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Phytochemistry 65 (2004) 71–80
www.elsevier.com/locate/phytochem

A rac-like small G-protein from *Brassica campestris* activates a PKC-dependent phospholipase D

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Received 19 March 2003; received in revised form 10 September 2003

Abstract

A cDNA clone encoding a rac-like small GTP binding protein was isolated from a cDNA library of Chinese cabbage (*Brassica campestris* L. ssp. *pekinensis*) flower buds and named *Brac1*. The *Brac1* cDNA contains an open reading frame encoding 198 amino acid residues with an estimated molecular mass of 21,690 Da and this coding region has conserved residues and motifs unique to the Rho subfamily of proteins. The deduced amino acid sequence of the Brac1 protein is closely related to that of *Arabidopsis thaliana* Arac3 (91%), but it shares relatively little homology with other members of the Ras superfamily (about 30% identity). To further characterize Brac1, a pGBrac1 expression vector consisting of PCR-amplified *Brac1* cDNA plus glutathione S-transferase (GST) and pBKS(+)II was used to purify the protein. Using a PEI-cellulose/TLC plate, GTPase activity of this protein was confirmed and competition binding studies, using the guanine nucleotides, ATP, UTP and CTP, revealed that the di- and triphosphate forms of guanine nucleotides strongly bind Brac1. Membrane-bound PLD activity was synergistically enhanced by Brac1 in the presence of protein kinase C, but not in the presence of ARF (ADP-ribosylation factor). Genomic analysis indicated that *Brac1* belongs to a multigene family. *Brac1* transcripts were expressed in all the organs of *Brassica*, but were especially prevalent in flower buds.
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Keywords: *Brassica campestris*; *Cruciferae*; Chinese cabbage; Rac-like small G-protein; Phospholipase D; Rho subfamily

1. Introduction

The enzyme phospholipase D (PLD, EC 3.1.4.4) is widely distributed in plants, fungi and bacteria (Exton, 1997a,b) and is generally thought to catalyze the hydrolysis of the terminal diester bond of membrane glycerophospholipids, resulting in the formation of phosphatidic acid and a related base (Gomez-Cambronero and Keire, 1998). Recent studies in animal systems indicate that PLD activation and formation of phosphatidic acid are involved in transducing signals from many extracellular stimuli. Various reports also indicate that the resulting phosphatidic acid functions in the regulation of protein kinases, actin assembly, vesicle trafficking and secretion (Exton, 1997a,b; Ktistakis et al., 1995, 1996; Chen et al., 1997).

The structural and biochemical properties of various plant PLDs, including castor bean (Wang et al., 1994), rice (Ueki et al., 1995), maize (Ueki et al., 1995), cowpea and *Arabidopsis* (Pappan et al., 1997a,b) have been described. Comparison of these properties indicated a high degree of similarity (except for *Arabidopsis* PLD) and these enzymes were named PLDα. On the other hand Pappan et al. (1997a,b) identified a PLD isoform, named PLDβ, which is different from PLDα in terms of cDNA length, Ca^{2+}-dependency and pH sensitivity.

The first indication for the involvement of a small G-protein in the regulation of PLD emerged from initial observations that a cytosolic factor was involved, which was subsequently identified as ARF (ADP-ribosylation factor) (Brown et al., 1993). In addition, other GTPases such as RhoA, Rac1 and Cdc42Hs were found to activate membrane-associated PLD in the presence of GTPγS (Malcolm et al., 1994; Siddiqui et al., 1995; Kwak et al., 1995). Among the five distinct families of small GTPases (Ras, Rab, Arf/Sar, Rho and Ran), the

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0031-9422/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2003.10.001

Rho family is known to play key roles in the regulation of focal adhesion complex and actin cytoskeleton formation. The Rho family can be further subdivided into three subfamilies: Rho, Rac and Cdc42.

Brown et al. (1995) showed that Rho acts synergistically with ARF to activate brain PLD. In addition, Luo et al. (1997) demonstrated using cell-free systems that some members of the small G-protein Ras superfamily, namely Rho, ARF, and RalA, stimulate PLD activity.

Although a relatively large number of cDNAs encoding Rho family small GTP-binding proteins have been isolated in plants, such as *Arabidopsis* (Winge et al., 1997; Xia et al., 1996), garden pea (Yang and Watson, 1993) and cotton (Delmer et al., 1995), the function of most of these plant genes has not been well characterized, except in the case of *Arabidopsis* (Vernoud et al., 2003). Within the Rho family, the Rac proteins have been reported to enhance superoxide generation as a response to microbial infection (Abo et al., 1991; Knaus et al., 1991). A similar role for Rac proteins is also observed in plants during hypersensitivity responses (Kawasaki et al., 1999; Park et al., 2000). In *A. thaliana*, although several new members of the Rab, Ran and Arf families have been isolated, to date no true homologues of the Ras-, Rho- and Cdc42-like proteins have been found. However, a large number of *rac*-like genes were identified in *Pisum sativum* (Yang and Watson, 1993), *Gossypium hirsutum* (Luo et al., 1997; Trainin et al., 1996) and *A. thaliana* (Xia et al., 1996).

In order to further elucidate the mechanism by which Rac proteins stimulate PLD activity in plants, we have isolated a *rac*-like cDNA clone from *Brassica campestris*, named *Brac1* (GenBank accession number AF042330). Here we report that the isolated clone is a member of the plant-specific Rho family, and its coding region has the ability to bind and hydrolyze GTP. We further propose that Brac1 can synergistically activate a PKC (protein kinase C)-dependent rat brain PLD in the presence of guanosine-5′-*O*-(3-thiotriphosphate) (GTPγS).

2. Results and discussion

2.1. Isolation and sequence analysis of a rac-like cDNA clone, Brac1

We partially sequenced 1216 individual cDNA clones from a Chinese cabbage flower bud cDNA library. Comparative analysis of the primary structures of the deduced amino acid sequences was carried out in PIR or Swissprot. Among the matched *Brassica* ESTs, we selected and further characterized a cDNA encoding a rac-like small GTP binding protein. The cDNA clone, designated as *Brac1* (*Brassica rac1*), encodes a protein of 198 amino acid residues with an estimated molecular mass of 21,690 Da and an isoelectric point of 9.6 (Fig. 1). A comparison of amino acid sequences with related proteins indicated that Brac1 is more similar to the Rho proteins with 60–80% identity as compared to other members of the Ras superfamily (30% or less in identity). Among the Rho family members, Brac1 is most closely related to Arac 3 (91%). In spite of its low amino acid sequence homology to other groups of small GTP binding proteins, the five characteristic motifs unique to the GTPase superfamily are well conserved, including the two phosphoryl group binding motifs, GNGAVGKTCLL (13–23 in Fig. 2) and DTAGQE (60–66 in Fig. 2), and the guanine nucleotide specificity motif, LVGTKLDL (115–122 in Fig. 2). Brac1 also has a conserved motif (35–43 in Fig. 2) equivalent to the ras effector domain that appears to interact with a GTPase activating protein. Importantly, Brac1 contains not only all the conserved residues or motifs unique to members of the Rho family, but also an Asn-42 residue, which is the site for specific ADP-ribosylation by the C3 exoenzyme toxin from *Clostridium botulinum* (Sekine et al., 1989), and the C-terminal CAAL (A is an aliphatic amino acid) of the signal sequence for post-translational modification by the enzyme geranylgeranyltransferase I (Reiss et al., 1991). Other Rho-specific residues are clustered between domains II, III and near the C terminus of the polypeptide. Brac1 also contains a typical poly(lysine) domain next to the CAAL motif. The CXXL motif is known to direct geranylgeranylation and methylesterification of the C-terminal cysteine in Rac and Rho proteins, a modification which is important for membrane localization (Trainin et al., 1996; Finegold et al., 1991). Furthermore, the amino acid sequences of the N-terminal region for GTP and its effector binding regions L1-L4 (positions 13–67) clearly indicate that this clone is more closely related to Rac proteins than to Rho or Cdc42 proteins.

2.2. GTP binding and GTPase activities of the Brac1 protein

To demonstrate the GTP-binding capability of the Brac1 protein, the corresponding cDNA was ligated into the pGEX2T-L1 expression vector thereby constructing pGBrac1. The protein expressed in *E. coli* cells transformed with the recombinant plasmid showed the expected molecular mass of 48 kDa (26 kDa for GST and 22 kDa for the Brac1) on SDS–PAGE by Coomassie Brilliant Blue staining as shown in Fig. 3A. Most of the proteins were found in the cytosolic fraction of *E. coli* (data not shown). The total cytosolic proteins obtained from the transformed *E. coli* cells were separated by SDS–PAGE, transferred onto a nitrocellulose membrane and incubated with [α-^{32}P]GTP using a method that would allow renaturation of small GTP binding proteins. Autoradiography indicated that the

```
-58    ggatcccccaaaaatccccaaatcttgaaaagtttaggttttgaagtttgagagagag
  1   ATGAGTGCTTCGAGGTTTATCAAGTGTGTCACCGTCGGCAACGGCGCTGTCGGAAAGACT
       M  S  A  S  R  F  I  K  C  V  T  V  G  N  G  A  V  G  K  T    20

 61   TGTCTGCTCATTTCCTACACTAGCAACACTTTCCCCACGGACTATGTGCCAACTGTGTTC
       C  L  L  I  S  Y  T  S  N  T  F  P  T  D  Y  V  P  T  V  F    40

121   GATAATTTAAGCGCCAATGTGATTGTGGATGGGAACACTATCAACTTGGGATTGTGGGAT
       D  N  L  S  A  N  V  I  V  D  G  N  T  I  N  L  G  L  W  D    60

181   ACTGCAGGACAAGAGGACTACAATAGACTAAGACCATTGAGCTATCGCGGCGCAGATGTC
       T  A  G  Q  E  D  Y  N  R  L  R  P  L  S  Y  R  G  A  D  V    80

241   TTCTTACTTGCCTTCTCTCTCGTCAGCAAAGCTAGCTATGAAAATGTTTCTAAAAAGTGG
       F  L  L  A  F  S  L  V  S  K  A  S  Y  E  N  V  S  K  K  W   100

301   GTGCCTGAACTGAGACATTATGCACCTGGTGTTCCCATCATCCTCGTTGGAACCAAGCTT
       V  P  E  L  R  H  Y  A  P  G  V  P  I  I  L  V  G  T  K  L   120

361   GATCTTCGAGATGACAAGCAATTCTTTGTTGAGCACCCTGGTGCTGTTCCTATCTCTACT
       D  L  R  D  D  K  Q  F  F  V  E  H  P  G  A  V  P  I  S  T   140

421   GCTCAGGGTGAAGAACTGAAGAAGCTGATTGGGGCACCTGCTTATATCGAATGCAGTGCA
       A  Q  G  E  E  L  K  K  L  I  G  A  P  A  Y  I  E  C  S  A   160

481   AAAACACAACAGAATGTAAAAGCGGTGTTTGATGCGGCTATCAAGGTAGTTCTCCAGCCA
       K  T  Q  Q  N  V  K  A  V  F  D  A  A  I  K  V  V  L  Q  P   180

541   CCCAAAAACAAGAAGAGGAAGAAGAGAAAGTCTCAGAAAGGTTGTTCTATATTGTGAttc
       P  K  N  K  K  R  K  K  R  K  S  Q  K  G  C  S  I  L  *       198

601   aatagagagagagagagagagagagagagagagagagaaggaatcaacatgaggattgaa
661   gtagctccacctgtttgtttccttcaagaaaggaaggaactgttgtttgtagcctttaaa
721   aatattcttgttttcattttgatctgtaaatataccatggatccttttatttctttgctt
781   cctagactcacgacttgtttagagattaatctccaataacgttttatgtggtttcagcca
841   catataatcagttatgaacaagtatattaaaaaaaaaaaaaaa
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Fig. 1. Nucleotide and predicted amino acid sequences of *Brac1* cDNA. The deduced amino acid sequence is shown using the single-letter code. Numbers on the right refer to the amino acid sequence; numbers on the left refer to the nucleotide sequence. The TGA stop codon at the end of the long open reading frame is marked with an asterisk.

48-kDa protein expressed in the bacterial strain did bind [α-^{32}P]GTP, as shown in Fig. 3B. These results demonstrate that pGBrac1 expressed in *E. coli* is an active GTP-binding protein.

The binding specificity of this protein was examined in a competition experiment as shown in Fig. 4. At all the concentrations of competitors examined, the guanine nucleotides as GTP, GTPγS or GDP, but not ATP, effectively competed with [α-^{32}P]GTP in the binding activity of Brac1. Incubation of the blots with only 50 μM of the guanine nucleotides completely blocked the binding of [α-^{32}P]GTP. However, ATP did not affect the binding of [α-^{32}P]GTP at all. In the case of CTP, under more than 100 μM concentration it slightly blocked its binding to Brac1. Although UTP almost blocked binding of [α-^{32}P]GTP at 200 μM, a labeled Brac1 band could be still detected. This assay clearly demonstrates that Brac1 had binding specificity for di- and triphosphate forms of guanine nucleotides.

In addition to GTP binding specificity, we examined the hydrolyzing activity of Brac1 since plant rho-related small GTP-binding proteins are known to have intrinsic GTPase activity. Analysis of the GTPase activity of this protein on a PEI-cellulose/TLC plate revealed that the free forms of GDP gradually increased as the incubation time progressed, whereas no such reaction was observed in the control samples in which the protein was absent (Fig. 5).

2.3. PLD activity regulation by the Brac1 protein

We used Brac1 to examine the detailed mechanism of one Rho subfamily small GTP-binding protein, to determine whether or not it stimulates PLD activity in plants. We assayed for PLD activity using rat brain PLD in the presence of guanosine-5′-*O*-(3-thiotriphosphate)(GTPγS), with the reaction being initiated by the addition of either PKC or ARF. Of the Rho subfamily proteins, studies using dominant negative forms of Rac1 have implicated this protein type in the regulation of PLD by growth factors (Hess et al., 1997), but there have been no reports of a similar cellular role for Cdc42Hs. To measure guanine nucleotide-sensitive or guanine nucleotide-dependent PLD activity, a simple assay system was devised. This assay is convenient in that it utilizes an exogenous radiolabeled substrate presented in the form of phospholipid vesicles and allows for simple analysis of hydrolytic products. GTPγS, in

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                        I                         E
                          *   *           *      ** N42
Brac1      MSASRFIKCVTVGNGAVGKTCLLISYTSNTFPTDYVPTVFDNLSANVI-VD   50
ARAC3 A    .............D............................F.....-..   50
Rho1P P    ...............................................V-.N   50
Rac9  C    .NT..................M..........................-.D   50
CDC42 S    .PT---.......................K..SD.........AVT..-IG   47
RhoA  H    .ATI.-K.L.I.....C.......VFSKDQ..EV......E.YV.DIE-.D   49
Ypt2  S    KSYDYLL..L.GAS.V...SALTLR..E.S.TPSFIT.IGID.KIRTIEL.   51
Rab2  H    MA.A..F.YI.IGATG........Q..DKR.Q.VHDL...VE.G..M.TI.   51

                      II
                 **       * *** ***     **   * *  *          *
Brac1      GNTINLGLWDTAGQEDYNRLRPLSYR-GADVFLLAFSLVSKASYENVSKKW  100
ARAC3 A    ..........................-........................  100
Rho1P P    .S.V......................-.....I.....I............  100
Rac9  C    ..........................-.............R......H...  100
CDC42 S    DE.YT...F.................-......V...VTS.......KE..   97
RhoA  H    DKQ.E.A..................P-DT..V.MC..ID.PD.L..IP...   99
Ypt2  S    ..RIKLQ.L......RFRTITTAYM.-TGMGI.LLYDVTDTK.FDD.-RTR  100
Rab2  H    ..Q....I.......S..S..RS...-..A.A..V..I.RRDT.NHL-T..  100

                             III
            **  *      *          ***             *
Brac1      VPELRHYAP-GVPIILVGTKLDLRDDKQFF--VEHPGAVPISTAQGEELKK  148
ARAC3 A    .........-....................--A..................  148
Rho1P P    I...K....-....................--VD.......T.......R.  148
Rac9  C    ....R....-N...V..............L--S.N...IS...S.......  148
CDC42 S    F...H....-GV.CLI...QI.....PSVQQ..ARQHQH.L.HE..ERL.R  147
RhoA  H    I..VT.FC.-N..F....N.K...N.EHTRRELAKMKQE.VKP.EARDMAN  149
Ypt2  S    LSN.EQHDSENVYKILIGNQCDCEAQRV------------.SFE..QAV.D  139
Rab2  H    .EDAR..SNS.M..M.....S..ESR.E------------.KK.....F.R  139

                     IV                                   CAAL
                *   *        * * * ** * * *          *       *
Brac1      LIGAPAYIECSAKTQQNVKAVFDAAIKVVLQPPK-NKKRKKRKSQKGCSIL  198
ARAC3 A    ..................................-...K............  198
Rho1P P    ..N........S.S............R.......-Q..-..S.AQKA....  197
Rac9  C    M.G.VT.......T.........V...IA.R...-P.R--.PIKRRS.AF.  196
CDC42 S    .L.....V...........N......VAA.D.PV-PH.K.S.-----.LV.  192
RhoA  H    R..SFG.M....L.KDGLRE..EM.TR..LEA-R-RGSR.-----SG....  193
Ypt2  S    ELGV-KFLEASALTNVGLDEAFFTLAREIK                 .C    170
Rab2  H    .H..-I.M.T...AS..E...INT.K..Y                  .C    170
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Fig. 2. Alignment of Brac1 amino acid sequence with those of other members of the Rho subfamily. Amino acid sequences were aligned using the PC/GENE software program. Conserved domains referred to as I, II, III, IV and E (effector domain) are shown above aligned sequences. Conserved amino acids unique to Rho proteins are marked with asterisks. CAAL is a motif recognized by an enzyme geranylgeranyl transferase I. N42 is the site for ADP-ribosylation by a C3 ribosyltransferase. Dashes represent gaps introduced into the alignment for maximizing sequence identity. Dots represent exact matches with the Brac1 sequence. Species abbreviations: A, *Arabidopsis thaliana*; P, *Pisum sativum*; C, *Gossypium hirsutum*; S, *Schizosaccharomyces pombe*; H, Human.

the absence of PMA (phorbol 12-myristate 13-acetate), did not stimulate PLD activity, even in the presence of ARF and Brac1, as shown in Fig. 6A. However, in the presence of both PMA and GTPγS, PKC-mediated PLD activity was synergistically potentiated by Brac1 (Fig. 6B). PMA has long been known to be one of the most potent agonists for PKC-mediated PLD activity and this was the case. Lee et al. (1997) also demonstrated that purified PKCα has functioned as a positive regulator of PLD activity only in the presence of PMA. Furthermore, when the amount of Brac1 protein was increased to 6 μg, it showed the same trend as that of the Rho protein, which was used as a positive control. Here we found that Brac1 could synergistically activate PLD with PKC, especially in the presence of PMA, but not with ARF under the same conditions except PMA. This suggests that Brac1 may be involved in the in vivo regulation of PLD. However, this is a tentative conclusion given the exclusive reliance on an in vitro PLD activity assay.

2.4. Genomic complexity of the Brac1 gene

To establish the copy number of the *Brac1* gene in the *Brassica* genome, genomic DNA digested with *Bam*HI, *Eco*RI and *Hin*dIII was hybridized using *Brac1* cDNA as a probe. Fig. 7 shows that the ^{32}P-labelled *Brac1* gene can hybridize to *Brassica* genomic DNA. In addition,

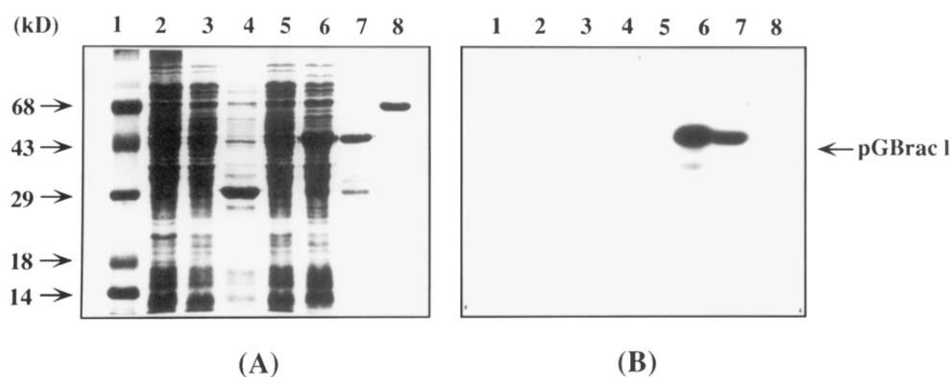

Fig. 3. GTP-binding assay for the pGBrac1 protein expressed in *E. coli*. (A) Each of total proteins (10 μg) from induced (+) and uninduced (–) *E. coli* carrying the pGEX2TL-Brac1 (see Fig. 3B legend) was analyzed by SDS–PAGE on a 12.5% polyacrylamide gel and stained with Coomassie Brilliant Blue. (B) The proteins shown in (A) were electroblotted onto a nitrocellulose filter and incubated with 60 μCi of [α-^{32}P]GTP (3000 Ci/nmol). Lane 1, protein molecular weight markers; Lane 2, *E. coli* BL21(DE3)pLys S; Lane 3, pGEX2TL1(–); Lane 4, pGEX2TL1(+); Lane 5, pGBrac1(–); Lane 6, pGBrac1(+); Lane 7, purified pGBrac1; Lane 8, BSA.

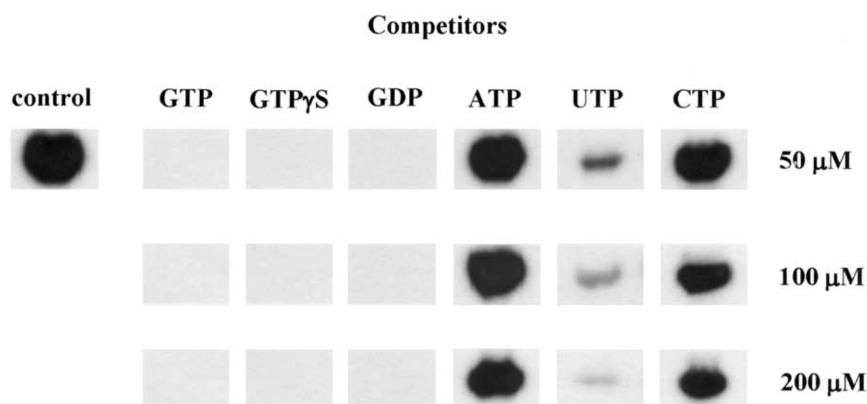

Fig. 4. Ability of various nucleotides to compete with Brac1 for binding of 10^{-9} M [α-^{32}P]GTP. The GTP-binding activity of the crude protein was assayed in the presence of each unlabeled nucleotide supplied as indicated concentrations of GTP, GTPγS, GDP, ATP, UTP and CTP. The control sample was incubated with 10^{-9} M [α-^{32}P]GTP only.

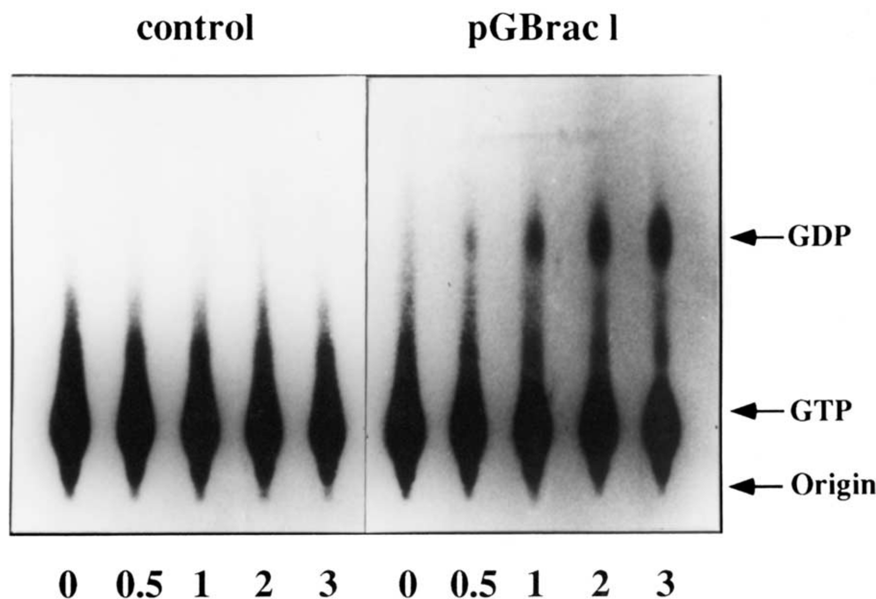

Fig. 5. GTP hydrolysis of the Brac1 protein. The purified protein (1 μmol) was incubated with 33 nM [α-^{32}P]GTP at 30 °C in the presence of 10 mM $MgCl_2$. Reaction mixtures were separated into GTP and GDP on a PEI-Cellulose TLC plate and visualized by autoradiography. The numbers under the autoradiogram indicate the incubation times in hourly units.

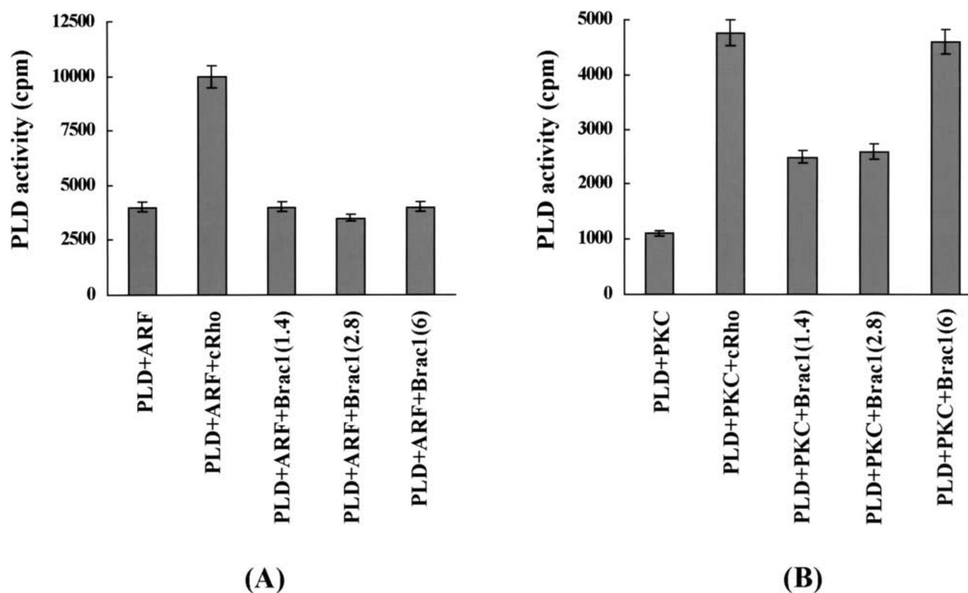

Fig. 6. Regulation of PLD activity by the Brac1 protein. (A) Effect of pGBrac1-throm on ARF-dependent phospholipase D activity. The concentrations of each reaction component were as follows; PLD, 50 pM; ARF, 750 nM; control Rho (cRho), 50 nM; GTPγS, 1 mM. (B) Effect of pGBrac1-throm on PKC-dependent phospholipase D activity. PLD, 50 pM; PKC, 10 nM; control Rho, 50 nM; GTPγS, 1 mM; PMA 100 nM. cRho was derived from human RhoA cDNA expressed in *E. coli*, used as control. ARF and PKC were prepared from rat brain cytosolic fraction according to Lambeth et al.'s (1995) and Lee et al.'s (1997) methods, respectively. The vertical axis indicates the PLD activity in a cpm unit as the mean (±S.D.) of three independent experiments. The amount (μg) of Brac1 used is shown in parentheses.

there are several strongly or weakly hybridizing fragments. From these, we deduced that the *Brac1* gene might be a member of a multigene family in the *Brassica* genome, as in *A. thaliana* (Winge et al., 1997), *Pisum sativum* (Yang and Watson, 1993), cotton (Delmer et al., 1995) and other species. However, it is not clear whether the weakly hybridized bands represent additional *Brac1* genes or genes encoding other closely related proteins in *Brassica*.

2.5. Analysis of Brac1 transcripts

Since a gene encoding a rac-like small GTP binding protein has been isolated from the *Brassica* cDNA library, we examined the level of its expression by northern blot analysis using total RNA isolated from leaves, flower buds and root organs of *Brassica campestris*. Hybridizing transcripts were more abundant in flower buds than any other organ (Fig. 8A). To investigate the distribution pattern of the *Brac1* mRNA accumulation, this assay was also applied to total RNA prepared from flower buds (F), anther (A), stigma (St), petal (P) and sepal (Se) (Fig. 8B). The transcripts were most abundant in flower buds and stigma. However, using this approach, we could not deduce the precise level of *Brac1* gene transcription, since a few other highly related genes are present in the genome of this plant, as revealed by the genomic Southern analysis.

To date, most plant small GTP-binding proteins have been isolated on the basis of sequence homology to those of other organisms, using either heterologous genes or oligonucleotides as probes or as PCR primers, respectively (Terryn et al., 1993). However, the functions of these plant proteins are largely unknown. One possible function of plant Rac protein could be regulation of cell wall synthesis. It is possible that this protein functions as a major organizer of the actin cytoskelton providing the necessary building blocks for cell wall assembly. Actin is also a major part of the phragmoplast and is thought to have key functions in the process of directing transport vesicles to the cell plate (Staehelin and Hepler, 1996).

Interestingly, ARF-responsive PLD activity has been found in the Golgi nuclei, and in plasma membranes of several cell types. It has been proposed that activation of PLD in the Golgi is important for vesicle trafficking in this organelle (Brown et al., 1993).

Another function of this protein is suggested by recent results from animal systems, where activated forms of Rac and Cdc42 proteins have been shown to control C-jun N-terminal kinases (JNKs) SAPKs (Coso et al., 1995), but so far there have been no reports of a similar Rac-regulated cascade in plants. However, given that striking parallels exist between the oxidative burst in animal phagocytes and in plant cells exposed to microbial elicitors, it is suggested that plant Rac proteins may have functions similar to their animal counterparts (Abo et al., 1991). Moreover, the expression of *A. thaliana Rac* gene in *Schizosaccharomyces pombe* has been shown to induce severe morphological changes

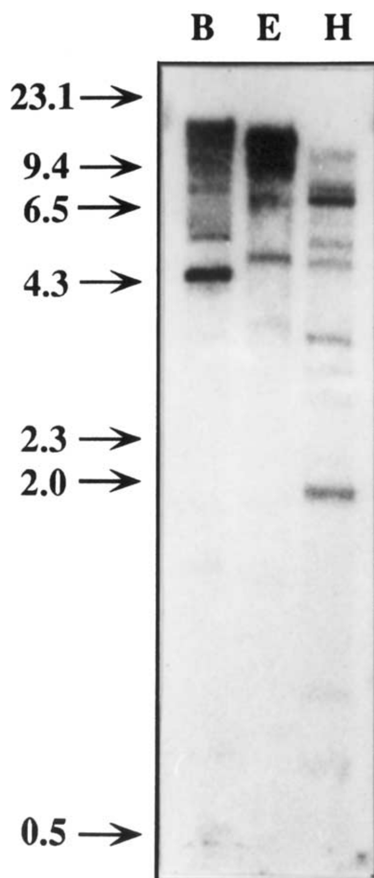

Fig. 7. Southern blot analysis of *Brassica campestris Brac1* gene. Chinese cabbage genomic DNA (10 μg) were digested with restriction endonucleases, *Bam*H1 (lane B), *Eco*R1 (lane E) and *Hin*dIII (lane H). Digested DNAs were subjected to electrophoresis on a 0.8% agarose gel, transferred to a nylon membrane and hybridized with ^{32}P-labeled *Brac1* cDNA under low stringency conditions.

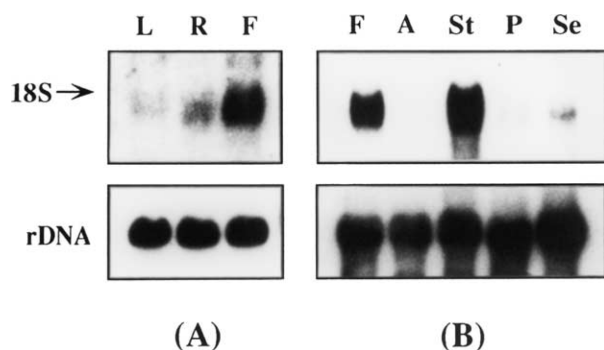

Fig. 8. Northern blot analysis of *Brac1* mRNA in different organs. (A) Total RNA (20 μg) was prepared from leaves (lane L), roots (lane R) and flower buds (lane F) of mature Chinese cabbage and were fractionated on a formaldehyde agarose gel. Each of the RNA blots was analyzed with a ^{32}P-labeled *Brac1*. (B) Spatial distribution of *Brac1* transcripts in mature Chinese cabbage flower buds. Aliquots of total RNA (20 μg) from flower buds (lane F), anthers (lane A), stigma (lane St), petal (lane P) and sepal (lane Se) of mature Chinese cabbage were electrophoresed, transferred to a nylon membrane and hybridized with ^{32}P-labeled *Brac1*. As a control, 18S rRNA was used.

(Xia et al., 1996), indicating that plant Rac proteins may have similar functions to Rac/Cdc 42 in yeast and animal cells.

In conclusion, our results here indicate that Brac1 plays a synergistically activating role in PKC-dependent, but not in ARF-dependent, PLD. Importantly, this distinction is a contrast to the similar functions of ARF and Rac proteins that have previously been observed in animal and plant systems.

To further elucidate the regulation of signal transduction by plant small G-proteins, more extensive work on the identification and characterization of additional factors that regulate PLD is required.

3. Experimental

3.1. Plant materials and construction of Chinese cabbage flower bud cDNA library

Chinese cabbage (*Brassica campestris* L. ssp. *pekinensis*) was grown in a growth chamber under various conditions. Seeds were aseptically sown on a mixed bed of vermiculite and peat moss (1:1, v/v) and grown for 21 days. Flower buds of approximately 5 mm in length were harvested from the seeds purchased at Seoul seeds Co. (Seoul, Korea). Different organs of each sample were dissected and used for the preparation of genomic DNA, RNA and a cDNA library. To construct a cDNA library we extracted total RNA from flower buds as described previously (Ausubel et al., 1991). Poly(A)$^+$ RNA was obtained using a commercially available kit [poly(A)$^+$RNA purification kit; Pharmacia]. cDNAs were made using a λ Zap cDNA synthesis kit (Stratagene) and cloned into pBluescript II KS(+) using unphosphorylated adaptors according to the manufacturer's instructions.

3.2. DNA sequence analysis

A cDNA encoding Brac1 was obtained from ESTs of a Chinese cabbage flower bud cDNA library. Nucleotide sequences were determined from both strands of cDNA using a Taq dye primer cycling sequencing kit on a 373A automatic DNA Sequencer (Applied Biosystems Inc.). Nucleotide and deduced amino acid sequences were analyzed using the GCG sequence analysis program. The phylogenetic relationship of *Brac1* and other known genes encoding small GTP binding proteins was analyzed using the PCGENE software program (Higgins and Sharp, 1988).

3.3. Southern and northern blot hybridization

Genomic DNA from *Brassica* leaves was prepared according to the CTAB method (Murray and Thompson,

1980). Three different restriction digests of genomic DNA were prepared, electrophoresed on a 0.8% agarose-TBE gel, and transferred onto a nylon membrane (GeneScreen Plus, Dupont). After UV crosslinking using a UV stratalinker 1800 (Stratagene), the membrane was hybridized with a ^{32}P-labeled *Brac1* cDNA clone. Hybridization was carried out for 24 h at 55 °C in a solution containing 6 × SSC, 5 × Denhardt's solution (1 × Denhardt's solution is 0.02% w/v PVP and 0.02% w/v BSA), 0.5% w/v SDS, and 100 μg/ml denatured calf thymus DNA and a 5×10^6 cpm/ml probe at 55 °C. The hybridized blots were finally washed in 2 × SSC, 0.1% w/v SDS at 65 °C. The filters were then dried and exposed to a photographic film for appropriate times at −70 °C. For northern blot analysis, tissues and organs were obtained from 14-day-old seedlings and flower buds of *Brassica*. Total RNA was extracted from the sections by the guanidium thiocyanate/phenol/chloroform extraction method, and further purified by ultracentrifugation (Sambrook and Russell, 2001). Twenty microgram-aliquots of total RNA were electrophoresed on a 1.4% gel, transferred and crosslinked to a nylon membrane by UV irradiation. The probe for hybridization was a ^{32}P-labeled *Brac1* cDNA clone. Hybridization conditions were the same as those used for southern blotting, except for the reaction and final washing temperatures, which were 53 and 60 °C, respectively.

3.4. Overexpression of a Brac1 cDNA in Escherichia coli

To overexpress a *Brac1* cDNA in *E. coli* strain BL21/DE3 (pLysS), we constructed an expression vector pGBrac1. The *Brac1* DNA segment for vector insertion was amplified by PCR using a recombinant derivative of pBKS(+)II containing the full length of the rac-like small G-protein. The forward and reverse primers were as follows; 5′-GTTTTGAAGCTTTGAGAG-3′ (designed to create a *Hin*dIII restriction site) and 5′-ATTAACCCTCACTAAAG-3′ (T3 primer), respectively. The PCR product digested with *Hin*dIII was purified and ligated into the same restriction site of the multicloning region of pGEX2T Linker1 (Pharmacia). The sequence fidelity of the PCR product was confirmed by DNA sequencing. Expression of these constructs would result in a fusion protein consisting of Brac1 ligated to the carboxyl terminus of glutathione *S*-transferase (GST). The construct was transformed into *E. coli* BL21/DE3 (pLysS). The transformants were inoculated into a 5-ml aliquot of the enriched medium containing 2% w/v tryptone, 1% w/v yeast extract, 0.2% w/v NaCl and 50 μg/ml ampicillin. The culture was grown at 37 °C overnight, and then supplemented with 500 ml of fresh enriched medium. IPTG was added to the culture when it reached an optical density of 0.6 at 590 nm to obtain a final concentration of 0.4 mM, and incubation was continued for a further 4 h at 37 °C. The bacterial cells were harvested by centrifugation at 6000 *g* for 10 min at 4 °C, resuspended in 15 ml of STE buffer consisting of 10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, and 100 μg/ml lysozyme was added. DTT was added to a final concentration of 5 mM and sonicated. Proteins were precipitated by centrifugation at 12,000 *g* for 30 min at 4 °C. The expressed plant GST fusion protein was mixed with 2 ml of 50% glutathione sepharose-4B column (Sigma) and incubated for 1 h at 4 °C with gentle shaking. The agarose beads were washed four times with 10 ml PBS containing 50 mM Na_2HPO_4/KH_2PO_4, pH 7.2 and 0.12 M NaCl. The fusion protein was eluted by competition with glutathione, that is, through 2 × 2-min-washes with 10 mM glutathione in 50 mM Tris–HCl, pH 8.0. Free glutathione was removed by dialysis against 50 mM Hepes (pH 7.5/ NaOH), 2 mM $MgCl_2$ and 2 mM DTT.

3.5. GTP binding and competition assay

To determine the GTP binding activity of this protein, samples from bacterial crude or purified extracts were separated by 12.5% SDS–PAGE. Gels were incubated for 45 min at 4 °C in the aforementioned PBS solution, and the proteins were electrophoretically transferred to nitrocellulose filters. After washing the membrane, incubation was performed with 60 μCi [α-^{32}P]GTP according to the method described previously (Schmitt et al., 1986). The radiolabelled band was detected by autoradiography. In competition experiments, the blots were first incubated with buffer A consisting of 20 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 2 mM DTT, 0.1% v/v Tween 20 and 0.3% w/v BSA including 100 μl each of unlabelled competing substances such as GTP, guanosine 5′-(γ-thio)triphosphate (GTPγS), GDP, ATP, UTP or CTP, and then incubated with buffer B consisting of buffer A with 1 nM [α-^{32}P]GTP under the same conditions.

3.6. GTPase assay

GTP hydrolytic activity was measured using a previously described method with minor modifications (Seo et al., 1997). Briefly, the reaction was carried out in a final volume of 100 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]GTP (3000 Ci/mM), 1 mM ATP and 1 μM of pGBrac1 protein at 30 °C. Five-microliter samples were collected at 1-h intervals followed by an addition of 5 μl of 0.5 M EDTA to stop the reaction. An aliquot (1 ml) was 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 X-ray film.

3.7. Assay of PLD activity

The fusion protein (~2 mg) was incubated with thrombin (4 μg) at 37 °C for 20 min in 5 ml reaction buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 2.5 mM $CaCl_2$ and 0.1% β-mercaptoethanol). The fusion protein cleaved by thrombin was used to assay PLD activity. This was determined by the method of Brown et al. (1993) with minor modifications. Phospholipid vesicles were prepared by mixing phospholipids in a molar ratio of PE: PIP_2: PC = 16: 1.4: 1, upon drying under a stream of nitrogen and sonication in a vesicle buffer (50 mM Hepes/NaOH, pH 7.5, 80 mM KCl, 3 mM EGTA). [Choline-methyl-^{3}H]dipalmitoyl-PC was added to allow about 150,000 cpm/0.25 nM per assay. The reaction was carried out at 37 °C for 15–20 min in a volume of 150 μl containing 50 mM Hepes/NaOH, pH 7.5, 5 mM $MgCl_2$, 3 mM $CaCl_2$, 3 mM EGTA, 80 mM KCl, phospholipid vesicles and PLD enzyme preparation. Other PLD regulating proteins such as ARF or the resolved cytosolic fractions and regulating ligands, for example, GTPγS, PMA or ATP, were also included as indicated in the figure legends (Fig. 6). The reactions were quenched by addition of 1 ml $CHCl_3$:MeOH: HCl = 50:50:0.3 and 0.35 ml of 1 N HCl. The mixture was shaken vigorously, and a 0.5-ml aliquot of the supernatant was withdrawn for liquid scintillation counting after centrifugation at 2000 *g* for 5 min.

Acknowledgements

PLD is a kind gift from Professor S.H. Ryu's lab at POSTEC in Korea. This work was supported by a grant (No. 2000-015-DP0314) from KRF to the Research Institute of Natural Sciences (RINS) at Gyeongsang National University and in part, by the Agricultural Plant Stress Research Center at Chonnam National University.

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