A Short Sequence Responsible for Both Phosphoinositide Binding and Actin Binding Activities of Cofilin*

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Cofilin is a widely distributed actin-modulating protein that has abilities to bind along the side of F-actin and to depolymerize F-actin. Both abilities of cofilin can be inhibited by phosphoinositides such as phosphatidylinositol, phosphatidylinositol 4-monophosphate, and phosphatidylinositol 4,5-bisphosphate (PIP2). We have previously shown that the synthetic dodecapeptide corresponding to Trp104-Met115 of cofilin is a potent inhibitor of actin polymerization (Yonezawa, N., Nishida, E., Iida, K., Kumagai, H., Yahara, I., and Sakai, H. (1991) J. Biol. Chem. 266, 10485–10489). In this study, we have found that the inhibitory effect of the synthetic dodecapeptide on actin polymerization is canceled specifically by phosphatidylinositol, phosphatidylinositol 4-monophosphate, and PIP2. We further show that the dodecapeptide as well as cofilin binds to PIP2 molecules and inhibits PIP2 hydrolysis by phospholipase C. Thus, the actin-binding dodecapeptide sequence of cofilin may constitute a multifunctional domain in cofilin.

Cofilin (1) is a widely distributed actin-binding protein with an apparent molecular mass of 21 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Cofilin binds not only along F-actin but also to G-actin and depolymerizes F-actin in a pH-dependent manner in vitro (1–3). Cofilin forms intranuclear and/or cytoplasmic actin/cofilin rods in cultured cells in response to various stimuli (4).

Cofilin comprises 166 amino acid residues as revealed by the analyses using cDNA cloning and sequencing (5). Our recent study has shown that Lys132 and/or Lys148 of cofilin can be cross-linked by a chemical zero length cross-linker to actin molecule and that the synthetic dodecapeptide, WAPECAPLKSKM, patterned on the sequence around the cross-linking site (corresponding to Trp104-Met115) of cofilin is a potent inhibitor of actin polymerization (6).

It has been reported that polyphosphoinositides such as phosphatidylinositol 4,5-bisphosphate (PIP2) and phosphatidylinositol 4-monophosphate (PIP) inhibit the actin-modulating activities of profilin, gelsolin, and villin in vitro (7–10). Polyphosphoinositides induce the dissociation of profilin and gelsolin-actin complex by interacting with profilin and gelsolin, respectively. In addition, we reported that cofilin also has the polyphosphoinositides sensitivity (11). In the case of cofilin, not only PIP and PIP2 but also phosphatidylinositol (PI) inhibited the interactions of cofilin with both G- and F-actin.

Previous studies using gelsolin fragments generated by the limited proteolysis or the gene truncation of plasma gelsolin cDNA revealed that the PIP2-binding site on gelsolin sequence is on residues 150–160 (12, 13). It is also suggested that the sequence of residues 150–160 may be the F-actin-binding site (12, 13). Cofilin does not have the sequence similar to this polyphosphoinositide binding sequence of gelsolin. The polyphosphoinositide binding sequences of other actin-binding proteins have not yet been identified. Here, we have shown that the actin binding sequence of cofilin (Trp104-Met115), previously identified by the chemical cross-linking study and by the use of the synthetic peptide, is a phosphoinositide binding sequence. Thus, inhibition of actin polymerization by the synthetic dodecapeptide corresponding to Trp104-Met115 of cofilin is canceled specifically by phosphoinositides such as PIP2, PIP, and PI.

Recent reports have revealed that profilin binds with high affinity to PIP2 molecules (7, 14) and that profilin inhibits the hydrolysis of PIP2 by unphosphorylated phospholipase C (PLC) but not the hydrolysis by PLC phosphorylated by epidermal growth factor receptor tyrosine kinase (14, 15). These results raise the interesting possibility that actin-modulating proteins function as a negative regulator of the phosphoinositide signaling pathway in quiescent cells (14, 15). In this report, we show that the dodecapeptide as well as cofilin binds to PIP2 molecules and inhibits PIP2 hydrolysis by PLC.

**MATERIALS AND METHODS**

**Proteins**—Recombinant cofilin was expressed in *Escherichia coli* and then purified to homogeneity as described previously (16). PLC-γ- and PLC-δ were purified from bovine thymus and PLC-γ- was from bovine spleen as described previously (17). PLC-δ, was purified from bovine brain as described previously (23).

**Synthetic Peptides**—A dodecapeptide, a pentapeptide, and a peptide corresponding to residues 150–160 of cofilin were synthesized as described previously (6).

**Chemicals**—Phosphatidylinositol (PI), phosphatidylinositol 4-monophosphate (PIP), phosphatidylinositol 4,5-bisphosphate (PIP2), phosphatidylinositol 4-monophosphate; PC, phosphatidyicholine; PS, phosphatidylycerine; IP3, inositol 1,4,5-trisphosphate; PLC, phospholipase C; Mes, 4-morpholineethanesulfonic acid.

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‡The abbreviations used are: PIP2, phosphatidylinositol 4,5-bisphosphate; Pipes, piperazine-N,N'-bis(2-ethanesulfonic acid); r-cofilin, recombinant cofilin; PI, phosphatidylinositol; PIP2, phosphatidylinositol 4,5-bisphosphate; PC, phosphatidylicholine; PS, phosphatidylycerine; IP3, inositol 1,4,5-trisphosphate; PLC, phospholipase C; Mes, 4-morpholineethanesulfonic acid.
Assay for Actin Polymerization Using Pyrene-labeled Actin—In the presence or absence of the synthetic peptide and either lipids or PI₂, polymerization of G-actin (5 μM, 3.6% pyrene actin) was monitored at 25 °C by the changes in fluorescence intensity of pyrene labeled to actin in a buffer containing 90 mM KCl, 20 mM ATP, 10 mM CaCl₂, and 4 mM Pipes, pH 7.0. The pyrene-labeled actin was prepared as described (1, 18). The fluorescence intensity was measured with a Hitachi 650–10S fluorescence spectrophotometer, and is shown as arbitrary units. The excitation and emission wavelengths were 365 and 407 nm, respectively.

Gel Filtration Chromatography—Gel filtration was performed on Sephadex G-25 (0.9 × 12 cm) at room temperature. The dodecapeptide (210 μM) was mixed with no phospholipid, PIP₂ (630 μM), PIP (630 μM), PS (630 μM), or PC (630 μM) in a solution consisting of 10 mM potassium phosphate and 100 mM NaCl, pH 7.0, and then passed through the column in the same buffer solution. The elution of the dodecapeptide was detected by measuring the absorbance at 215 nm. PIP₂ Hydrolysis by Phospholipase C—PIP₂ hydrolysis by PLC was performed at 37 °C for 10 min with small unilamellar vesicles consisting of [³H]PIP₂ (20 μM) and phosphatidylethanolamine (20 μM) in 50 mM Mes, pH 6.5, 0.1 mM CaCl₂, and 1 mg/ml of bovine serum albumin. The [³H]PIP₂ generated was separated from [³H]PIP₁ and then the amount of [³H]IP₁ was measured by liquid scintillation counting as described previously (17).

RESULTS AND DISCUSSION

The synthetic dodecapeptide, WAPECAPLKSKM, corresponding to Trp¹⁰⁴-Met¹⁵ of cofilin inhibited actin polymerization as previously reported (Fig. 1, and Ref. 6). We have found that the inhibition of actin polymerization by the dodecapeptide is canceled by PIP₂ in a dose-dependent manner as shown in Fig. 1a. About an equimolar PIP₂ to the dodecapeptide was required for the complete cancellation (Fig. 1a).

Fig. 1b shows the effects of various lipids and IP₁ on the inhibitory effect of the dodecapeptide on actin polymerization. In addition to PIP₂, PIP (●) and PI (■) canceled the inhibition of actin polymerization by the dodecapeptide, while PC (○), PS (△), 1-oleoyl-3-acetylglycerol (◇), or IP₁ (▲) did not affect the activity of the dodecapeptide at all. Thus, the inhibitory effect of the dodecapeptide on actin polymerization was inhibited specifically by phosphoinositides. Because phosphoinositides do not affect the actin polymerization and do not bind to actin (11), these results suggest that the phosphoinositides block the interaction of the dodecapeptide with actin by binding to the dodecapeptide.

To show the binding of the dodecapeptide to the phosphoinositides directly, we carried out the gel filtration chromatography. In gel filtration on the Sephadex G-25 column, PIP₂ or PIP eluted at void volume while the dodecapeptide alone eluted at the position corresponding to an apparent molecular weight of ~1000 (Fig. 2, ○). When the dodecapeptide mixed with PIP₂ or PIP was passed through the column, however, the dodecapeptide co-eluted with PIP₂ or PIP at void volume (Fig. 2, ●). This indicated that the dodecapeptide binds to PIP₂ or PIP. In contrast, neither PC nor PS affected the elution position of the dodecapeptide (Fig. 2, ▲). A synthetic peptide corresponding to residues 150–166 of cofilin, that does not inhibit actin polymerization at all (6), eluted itself at the position corresponding to an apparent molecular weight of ~2000. Neither PIP₁ nor PIP affected the elution position of this 17-amino acid synthetic peptide (data not shown). These results suggest that phosphoinositides such as PIP₂ and PIP specifically bind to the dodecapeptide, that is, the actin-binding sequence of cofilin. Thus, the inhibition of interaction of cofilin with actin by phosphoinositides may be accounted for by assuming that phosphoinositides and actin compete for binding to the same site, the dodecapeptide sequence, in cofilin.

As the dodecapeptide as well as cofilin was found to bind to PIP₂ tightly, we tested the effect of cofilin and the dodecapeptide on PIP₂ hydrolysis by PLC. Cofilin inhibited the hydrolysis of PIP₂ by PLC-γ₂ in a dose-dependent manner (Fig. 3a). The rate of hydrolysis was 0 at molar ratios of 1 cofilin per 2.5–3 PIP₂ molecules. The stoichiometry of cofilin

![Fig. 1. Phosphoinositide sensitivity of the synthetic dodecapeptide.](image)

![Fig. 2. PIP₂ specifically binds to the dodecapeptide.](image)
Phosphoinositide Binding Sequence of Cofilin

The concentrations of the synthetic peptides required for the half-maximal inhibition of the initial rate of actin polymerization (assay conditions were the same as in Fig. 1 except for 3 μM actin containing 6% pyrene actin) and for the half-maximal inhibition of PIP₂ hydrolysis by PLC-γ₂ (as in Fig. 3) were determined. As both abilities of the synthetic pentapeptide, LKSKM, were weak, the data for the pentapeptide cannot be accurately determined and are rough estimates.

**TABLE II**

| Comparison of activities of the dodecapeptide and the pentapeptide |
|---------------------------------------------------------------|
| Half-maximal inhibition of PIP₂ hydrolysis by PLC          |
| PLC-γ₂ activity by cofilin                                  |
| Half-maximal inhibition of                                  |
| PLC-γ₂ activity by the dodecapeptide                        |
| μM                                                           |
| 250                                                        |
| 200                                                        |
| 150                                                        |
| 100                                                        |
| 50                                                         |
| 20                                                         |
| 10                                                         |
| 5                                                          |
| 2                                                          |

**REFERENCES**

1. Nishida, E., Maekawa, S., and Sakai, H. (1984) Biochemistry 23, 5307-5313
2. Nishida, E. (1985) Biochemistry 24, 1160-1164
3. Yonezawa, N., Nishida, E., and Sakai, H. (1985) J. Biol. Chem. 260, 14410-14412
4. Nishida, E., Iida, K., Yonezawa, N., Koyasu, S., Yahara, I., and Sakai, H. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 5262-5266
5. Matsuzaki, F., Matsumoto, S., Yahara, I., Yonezawa, N., Nishida, E., and Sakai, H. (1988) J. Biol. Chem. 263, 11564-11568

**FIG. 3.** The dodecapeptide as well as cofilin inhibits PIP₂ hydrolysis by phospholipase C (PLC). a, dose-dependent inhibition of PLC-γ₂ activity by cofilin. b, dose-dependent inhibition of PLC-γ₂ activity by the dodecapeptide. The activity of PLC-γ₂ in the absence of cofilin or the dodecapeptide was regarded as 100%.
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6. Yonezawa, N., Nishida, E., Iida, K., Kumagai, H., Yahara, I., and Sakai, H. (1991) J. Biol. Chem. 266, 10485–10489
7. Lassing, I., and Lindberg, U. (1985) Nature 314, 472–474
8. Janmey, P. A., and Stossel, T. P. (1987) Nature 325, 362–364
9. Janmey, P. A., Iida, K., Yin, H. L., and Stossel, T. P. (1987) J. Biol. Chem. 262, 12228–12236
10. Janmey, P. A., and Matsuda, P. T. (1988) J. Biol. Chem. 263, 16738–16743
11. Yonezawa, N., Nishida, E., Iida, K., Yahara, I., and Sakai, H. (1990) J. Biol. Chem. 265, 5771–5773
12. Yin, H. L., Iida, K., and Janmey, P. A. (1988) J. Cell Biol. 106, 805–812
13. Kwiatkowski, D. J., Janmey, P. A., and Yin, H. L. (1989) J. Cell Biol. 108, 1717–1726
14. Goldschmidt-Clermont, P. J., Machesky, L. M., Baldassare, J. J., and Pollard, T. D. (1990) Science 247, 1575–1578
15. Goldschmidt-Clermont, P. J., Kim, J. W., Machesky, L. M., Rhee, S. G., and Pollard, T. D. (1991) Science 251, 1231–1233
16. Moriyama, K., Nishida, E., Yonezawa, N., Sakai, H., Matsumoto, S., Iida, K., and Yahara, I. (1990) J. Biol. Chem. 265, 5771–5773
17. Homma, Y., Emori, Y., Shibasaki, F., Suzuki, K., and Takenawa, T. (1990) Biochem. J. 269, 13–18
18. Brenner, S. L., and Korn, E. D. (1983) J. Biol. Chem. 258, 5013–5020
19. Nishida, E., Maekawa, S., Muneyuki, E., and Sakai, H. (1984) J. Biochem. (Tokyo) 95, 387–398
20. Nishida, E., Muneyuki, E., Maekawa, S., Ohta, Y., and Sakai, H. (1985) Biochemistry 24, 6624–6630
21. Mabuchi, I. (1983) J. Cell Biol. 97, 1612–1621
22. Bamburg, J. R., Harris, H. E., and Weeds, A. G. (1980) FEBS Lett. 121, 178–182
23. Ryu, S. H., Cho, K. S., Lee, K.-Y., Suh, P.-G., and Rhee, S. G. (1987) J. Biol. Chem. 262, 12511–12518