Pleckstrin Induces Cytoskeletal Reorganization via a Rac-dependent Pathway*

(Received for publication, March 5, 1999, and in revised form, July 16, 1999)

Alice D. Ma‡ and Charles S. Abrams§
From the Department of Medicine, University of Pennsylvania Medical School, Philadelphia, Pennsylvania 19104

Pleckstrin homology (PH) domains are present in over one hundred signaling molecules, where they are thought to mediate membrane targeting by binding to phosphoinositides. They were initially defined at the NH2 and COOH termini of the molecule, pleckstrin, a major substrate for protein kinase C in platelets. We have previously reported that pleckstrin associates with the plasma membrane, where it induces the formation of villous and ruffled structures from the surface of transfected cells (1). We now show that overexpression of pleckstrin results in reorganization of the actin cytoskeleton. This pleckstrin effect is regulated by its phosphorylation and requires the NH2-terminal, but not the COOH-terminal, PH domain. Overexpression of the NH2-terminal PH domain alone of pleckstrin is sufficient to induce the cytoskeletal effects. Pleckstrin-induced actin rearrangements are not inhibited by pharmacologic inhibition of phosphatidylinositol 3-kinase, nor are they blocked by co-expression of a dominant negative phosphatidylinositol 3-kinase. The cytoskeletal effects of pleckstrin can be blocked by co-expression of a dominant negative Rac1 variant, but not wild-type Rac and not a dominant negative Cdc42 variant. These data indicate that the NH2-terminal PH domain of pleckstrin induces reorganization of the actin cytoskeleton via a pathway dependent on Rac but independent of Cdc42 and phosphatidylinositol 3-kinase.

PH1 domains are a family of protein motifs present in more than one hundred signaling proteins. PH domains are thought to mediate intramolecular interactions, bringing their proteins into proximity with critical upstream or downstream targets. NMR and x-ray crystallography studies have revealed a remarkable similarity in the three-dimensional structures of all PH domains studied to date, despite divergence in their amino acid sequence (2–8). Harlan and co-workers tested PH domains for their ability to bind lipids and demonstrated that they bind to phosphoinositides (9). Since then, a number of reports have shown that the binding of PH domains to inositol phospholipids regulates protein function (1, 5, 10–14). In addition, some PH domains may interact with other targets such as the βγ subunits of heterotrimeric G proteins (15–18) or protein kinase C (19–21).

Despite the structural similarity of PH domains, they exhibit heterogeneity in their specificity and selectivity for inositol phospholipids (22, 23), as well as in their function (1, 24, 25). Moreover, the function of the protein may be regulated by the specific phosphoinositide bound to the PH domain. For example, the PH domain of Vav appears to regulate the guanosine exchange activity of Vav. This PH domain-mediated inhibition is augmented by the binding of the PH domain to phosphoinositide 4,5-bisphosphate (PIP2). Alternatively, this effect is decreased by the binding of the PH domain to phosphoinositide 3,4,5-trisphosphate (11). The structure of the tandem Dbl homology (DH) exchange motif/PH domain of a related protein, Sos, has recently been solved and confirms that lipid binding to the PH domain should be functionally coupled to the GTPase binding site of the DH domain (26).

Many signal transduction pathways lead to reorganization of the actin cytoskeleton, thus mediating motility, shape change, and attachment to substrate. Several proteins involved in these pathways are known to contain PH domains, and the PH domain may be critical in the regulation in many of these proteins, perhaps by affecting locally available levels of specific phosphoinositides (11, 12, 24, 27–29). Pleckstrin is a 40–47 kDa protein which contains the two prototypic PH domains at its NH2 and COOH termini. It was first described as a major substrate for protein kinase C in platelets and leukocytes, and its phosphorylation has long been used as a marker for platelet activation. Though its function in vivo remains unclear, we have shown that expressed pleckstrin can inhibit PIP2-based signaling mediated by phospholipase C, a 5-inositol phosphatase and PI3K (30–32). Ser113, Thr114, and Ser117, the three residues phosphorylated by PKC, lie adjacent to, but not within the NH2-terminal PH domain, and their phosphorylation appears to regulate the function of this domain (33). We have previously demonstrated that pleckstrin will bind to plasma membranes, where it induces the formation of ruffled and villous structures at the surface of transfected cells. These effects require pleckstrin to be phosphorylated and are dependent on the presence of the amino-terminal of pleckstrin but not the carboxyl-terminal, PH domain (1).

In this report, we demonstrate that transient overexpression of pleckstrin in COS-1 cells leads to reorganization of the actin cytoskeleton, with dissolution of central actin cables and reappear-
and are at least partially dependent on Rac because co-expressing a dominant negative variant of Rac1 (V12N17 Rac1) along with pleckstrin blocked most of the cytoskeletal changes.

MATERIALS AND METHODS

Expression Vectors—The generation of expression plasmids encoding hemagglutinin antigen (HA) epitope-tagged full-length human pleckstrin, and pleckstrin variants missing the amino-terminal PH domain (AN-PH), the carboxyl-terminal PH domain (AC-PH), and both the amino- and the carboxyl-PH domain (Δ2PH), as well as pleckstrin variants with Ser\(^{113}\), Thr\(^{114}\), and Ser\(^{117}\) mutated to either glutamates (3 Phos Gly) or to glycines (3 Phos Gly) has been described previously (1, 30, 33). Vectors encoding either the amino-terminal PH domain alone (AN-PH) or the carboxyl-terminal PH domain alone (C-PH) with the HA epitope tag fused to the carboxyl-terminal of the molecule were generated by PCR overlap extension according to the technique of Ho et al. (34). These cDNAs were cloned into pCMV5, and the full-length sequences were confirmed. Plasmids encoding Myc-tagged V12N17 Rac and Myc-tagged N17 Cdc42 were a gift of Dr. Alan Hall (University College, London, UK). The plasmid directing the synthesis of the dominant negative p85 \((Δp85/pCMV5)\) was subcloned from the EcoRI- and BamHI-digested cDNA insert of pSG5 bovine p85 \(Δ478–533\) (a generous gift of Julian Downward, Imperial Cancer Research Fund, UK).

Cell Culture, Transfection, and Immunofluorescence—COS-1 cells (American Type Cell Culture, Manassas, VA) were grown in Dulbecco’s Eagle medium (Life Technologies, Inc., Gaithersburg, MD) under 5.5% CO\(_2\). Cells in 100-mm plates were transfected with 20 μg of plasmid DNA using DNA-calcium phosphate co-precipitation. Twenty-four hours after transfection, cells were shocked with 10% glycerol. After glycerol shocking, cells were trypsinized and replated onto 2-well chamber slides (Becton Dickinson, Franklin Lakes, NJ). For PI3K inhibition, cells were treated overnight with either Me\(_2\)SO alone (Fisher Scientific, Pittsburgh, PA), 50 μM wortmannin, or 100 μM LY294002 (Biomol, Plymouth Meeting, PA). Twenty-four hours later, slides were fixed and stained as described previously (1, 12). Actin staining was performed using rhodamine-labeled phalloidin (Molecular Probes, Eugene, OR) according to the protocol of the manufacturer.

Antibodies—For Western blotting, antibodies used include HA.11, a murine monoclonal antibody directed against the hemagglutinin HA tag (BAbCO, Richmond, CA), 9E10, a murine monoclonal antibody directed against the Myc epitope (BAbCO, Richmond CA), and A431, a monoclonal antibody directed against the p85 subunit of PI3K (Transduction Laboratories, San Diego, CA).

RESULTS

Pleckstrin Leads to Reorganization of the Actin Cytoskeleton—Having determined that expressed pleckstrin could alter the surface membrane architecture of transfected cells (1), we wished to determine whether pleckstrin could induce reorganization of the actin cytoskeleton. Based on the observation that transfected cells expressing pleckstrin developed ruffle-like membrane projections, we hypothesized that pleckstrin might induce the formation of cortical actin. To test this hypothesis, we transiently transfected COS-1 cells with either empty vector or with a plasmid directing the expression of HA epitope-tagged wild-type pleckstrin. We stained the cells with 12CA5, a monoclonal antibody directed against the HA epitope, FITC-labeled goat anti-mouse secondary antibody, and rhodamine-labeled phalloidin. Cells expressing HA-tagged pleckstrin were identified by the presence of FITC staining, and their pattern of F-actin polymerization was assayed by visualization of rhodamine staining. Rhodamine staining is shown in panels a and c. FITC staining is shown in panel b. a, more than 90% of cells transfected with empty vector or untransfected cells showed thick F-actin cables in the center of the cell. Rather, the F-actin had reorganized around the peripheral actin cables. Rather, the F-actin had reorganized around the peripheral actin cables. c, rhodamine phalloidin staining of these pleckstrin-expressing cells showed no central actin cables. Rather, the F-actin had reorganized around the peripheral actin cables. d, more than 95% of cells expressing HA-tagged wild-type pleckstrin had the pleckstrin concentrated within membrane ruffles at the surface of the cell. e, rhodamine phalloidin staining of these pleckstrin-expressing cells showed no central actin cables. Rather, the F-actin had reorganized around the peripheral actin cables. Bars represent 38 microns. In the bottom panel, an anti-HA immunoblot demonstrates expression of HA-tagged pleckstrin in lane 1, but none in mock-transfected cells (lane 2).

Pleckstrin Must be Phosphorylated to Cause Actin Reorganization—Having determined that pleckstrin was capable of inducing actin reorganization, we next asked if the effects of pleckstrin on the cytoskeleton required pleckstrin phosphorylation. To do this, we examined the pattern of F-actin polymerization in cells expressing a HA-tagged pleckstrin variant which had Ser\(^{113}\), Thr\(^{114}\), and Ser\(^{117}\) residues phosphorylated by PKC (33), mutated to either glycines (3 Phos Gly) or glutamates (3 Phos Gly). We have previously shown that replacing these three residues with glycines results in a pleckstrin variant that is inactive with regard to inhibition of PLC or PI3K (31, 35, 36). By contrast, substitution of these residues with glutamate through changes in the actin cytoskeleton. These effects were seen in >90% of pleckstrin-expressing cells. Additional experiments were performed to study the pleckstrin-mediated effects on the cytoskeleton in a wide variety of different tissue culture cells. We have seen a similar phenotype in all other cell lines we have tested, including transformed cell lines such as COS7, HEK 293, CHO, NIH 3T3 fibroblasts, HepG2 hepatoma cells, and primary cells including REF cells and WRTL thyroid cells (data not shown).
Pleckstrin must be phosphorylated to induce actin reorganization. This figure shows that charged glutamate residues in place of the phosphorylation sites of pleckstrin lead to a constitutively active variant which induces actin changes, and neutral glycine residues in those sites leads to an inactive pleckstrin variant. COS-1 cells were transiently transfected with plasmids directing the expression of pleckstrin variants that had the residues normally phosphorylated by PKC (Ser113, Thr114, Ser117) collectively mutated to either glutamates (3 Phos Glu) (panels a and b) or to glycines (3 Phos Gly) (panels c and d) and were then fixed and stained as described in Fig. 1. Panels a and c show FITC staining. Panels b and d represent rhodamine staining. a, FITC staining shows the 3 Phos Glu variant within membranous projections from the surface of transfected cells. b, rhodamine phalloidin staining shows cortical actin reorganization around the cell periphery. c, cells expressing the 3 Phos Gly variant do not have membrane ruffles. d, the actin cytoskeleton in cells expressing the 3 Phos Gly variant is not distinguishable from that of nontransfected cells. There are still central actin cables and no cortical actin. Bottom panel, Western blotting confirmed equal levels of expression of the 3 Phos Glu (lane 1) and the 3 Phos Gly variant (lane 2). Lane 3 is from vector-only transfected cells. Bars represent 38 microns.

mates, which mimics the charged state of phosphorylation, creates a pleckstrin variant that is constitutively active. More than 90% of cells expressing the 3 Phos Glu pleckstrin variant showed a cortical pattern of F-actin polymerization around the periphery. In addition, there were smaller actin bundles underly ing the surface membrane projections (Fig. 2b). This pattern is similar to that produced by wild-type pleckstrin because overexpressed pleckstrin in COS-1 cells exists predominantly in the phosphorylated state (33). By contrast, all cells expressing the 3 Phos Gly pleckstrin variant, which is not phosphorylated, continue to demonstrate central F-actin cables and neither exhibit cortical actin nor contain actin bundles in membrane projections (Fig. 2d). This pattern is similar to that of nontransfected cells, indicating that phosphorylation is required for pleckstrin to induce cytoskeletal reorganization.

The NH2-terminal PH Domain Is Required for Actin Reorganization—To determine whether the PH domains of pleckstrin were responsible for its ability to induce actin rearrangements, we expressed an HA-tagged variant of pleckstrin missing both of its PH domains (Δ2-PH) in COS-1 cells and stained the cells for the HA epitope and actin. Cells expressing the PH domain-deficient pleckstrin showed no dorsal projections, and the pleckstrin was diffusely distributed within the cell (Fig. 3a). All of these cells had absent cortical actin staining, and they continued to show central actin cables, indistinguishable from cells which were not transfected (Fig. 3b), arguing that the presence of the PH domains was required for the ability of pleckstrin to reorganize the cytoskeleton.

To determine the relative contribution of the individual PH domains on pleckstrin-induced actin rearrangements, we analyzed the pattern of F-actin polymerization in cells expressing pleckstrin variants which were missing either the NH2-terminal or the COOH-terminal PH domain. More than 90% of cells transfected with a pleckstrin variant missing the NH2-terminal PH domain (ΔN-PH) failed to rearrange their actin. Their F-actin remained within central bundles and did not appear around the periphery, demonstrating that the NH2-terminal was critical for cytoskeletal reorganization (Fig. 3d). By contrast, all cells expressing a pleckstrin variant missing the COOH-terminal PH domain (ΔC-PH) showed actin rearrangements indistinguishable from those produced by wild-type pleckstrin. There was dissolution of the central F-actin, and a redistribution of actin around the periphery (Fig. 3f), indicating that the COOH-terminal PH domain, unlike the NH2-terminal PH domain, was not required for the effects of pleckstrin on the cytoskeleton.

Expression of the NH2-terminal PH Domain Alone Can Induce Actin Rearrangement—Once we had determined that the NH2-terminal PH domain was critical in mediating the actin reorganization of pleckstrin, we next asked whether this domain by itself could lead to actin rearrangements or whether...
Pleckstrin and Actin Reorganization

the presence of the remainder of the molecule was required for the cytoskeletal effects of pleckstrin. We therefore generated plasmids encoding an HA-tagged NH2-terminal PH domain and an HA-tagged COOH-terminal PH domain and examined the pattern of F-actin polymerization in COS-1 cells expressing these lone PH domains. Expressing the NH2-terminal PH domain led to a pattern of F-actin staining that looked identical to that induced by expressing wild-type pleckstrin. The NH2-terminal PH domain was membrane-associated, and all of the cells expressing this domain had surface membrane projections (Fig. 4a). Phalloidin staining revealed that the actin has polymerized around the periphery of the cell and is also found in smaller bundles underlying the surface membrane ruffles. Bottom panel, Western blotting shows equal levels of expression of the NH2-terminal PH domain (lane 1) and the COOH-terminal PH domain (lane 2). Lane 3 is from vector-only transfected cells. Bars represent 38 microns.

with pleckstrin in COS-1 cells and examined the pattern of phalloidin staining. As controls, we used a Myc-tagged, dominant negative variant of a similar protein Cdc42 (Myc-N17 Cdc42) and also wild-type Rac co-expressed with pleckstrin.

We determined that Myc-V12N17 Rac co-expression was able to block the cytoskeletal effects of pleckstrin. Cells expressing the dominant negative Rac variant had no cortical actin, and the central stress fibers remained intact (Fig. 5b), arguing that the effects of pleckstrin on the actin cytoskeleton required the activity of Rac. Dramatic formation of ruffled projections was not seen in cells coexpressing pleckstrin and dominant negative Rac, but occasional spikes that were shorter
and flatter could sometimes be seen on the dorsal surface.

By contrast, co-expression of a Myc-tagged, dominant negative Cdc42 variant (Myc N17 Cdc42) had no effect on the pleckstrin-induced actin rearrangements. Cells co-expressing pleckstrin and Myc N17 Cdc42 remained with intact cortical actin and small actin bundles; no central actin cables were seen (Fig. 5d). As a further control, we show that co-expression of wild-type Rac also had no effect on pleckstrin-mediated cytoskeletal reorganization, thus arguing that the inhibitory effect of the Myc V12N17 Rac could be attributed to the dominant negative effects of Rac and not to the sequestering of a binding protein (Fig. 5f). The data thus indicate that the effects of pleckstrin on the actin cytoskeleton are at least partially dependent on Rac, but are independent of Cdc42. The effect of pleckstrin on the cytoskeleton also appears to involve pathways that are independent of Rac because (i) dominant negative Rac only inhibits some of the pleckstrin effect, and (ii) constitutively active Rac alone does not completely mimic the pleckstrin effect.

The Effects of Pleckstrin on the Cytoskeleton Do Not Depend on PI3K—PI3K enzymes phosphorylate inositol lipids at the D3 position of the inositol ring, leading to the formation of lipid second messengers which are known to play a role in cytoskeletal regulation. The assembly of cortical actin mediated by platelet-derived growth factor or insulin requires PI3K and examined their pattern of F-actin polymerization. Neither overnight treatment with 50 nM wortmannin, 100 μM LY294002, nor Me2SO diluent alone could reverse the cytoskeletal effect of pleckstrin (data not shown). Pleckstrin-expressing cells treated with these inhibitors still demonstrated cortical actin and smaller bundles underlying the membrane projections. As a further test for PI3K involvement in this pathway, we co-expressed pleckstrin with a dominant negative variant of PI3K (Ap85) and assayed for cytoskeletal rearrangement. Cells co-expressing pleckstrin and Ap85 were still capable of reorganizing their actin into the small bundles within the dorsal membrane projections and at the periphery in a cortical pattern (data not shown). Similarly, coexpression of dominant negative PI3K (p110, Ap85) also had no effect on the pleckstrin phenotype (data not shown.) Collectively, these data indicate that PI3K is not directly involved in the pleckstrin-induced cytoskeletal reorganization.

**DISCUSSION**

Though pleckstrin has been known for decades as the major substrate for protein kinase C in platelets, its function in vivo remains uncertain. Studies from our laboratory have demonstrated a role for expressed pleckstrin in (i) inhibiting the PLC-mediated hydrolysis of PIP2 (30), (ii) inhibiting the phosphorylation of PIP2 by the Gαi-activable PI3K (36), and (iii) accelerating the hydrolysis of inositol 3,4,5-trisphosphate by a 5-inositol phosphatase (32), implicating pleckstrin in the regulation of inositol-based cell signaling. We have also shown that pleckstrin will bind to plasma membranes and induce the formation of membrane projections from the dorsal surface of transfected cells (1). This report builds on the previous work and demonstrates that expressed pleckstrin can lead to reorganization of the actin cytoskeleton. Though the cytoskeletal effects are phosphorylation-dependent, they appear to require only the NH2-terminal PH domain because expression of this domain alone can induce the actin rearrangements. We demonstrate that the pathway between pleckstrin and the cytoskeleton at least partially depends on Rac1, but is independent of PI3K and Cdc42. These observations raise several questions, including the function of the remainder of the pleckstrin molecule, especially the COOH-terminal PH domain and the newly described DEP domain, the true in vivo ligand for the amino PH domain of pleckstrin, and the mechanism by which the NH2-terminal PH domain leads to cytoskeletal reorganization.

The conventional model for PH domain action is that the PH domain serves to localize its protein to the membrane where the rest of the molecule then performs its function. However, this model does not hold for the cytoskeletal effects of pleckstrin. Here, the membrane-associated, NH2-terminal PH domain of pleckstrin is capable of acting alone to induce actin reorganization. What then is the role of the COOH-terminal PH domain of pleckstrin? In Tiam1, another molecule with two PH domains, the NH2-terminal PH domain is required for membrane localization, Rac-dependent membrane ruffling, and c-Jun NH2-terminal kinase activation (24), and the COOH-terminal PH domain has uncertain function. The COOH-terminal PH domain alone of pleckstrin is not membrane-associated and does not induce cytoskeletal reorganization, and pleckstrin variants missing this PH domain are able to inhibit PLC (30). Together, this leaves the function of the C-terminal PH domain unknown.

A third functional domain has recently been described within pleckstrin, lying between the two PH domains and encompassing much of the remainder of the molecule (41). Termed the DEP domain, this region is homologous to sequences within Dishevelled and proteins in the RGS (Regulators of G protein Signaling) family. Though the DEP domains of Egl-10 and Dishevelled have been shown to play a role in membrane targeting (42, 43), pleckstrin does not appear to use this domain in a similar fashion because the NH2-terminal PH domain requires the DEP domain for neither membrane localization nor cytoskeletal reorganization. Preliminary data from our laboratory indicate that this domain may act to inhibit the rhoA-stimulated activity of phospholipase D (44), and at this point, the function of the remainder of the pleckstrin molecule remains undetermined.

Fesik and co-workers (9) demonstrated that the amino-terminal PH domain from pleckstrin can bind PIP2 and phosphoinositide 3-phosphate in vitro. More recent work from the Lemmon laboratory (22) revealed that, again in vitro, the amino-terminal PH domain from pleckstrin was remarkably promiscuous in its phospholipid-binding specificity. Our work in vivo suggests that the amino-PH domain is capable of recognizing a ligand that is not recognized by the carboxyl-terminal PH domain in this study) or the PH domains of βARK or dynamin (1). Mutations in the phospholipid-binding pocket of the amino-PH domain of pleckstrin disrupt its ability to regulate the formation of membrane ruffles. Together this suggests, but does not definitively prove, that the NH2-terminal PH domain of pleckstrin is recognizing a specific phospholipid in vivo, and binding of the PH domain to that phospholipid alters intracellular signaling to induce cytoskeletal changes.

Given that pleckstrin’s NH2-terminal PH domain can lead to cytoskeletal reorganization, independent of the remainder of the molecule, the next question is the mechanism by which this actin rearrangement occurs. Our results indicate that Rac, but not Cdc42 and not PI3K, is involved in this pathway, but how pleckstrin may activate Rac is less clear. Our attempts to demonstrate a direct interaction between pleckstrin and Rac by coimmunoprecipitation studies were unsuccessful. This could imply that an intermediate protein is involved. Rac is a GTPase in the Ras superfamily, and its function is regulated by three
classes of proteins. Guanosine exchange factors (GEFs) activate Rac by catalyzing the exchange of GDP for GTP, guanine dissociation inhibitors (GDIs) inhibit Rac by inhibiting GDP dissociation. GTPase activating proteins (GAPs) also inhibit Rac by stimulating GTP hydrolysis. Pleckstrin itself does not dissociation inhibitors (GDIs) inhibit Rac by inhibiting GDP, and probably function analogously. This hypothesis, as well as other potential interactions between pleckstrin and Rac are currently under investigation in our laboratory.

REFERENCES
1. Ma, A. D., Brass, I. F., and Abrams, C. S. (1997) J. Cell Biol. 136, 1071–1079
2. Downing, A. K., Driscoll, P. C., Gout, I., Salim, K., Zvelebil, M. J., and Waterfield, M. D. (1994) Curr. Biol. 4, 884–891
3. Ferguson, K., Lemmon, M. A., Schlessinger, J., and Sigler, P. B. (1994) Cell 79, 199–209
4. Fushman, D., Cahill, S., Lemmon, M. A., Schlessinger, J., and Cowburn, D. (1995) Proc. Natl. Acad. Sci., U. S. A. 92, 816–820
5. Hyväsien, M., Mancias, M. J., Nilges, M., Oschkinat, H., Saraste, M., and Wilmanns, M. (1995) EMBO J. 14, 4676–4685
6. Koshiba, S., Kigawa, T., Kim, J.-H., Shirouzu, M., Bowtell, D., and Yokoyama, S. (1997) J. Mol. Biol. 269, 575–591
7. Mancias, M., Musacchio, A., Ponstingl, H., Nilges, M., Saraste, M., and Oschkinat, H. (1994) Nature 369, 675–677
8. Timm, D., Salim, K., Gout, I., Guruprasad, L., Waterfield, M., and Blundell, T. (1994) Nat. Struct. Biol. 1, 782–788
9. Harlan, J. E., Hajduk, P. J., Yoon, H. S., and Fesik, S. W. (1994) Nature 369, 168–170
10. Ferguson, K. M., Lemmon, M. A., Schlessinger, J., and Sigler, P. B. (1995) Cell 83, 1037–1046
11. Han, J., Luby-Phelps, K., Das, B., Shu, X., Xia, Y., Mosteller, R. D., Krishna, U. M., Falck, J. R., White, M. A., and Broek, D. (1998) Science 279, 558–560
12. Ma, A. D., Metjian, A., Bagrodia, S., Taylor, M. A., and Abrams, C. S. (1998) Mol. Cell Biol. 18, 4744–4751
13. Miki, H., Sasaki, T., Takai, Y., and Takenawa, T. (1998) Nature 393, 93–96
14. Pitcher, J. A., Tsuchiya, K., Payne, E. S., and Leikowitz, R. J. (1995) J. Biol. Chem. 270, 11707–11710
15. Abrams, C. S., Zhao, W., and Brass, L. F. (1996) Biochim. Biophys. Acta 1314, 233–238
16. Mahadevan, D., Thanki, N., Singh, J., McPhee, P., Zangrilli, D., Wang, L.-M., Guerrero, C., LeVine, H., Humblet, B., Saldana, J., Gotkin, S., and Najmahadi-Halke, T. (1995) Biochemistry 34, 9111–9117
17. Touhara, K., Inglese, J., Pitcher, J. A., Shaw, G., and Leikowitz, R. (1994) J. Biol. Chem. 269, 10217–10220
18. Tsukada, S., Simon, M. I., Witte, O. N., and Katz, A. (1994) Proc. Natl. Acad. Sci., U. S. A. 91, 11256–11260
19. Konishi, H., Kuroda, S. I., and Kikkawa, U. (1994) Biochem. Biophys. Res. Commun. 205, 1770–1775
20. Yao, L., Kawakami, Y., and Kawakami, T. (1994) Proc. Natl. Acad. Sci., U. S. A. 91, 9175–9179
21. Yao, L., Suzuki, H., Ozawa, K., Deng, J., Lehel, C., Fukamachi, H., Anderson, W. B., Kawakami, Y., and Kawakami, T. (1997) J. Biol. Chem. 272, 15033–15037
22. Kavan, J. M., Klein, D. E., Lee, A., Falasca, M., Isakoff, S. J., Skelnik, E. Y., and Lemmon, M. A. (1998) J. Biol. Chem. 273, 30547–30550
23. Rameh, L. E., Arvidsson, A., Carraway, K. W., III, Courvillon, A. D., Rathbun, G., Crompton, A., Vanhouttepen, B., Czech, M. P., Ravichandran, K. S., Burakoff, S. J., Wang, D. S., Chen, C. S., and Cantley, L. C. (1997) J. Biol. Chem. 272, 22059–22066
24. Michiels, F., Stam, J. C., Hordijk, P. L., van der Kammen, R. A., Ruuls-van-Stalle, L., Feltkamp, C. A., and Collard, J. G. (1997) J. Cell Biol. 137, 387–398
25. Zheng, Y., Zangrilli, D., Cerione, R. A., and Eva, A. (1996) J. Biol. Chem. 271, 19017–19020
26. Sisson, S. M., Ninman, A. S., Uy, M., Bar-Sagi, D., and Kuriyan, J. (1998) Cell 95, 259–268
27. Leung, T., Chen, X.-Q., Manser, E., and Lim, L. (1996) Mol. Cell Biol. 16, 5313–5327
28. Nakagawa, T., Goto, K., and Kondo, H. (1996) J. Biol. Chem. 271, 12088–12094
29. Stevenson, J. M., Perera, I. Y., and Boss, W. F. (1998) J. Biol. Chem. 273, 17650–17657
30. Abrams, C. S., Wang, D., Belmonte, E., and Brass, L. F. (1995) J. Biol. Chem. 270, 14485–14492
31. Banin, S., Truong, O., Katz, D. R., Waterfield, M. D., Brickell, P. M., and Gout, I. (1996) Curr. Biol. 6, 961–965
32. Aeuthavekiat, V., Abrams, C. S., and Majerus, P. W. (1997) J. Biol. Chem. 272, 1786–1790
33. Abrams, C. S., Zhao, W., Belmonte, E., and Brass, L. F. (1995) J. Biol. Chem. 270, 23517–23521
34. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) Gene 7, 51–59
35. Baldassare, J. J., Henderson, P. A., Burns, D., Loomis, C., and Fisher, G. J. (1992) J. Biol. Chem. 267, 15585–15590
36. Abrams, C. S., Zhang, J., Downes, C. P., Tang, X.-w., Zhao, W., and Rittenhouse, S. (1996) J. Biol. Chem. 271, 25192–25197
37. Hawkins, P. T., Eguinoa, A., Qiu, R.-G., Stokoe, D., Cooke, F. T., Walters, R., Wennstrom, S., Claesson-Welsh, L., Evans, T., Symons, M., and Stephens, L. (1995) Curr. Biol. 5, 393–403
38. Kotani, K., Yonezawa, K., Hara, K., Ueda, H., Kitamura, Y., Sakaue, H., Ando, A., Chavanieu, A., Calas, B., Grigorescu, F., Nishiyama, M., Waterfield, M. D., and Kasuga, M. (1994) EMBO J. 13, 2313–2321
39. Wennstrom, S., Hawkins, P., Cooke, F., Hara, K., Yonezawa, K., Kasuga, M., Jackson, T., Claesson-Welsh, L., and Stephens, L. (1994) Curr. Biol. 4, 385–393
40. Hall, A. (1994) Annu. Rev. Cell Biol. 10, 31–54
41. Ponting, C. P., and Bork, P. (1996) Trends Biochem. Sci. 21, 245–246
42. Azelrod, J. D., Miller, J. R., Shulman, J. M., Moon, R. T., and Perrimon, N. (1998) Genes Dev. 12, 2610–2622
43. Koelle, M. R., and Horvitz, H. R. (1996) Cell 84, 115–125
44. Ma, A. D., and Abrams, C. S. (1998) Blood 92, 363 (abstr.)