250**, 1413–1415.

Received on July 18, 1994; revised on November 14, 1994