The Rho family GTP-binding proteins have been known to mediate extracellular signals to the actin cytoskeleton. Although several Rho interacting proteins have been found, downstream signals have yet to be determined. Many actin-binding proteins are known to be regulated by phosphatidylinositol 4,5-bisphosphate in vitro. Rho has been shown to enhance the activity of phosphatidylinositol-4-phosphate 5-kinase (PI4P5K), the phosphatidylinositol 4,5-bisphosphate synthesizing enzyme. Recently we isolated several isoforms of type I PI4P5K. Here we report that PI4P5K Iα induces massive actin polymerization resembling “pine needles” in COS-7 cells in vivo. When truncated from the C terminus to amino acid 308 of PI4P5K Iα, both kinase activity and actin polymerizing activity were lost. Although the dominant negative form of Rho, RhoN19, alone decreased actin fibers, those induced by PI4P5K Iα were not affected by the coexpression of RhoN19. These results suggest that PI4P5K Iα is located downstream from Rho and mediates signals for actin polymerization through its phosphatidylinositol-4-phosphate 5-kinase activity.

The stimulation of cells by growth factors and other agonists is mediated by intracellular signal transduction pathways. In response to environmental signals, a cell changes both its shape and its degree of attachment to the substrate. These changes are caused, at least in part, by the polymerization and rearrangement of the actin into sheet-like structures known as lamellipodia and bundle-like stress fibers. The Rho family GTP-binding proteins (Cdc42, Rac, and Rho) play key roles in this process. Whereas constitutive GTP-binding proteins bind to barbed ends to mask them, and monomer-stabilizing proteins bind to barbed ends to sequester them, several ABPs can bind PIP2 and release the free barbed end or free G-actin to polymerize. Whereas ABPs can bind PIP2 and release the free barbed end or free G-actin to polymerize, PI4P5K has been shown to stimulate PI4P5K activity. Therefore PI4P5K is a suitable link between the Rho family and actin polymerization. Mammalian type 2 PI4P5K has been cloned (12). We recently cloned several isoforms of type I PI4P5K (4). Here we report its important role in actin polymerization in vivo.

**Materials and Methods**

DNA Constructs—The wild type PI4P5K Iα construct with hemagglutinin (HA) epitope used was described previously (4). C-terminal deletion mutants were constructed by appropriate endonuclease digestion (BamHI and NcoI), designated 5K456S and 5K308M, and subcloning into the pAdexICA vector (13). C-terminal sequences were confirmed by DNA sequencing. The amino acids of constructs are: WT, 1–539; 5K456S, 1–456 with extra Asn; and 5K308M, 1–308 with extra LKIKLV due to the polylinker sequence of the vector. Partial OCRL cDNA was isolated by polymerase chain reaction using primers corresponding to nucleotides 806–828 (sense) and the complement of nucleotides 1520–1550 (antisense) based on the published sequence and tagged with HA Iα. The RhoN19 cDNA (threonine at codon 19 of Rho A was replaced with asparagine) was constructed by polymerase chain reaction, tagged with the Myc epitope at their N terminus and cloned into the pAdex1CA vector.

**Results**

C-terminal Truncated Forms of PI4P5K Iα—C-terminal truncations of PI4P5K Iα were made by endonuclease digestion using unique site with BamHI (amino acids 1–456) or NcoI (amino acids 1–308) and designated 5K456S and 5K308M, respectively (Fig. 1A). Wild type and truncated constructs were tagged with the HA epitope and expressed in COS-7 cells.

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1 The abbreviations used are: PtdIns4P, phosphatidylinositol 4-phosphate; ABPs, actin binding proteins; PtdIns, phosphatidylinositol; PIP2, phosphatidylinositol 4,5-bisphosphate; PI4P5K, phosphatidylinositol-4-phosphate 5-kinase; FITC, fluorescein isothiocyanate; HA, hemagglutinin; WT, wild type; FA, focal adhesion; PLC, phospholipase C; OCRL, ocuolocerebrorenal syndrome of Lowe.
Control cells were infected with the vector containing lacZ insert. The size of overexpressed proteins revealed by anti-HA antibody (12CA5) were: WT, 68 kDa (Fig. 1, lane 2); 5K456S, ~58 kDa (lane 3); and 5K308M, ~38 kDa (lane 4). Although WT and 5K456S retained PtdIns4P 5-kinase activity, 5K308M revealed little kinase activity (Fig. 1B). This was observed in several independent kinase assays and was not due to low expression levels.

Because 12CA5 and anti-PI4P5K Iα antibody (4) yielded identical patterns in overexpressed cells under immunofluorescence, we used 12CA5 in subsequent investigations. Wild type PI4P5K Iα showed cytosolic as well as punctate distribution, and the plasma membrane also appeared to be stained (Fig. 2A). This is consistent with the results reported previously (15). Although 5K456S showed a pattern similar to WT cells, 5K308M showed diffuse but little marginal staining, which is not typical for plasma membrane localization (Fig. 2B). Between amino acids 309 and 456, there is a sequence (YRXXXXXXSWK) similar to the putative PIP2-binding site of α-actinin (16). The detailed analysis of the relationship between kinase activity and the plasma membrane association will be published elsewhere.2

Effect of PI4P5K Overexpression on Actin Polymerization—If PI4P5K Iα is an effector for actin polymerization, increased levels of PI4P5K would be expected to result in increased actin polymerization. In control cells, actin filaments were organized in parallel bundles at the bottom of the cells, and peripheral actin filaments formed lamellipodia (Fig. 3A). However, the amount of F-actin in PI4P5K WT (5K456S; data not shown) cells increased as shown in Fig. 3C. Typical stress fibers and lamellipodia appeared to decrease, but relatively short actin fibers were observed in random array even in aggregated fashion like "pine needles." In kinase-negative 5K308M cells, actin fibers did not show such a pattern but instead were arranged as typical stress fibers as in control cells (Fig. 3E). With double labeling of 12CA5 and rhodamine-phalloidin, we confirmed this result at single cell level even in 5K308M high expressing cells. Therefore the lack of the effect was not due to low level expression. We then overexpressed partial OCRL protein (amino acids 264–506), known to retain a PtdIns 5-phosphatase activity, in COS-7 cells (OCRL cells). This protein was found in the cytoplasm unlike the Golgi pattern of full-length OCRL (14). In contrast to the cells expressing PI4P5K, there were few actin fibers in OCRL cells (Fig. 3F). There was little change in the total amount of actin between control and PI4P5K and OCRL overexpressed cells by Western blotting (data not shown). These results strongly suggest that PI4P5K induces actin polymerization through its kinase activity by elevating levels of PIP2. These results were also observed in other cell lines (data not shown).

Rho has been known to regulate actin-based cytoskeleton (1, 7). The dominant negative form of RhoA, RhoN19, was made by replacing the threonine in a position analogous to codon 17 of Ras with asparagine. When RhoN19 was expressed in COS-7

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2 Y. Shibasaki, H. Ishihara, N. Kizuki, T. Asano, Y. Oka, and Y. Yazaki, manuscript in preparation.

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**FIG. 2. Immunofluorescence of PI4P5K Iα.** HA tagged PI4P5K constructs were expressed in COS-7 cells. Cells were fixed with paraformaldehyde and stained with anti-HA antibody (12CA5) followed by FITC-conjugated anti-mouse IgG. A, Wild type PI4P5K. B, 5K308M. Bar, 10 μm.

**FIG. 3. Effect of PI4P5K Iα on actin polymerization.** Control (A and B) and PI4P5K(WT) expressed COS-7 cells (C and D) maintained in the presence of 10% fetal bovine serum were fixed and double-stained with anti-phosphotyrosine antibody (4G10) followed by FITC-conjugated anti-mouse IgG and rhodamine-phalloidin. 5K308M (E) or OCRL (amino acids 264–968) (F) was expressed in COS-7 (control) cells and stained with rhodamine-phalloidin. A, C, E, and F, actin fibers. B and D, phosphotyrosine staining of same cell as in A or C. The arrowheads indicate adhesion plaques. Bar, 5 μm.
cells (Fig. 4B) stress fibers decreased (Fig. 4A). We then expressed RhoN19 and PI4P5K (WT) simultaneously in COS-7 cells. Unlike RhoN19 cells, the actin fiber pattern was very similar to that in PI4P5K WT cells (Fig. 4C). Thus PI4P5K is probably located downstream from Rho, consistent with previous results (3).

**Effect on Focal Adhesion**—When COS-7 cells were infected with kinase active constructs (WT and 5K456S), we noticed that the degree of adhesion to the substrate decreased. This phenotype correlated with the expression levels of PI4P5K as judged by immunofluorescence. This change did not occur with expression of the kinase-negative construct (5K308M). It has been known that introduction of active Rho into Swiss 3T3 cells induces both stress fibers and focal adhesion (FA). This indicates that these two phenomena are located downstream from Rho. We stained control and PI4P5K-overexpressed cells with anti-phosphotyrosine (Tyr(P)) antibody (4G10) and anti-vinculin antibody (9E10) (same cell as in A). D, actin fibers enlarged from C. Bar, 10 μm.

Fig. 4. Coexpression of RhoN19 and PI4P5K Iα. RhoN19 was tagged with Myc epitope and expressed alone (A and B) or together with PI4P5K (C and D) in COS-7 cells. A, C, and D, cells were stained with rhodamine-phalloidin. B, cells were stained with anti-Myc antibody (9E10) (same cell as in A). D, actin fibers enlarged from C. Bar, 10 μm.

**DISCUSSION**

In this paper we described the effects of kinase-active and -negative forms of PI4P5K Iα on actin polymerization using immunofluorescence. Although several proteins that interact with Rho have been found recently (8–11), it is not clear which candidate is responsible for actin polymerization. Rho kinase is a candidate because it phosphorylates myosin phosphatase and induces a stress fiber-like pattern (17, 18). Although this indicates a pathway through myosin, it has been strongly suggested that there exists a mechanism acting directly on actin (19). Our results showed that PI4P5K plays an important role in actin polymerization. It is possible that the polymerization of actin fibers and their organization into stress fibers are distinct steps and that PI4P5K is important in the former process and Rho kinase is required in the latter process. The Rho family GTPases have been known to activate PtdIns4P 5-kinase activity (19, 20). Moreover, several recent reports have suggested that they interact with PI4P5K directly. Rac physically interacted with PI4P5K (20), and Rho binds to 68-kDa PI4P5K (21). It is possible that each member of Rho protein interacts with a different isoform of PI4P5K.

Actin polymerization is dependent on multiple ABPs. Many ABPs have been known to be regulated by PIP2, including profilin, thymosin (22), gelsolin, actinin (23), capping protein, and vinculin (24). For example, thymosin releases free G-actin when it binds to PIP2. Binding of vinculin to actin is regulated by PIP2 (24). Rac (and/or Rho) increase free actin barbed ends through PIP2 (19). Most of these results on ABPs in relation to PIP2 were obtained in vitro because it has been difficult to prove their effects in vivo. Our system is suitable for this purpose, and the results were striking.

PI4P5K probably acts to increase PIP2 levels. PIP2 is hydrolyzed by phospholipase C (PLC) to generate inositol (1,4,5)-P3 and diacylglycerol, which are well known second messengers. Our results might include these secondary effects. However, multivalent antigen or phorbol myristate acetate causes the polymerization of actin in basophilic leukemia cells. Although multivalent antigen activates PLC, phorbol myristate acetate does not. However, in both cases, there is good correlation between F-actin levels, PtdIns kinase activity, and the increased production of PIP2 (25). Therefore, activation of PI4P5K itself does not activate PLC. Our result showing that PI4P5K-induced actin polymerization was most likely due to elevated PIP2 levels. Because OCRL overexpression decreases PIP2 levels (14), the results in OCRL cells are consistent with our model in which signals to actin are mediated by PI4P5K through elevated PIP2 levels.

**REFERENCES**

1. Hall, A. (1994) *Annu. Rev. Cell Biol.* 10, 31–54
2. Janmey, P. A. (1994) *Annu. Rev. Physiol.* 56, 169–191
3. Chong, L. D., Traynor-Kaplan, A., Bokoch, G. M., and Schwartz, M. A. (1994) *Cell* 79, 507–513
4. Ishihara, H., Shibusaki, Y., Kimura, K., Iwamori, T., Yoneda, Y., Yasuda, Y., and Oka, Y. (1996) *J. Biol. Chem.* 271, 23611–23614
5. Nohes, C. D., and Hall, A. (1995) *Cell* 81, 53–62
6. Takai, Y., Sasa, T., Tanaka, K., and Nakanishi, H. (1995) *Trends Biochem. Sci.* 20, 227–231
7. Machesky, L. M., and Hall, A. (1996) *Trends Cell Biol.* 6, 304–310
8. Amano, M., Mukai, H., Ono, Y., Chihara, K., Matsui, T., Hamajima, Y., Okawa, K., Iwamutsu, A., and Kishida, K. (1996) *Science* 271, 648–650
9. Watanabe, G., Saito, Y., Maeda, H., Ishizaki, T., Fujisawa, K., Morii, N., Mukai, H., Ono, Y., Kakizuka, A., and Narumiya, S. (1996) *Science* 271, 645–648
10. Ishizaki, T., Maekawa, M., Fujisawa, N., Okawa, K., Iwamutsu, A., Fujita, A., Watanabe, N., Saito, Y., Kakizuka, A., Morii, N., and Narumiya, S. (1996) *EMBO J.* 15, 1885–1893
11. Matsui, T., Amano, M., Yamamoto, T., Chihara, K., Nakafuku, M., Ito, M., Nakano, T., Okawa, K., Iwamutsu, A., and Kaibuchi, K. (1996) *EMBO J.* 15, 2208–2216
12. Boronenkov, I. V., and Anderson, R. A. (1995) *J. Biol. Chem.* 270, 2881–2884
13. Kanegae, Y., Lee, G., Sato, Y., Tanaka, M., Nakai, M., Sakaki, T., Sugano, S., and Saito, I. (1995) *Nucleic Acids Res.* **23**, 3816–3821
14. Zang, X., Jefferson, A. B., Aueathavekiat, V., and Majerus, P. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 4853–4856
15. Jenkins, G. H., Fisette, P. L., and Anderson, R. A. (1994) *J. Biol. Chem.* **269**, 11547–11554
16. Fukami, K., and Takenawa, T. (1996) *J. Biol. Chem.* **271**, 2646–2650
17. Kimura, K., Ito, M., Amano, M., Chihara, K., Fukata, Y., Nakafuku, M., Yamamori, B., Feng, J., Nakano, T., Okawa, K., Iwamatsu, A., and Kaibuchi, K. (1996) *Science* **273**, 245–248
18. Leung, T., Chen, X-Q., Manser, E., and Lim, L. (1996) *Mol. Cell. Biol.* **16**, 5313–5327
19. Hartwig, J. H., Bokoch, G. M., Carpenter, C. L., Janmey, P. A., Taylor, L. A., Toker, A., and Stossel, T. P. (1995) *Cell* **82**, 643–653
20. Tolias, K., Cantley, L. C., and Carpenter, C. L. (1995) *J. Biol. Chem.* **270**, 17656–1770
21. Ren, X.-D., Bokoch, G. M., Traynor-Kaplan, A., Jenkins, G. H., Anderson, R. A., and Schwartz, M. A. (1996) *Mol. Biol. Cell* **7**, 435–442
22. Sun, H.-Q., Kwiatkowska, K., and Yin, H. L. (1995) *Curr. Opin. Cell Biol.* **7**, 102–110
23. Fukami, K., Endo, T., Imamura, M., and Takenawa, T. (1994) *J. Biol. Chem.* **269**, 1518–1522
24. Gilmore, A. P., and Burridge, K. (1996) *Nature* **381**, 531–535
25. Aggar, J. R. (1996) *Mol. Biol. Cell* **6**, 97–108
26. Clark, E. A., and Brugge, J. S. (1995) *Science* **268**, 233–239
27. Carvajal, J. J., Pook, M. A., Santos, M., Doudney, K., Hillermann, R., Minogue, S., Williamson, R., Hsuan, J. J., and Chamberlain, S. (1996) *Nat. Genet.* **14**, 157–162
28. Harlan, J. E., Hajduk, P. J., Yoon, H. S., and Fesik, S. W. (1994) *Nature* **371**, 168–170