Inhibition of Actin Polymerization by a Synthetic Dodecapeptide Patterned on the Sequence around the Actin-binding Site of Cofilin*

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Cofilin is an F-actin side-binding and -depolymerizing protein with an apparent molecular mass of 21 kDa. By means of the end label fingerprinting method, the amino acid residue on cofilin sequence cross-linked to actin by zero length cross-linker, 1-ethyl-3-(3-dimethylamino propyl)carbodiimide, was identified as Lys\(^{12}\) and/or Lys\(^{14}\). A synthetic dodecapeptide patterned on the sequence around the actin-cross-linking site of cofilin (Trp\(^{104}\)-Met\(^{115}\)) inhibited the binding of cofilin to actin. Moreover, the dodecapeptide was found to be a potent inhibitor of actin polymerization. Thus, we conclude that the dodecapeptide sequence constitutes the region essential for the actin-binding and -depolymerizing activity of cofilin. A sequence similar to the dodecapeptide is found in other actin-depolymerizing proteins, destrin, actin-depolymerizing factor, and depactin. Therefore, the dodecapeptide sequence may be a consensus sequence essential for actin-binding and -depolymerizing activity in actin-depolymerizing proteins.

Cofilin (1) is a widely distributed actin-binding protein with an apparent molecular mass of 21 kDa on SDS\(^{-}\)-polyacrylamide gel electrophoresis. Cofilin not only binds along F-actin with a 1:1 molar ratio of cofilin to actin protomer but also binds to G-actin in the same 1:1 molar ratio with a dissociation constant of 0.1–0.2 \(\mu\)M (1, 2) and depolymerizes F-actin in a pH-dependent manner \(\textit{in vitro}\) (3). The activity of cofilin \(\textit{in vitro}\) is regulated by phosphoinositides as well (4). In cultured cells, cofilin forms intranuclear and cytoplasmic actin/cofilin rods under specific conditions (5).

Cofilin comprises 186 amino acids as revealed by cDNA cloning and sequencing (6). Our recent study has shown that destrin (7, 8), a mammalian actin-depolymerizing protein with an apparent molecular mass of 19 kDa, consists of 165 amino acid residues and has a sequence highly homologous (71% identical) to that of cofilin (9). Destrin can depolymerize F-actin rapidly and completely in a \(\text{pH}\)-independent manner (7, 8). Low molecular mass (15–19 kDa) actin-depolymerizing proteins whose activity is similar or identical to that of destrin have been identified in other organisms: actin-depolymerizing factor (ADF) in chick embryo brain (10, 11), depactin in echinodermatous oocytes and eggs (12), and actophorin in \textit{Acanthamoeba} (13). The sequence of ADF shows extensively high homology with that of destrin or cofilin (14, 15) whereas depactin shows no significant overall homology with destrin or cofilin (16).

The N and C termini of actin have been shown to interact with actin-binding proteins such as myosin (17), depactin (18, 19), fragmin (20), gelsolin (21), caldesmon (22), and profilin (23). Cofilin is also cross-linked to the N-terminal region (residues 1–12) of actin by a zero length cross-linker (24). On the other hand, the identification of an actin-binding sequence in actin-binding proteins is also an important and critical step for the elucidation of interactions between actin and actin-binding proteins. Recently, a sequence essential for actin binding was identified in \textit{Dictyostelium} ABP-120 (25). Cofilin has a hexapeptide sequence identical to the N-terminal portion (residues 2–7) of tropomyosin (6). This region may be part of the actin-binding domain of cofilin (26). To further investigate the actin-binding domain of cofilin, we have identified the amino acid residues of cofilin cross-linked to actin by a chemical cross-linker. Furthermore, we have shown that a synthetic dodecapeptide patterned on the sequence around the actin-cross-linking site of cofilin is a potent inhibitor of actin polymerization.

MATERIALS AND METHODS

Proteins—Recombinant cofilin (r-cofilin) was expressed in \textit{Escherichia coli} and then purified to homogeneity as described previously (9). Porcine brain cofilin was purified as described previously (27). Rabbit skeletal muscle actin was prepared according to the method of Spudich and Watt (28) and further purified by gel filtration on Sephadex G-100 in 2 mM Hepes, 0.1 mM CaCl\(_2\), 0.2 mM ATP, 0.05 mM dithiothreitol, and 0.01% NaN\(_3\), pH 7.9.

Polyclonal antibodies against r-cofilin were raised in rabbits. The antibody against the N-terminal extrapeptide of r-cofilin (Fig. 1, antiepiprodpeptide antibody) was purified by sequential column chromatography on DEAE-cellulose and EAH-Sepharose column (Pharmacia LKB Biotechnology Inc.) conjugated with the synthetic peptide corresponding to the N-terminal extrapeptide of r-cofilin.

\textit{Synthetic Peptides}—A dodecapeptide (see text) and a synthetic peptide corresponding to residues 150–166 of cofilin were synthesized by an automatic peptide synthesizer (Milligen model p50). The synthetic peptides were purified by preparative reverse-phase high pressure liquid chromatography on \textit{μBondaspher} \(5 \mu\) (C\(_18\), 100 Å) with a linear gradient of 0–40% acetonitrile in 0.1% trifluoroacetic acid. The amino acid composition of the synthetic peptides was determined by a Pico-tag work station (hydrolysis) and an amino acid analyzer JEOL JLC-300. The results were consistent with theoretical yields.
Preparation of the Complex of r-Cofilin and the N-terminal Segment of Actin—A 1:1 complex of actin and r-cofilin formed in 0.1 M NaCl, 1 mM Hepes, 10 mM imidazole, and 0.1 mM dithiothreitol, pH 7.0, was cross-linked with 20 mM EDC for 2 hr at 25°C. The cross-linked complex of actin and r-cofilin and the noncross-linked r-cofilin were separated on an SDS slab gel (13%) according to Laemmli (29). The gel slices were cut out, washed with methanol, and finally dried in vacuo according to Sutoh and co-workers (19–21,30). The hydrolysis of actin (rCA1-12) were cut out, washed with methanol, and dried in vacuo.

Mapping of the Actin-Cross-linking Site of Cofilin by the End Label Fingerprinting—The partial hydrolysis with either BNPS-skatole or CNBr of the noncross-linked r-cofilin or rCA1-12 within gel slices was performed essentially according to Sutoh and co-workers (19–21,30). The hydrolysis of actin along the cofilin sequence by partial digestion with either BNPS-skatole or CNBr of the noncross-linked r-cofilin (lanes 1, 2) or rCA1-12 (lane 2) were immunoblotted with the antiextrapeptide antibody. The fragments stained with the antiextrapeptide antibody are indicated by arrows (see Fig. 5a).

Effect of the Dodecapeptide on the Actin-Cofilin Cross-linking—r-Cofilin (final concentration 9.4 μM) and actin (final concentration 8.3 μM) were incubated with 14 mM EDC at 25°C for 2 hr in the presence of various concentrations of the dodecapeptide or the synthetic peptide corresponding to residues 150–166 of cofilin, polymers of actin along the cofilin sequence by partial digestion with BNPS-skatole (a) or CNBr (b). Fragments generated from either the noncross-linked r-cofilin (lane 1) or rCA1-12 (lane 2) were partially digested with BNPS-skatole (a) or CNBr (b). The fragments stained with the antiextrapeptide antibody are indicated by arrows with either letters (lane 1) or letters with primes (lane 2) Band A, r-cofilin; Band A’, rCA1-12. c, schematic diagram of maps of the cross-linking site of the N-terminal segment of actin along the cofilin sequence. The top and middle rows represent the results obtained from BNPS-skatole mapping and CNBr mapping, respectively. Indicates the N-terminal extrapeptide of r-cofilin. Positions of tryptophan (W) and methionine (M) are shown. Boxes shaded with dots represent the fragments identified as containing the actin-cross-linking site. From these maps the actin-cross-linking site has been determined to be located within residues 105–115 (bottom closed box).

Pelleting Assay—r-Cofilin (3.4 μM) and F-actin (3.4 μM) were reacted in the presence of various amounts of the dodecapeptide in a solution consisting of 35 mM KCl, 2 mM MgCl₂, 16 mM Hepes, and 4 mM Pipes, pH 7.0, at room temperature for 20 min. After centrifugation at 356,000 × g for 10 min, the pellet and supernatant were electrophoresed on SDS-polyacrylamide gel as described (27). The amounts of F-actin and r-cofilin bound to F-actin were quantitated by densitometry of SDS-polyacrylamide gels as described (27).

Assay for Actin Polymerization by Using Pyrene-labeled Actin—In the presence of various concentrations of the dodecapeptide or the synthetic peptide corresponding to residues 150–166 of cofilin, polymerization (see Fig. 5a) of G-actin (3 μM, 6% pyrene actin) or depolymerization (see Fig. 5b) of F-actin (3 μM, 6% pyrene actin) was monitored at 25°C by the changes in fluorescence intensity of pyrene labeled to actin in a buffer solution containing 85 mM KCl, 18 μM ATP, 3 μM CaCl₂, and 8.5 mM Pipes, pH 7.0. The pyrene-labeled actin was prepared as described (1, 32). The fluorescence intensity was measured with a Hitachi 650-10S fluorescence spectrophotometer.
cofilin to actin by EDC in the presence of various concentrations of the dodecapeptide (O), or a synthetic peptide corresponding to residues 150-166 of cofilin (C) are shown. The cross-linking efficiency in the absence of synthetic peptides was regarded as 100%. b, SDS-polyacrylamide gel showing the effect of the dodecapeptide on the cross-linking. r-Cofilin and actin were cross-linked by EDC in the absence (lane 1) or presence of either the dodecapeptide (1.5 mM, lane 2) or the synthetic peptide corresponding to residues 150-166 of cofilin (3 mM, lane 3). The positions of r-cofilin-actin complex, the dodecapeptide-actin complex, and actin are indicated by an arrow, an arrowhead, and an asterisk, respectively.

**Table I**

The dodecapeptide has no effect on the binding of cofilin to F-actin

The molar ratios of r-cofilin bound to F-actin to actin molecule in F-actin were determined by the pelleting assay as described under “Materials and Methods” in the presence of various concentrations of the dodecapeptide.

| Concentration of dodecapeptide (mM) | Molar ratio to F-actin |
|-------------------------------------|-----------------------|
| 0                                   | 1.0                   |
| 0.018                               | 0.99                  |
| 0.045                               | 1.1                   |
| 1.8                                 | 1.0                   |
| 2.5                                 | 1.1                   |
| 5.0                                 | 0.97                  |

and is shown in Fig. 5 in an arbitrary unit. The excitation and emission wavelengths were 365 and 407 nm, respectively.

**RESULTS AND DISCUSSION**

An Actin-Cross-linking Site on Cofilin Sequence—Cofilin has been expressed in E. coli and purified to homogeneity (9). This purified recombinant cofilin has an N-terminal extra-peptide containing nine amino acids (see Fig. 1) but is indistinguishable from the authentic cofilin purified from porcine brain in its interaction with actin in vitro (4, 9). Because cofilin is covalently cross-linked to an N-terminal segment (residues 1–12) of actin by a zero length cross-linker EDC (24) and an Asn-Gly sequence exists only at residues 12–13 of actin, digestion by hydroxylamine of the cross-linked product of r-cofilin and actin produced the complex of r-cofilin and the N-terminal segment of actin (we call this complex rCA1-12). rCA1-12 moved, as expected, slower than noncross-linked r-cofilin in SDS-polyacrylamide gels as shown in Fig. 2, a and b (compare bands A and A’). The cross-linking site of the N-terminal segment of actin on cofilin sequence was determined by means of the end label fingerprinting method, for which we made a rabbit polyclonal antibody against the N-terminal extrapeptide of r-cofilin (Fig. 1). The specificity of the antibody (antiextrapeptide antibody) is shown in Fig. 1. The antiextrapeptide antibody reacted only with r-cofilin and the cross-linked complex of actin and r-cofilin but did not react with porcine brain cofilin, actin, or the cross-linked complex of actin and porcine brain cofilin.

A fragment that comprises the amino acid residues from the N terminus to Trp\(^{104}\) of cofilin was not cross-linked to the N-terminal segment of actin (Fig. 2c) since treatment of rCA1-12 with BNPS-skatolone produced a fragment containing the extrapeptide of r-cofilin, whose mobility on SDS gels was completely identical with that of a fragment generated from noncross-linked r-cofilin (Fig. 2a, bands B and B’). CNBr mapping showed that the actin-cross-linking site is located in the segment of cofilin from Leu\(^{97}\) to Met\(^{115}\) (Fig. 2c) since a fragment from the N terminus to Met\(^{125}\) derived from
rCA1-12, moved slower than the corresponding fragment derived from noncross-linked r-cofilin (Fig. 2b, bands B and B’) whereas a fragment from the N terminus to Met155 derived from both rCA1-12 and noncross-linked r-cofilin, exhibited the same electrophoretic mobility (Fig. 2b, bands C and C’). From these maps we concluded that the actin-cross-linking site of cofilin is located within Ala103-Met115 (Fig. 2c). Because an N terminus of actin is blocked and the N-terminal segment (residues 1–12) of actin has five carboxyl groups but no amino groups, the candidates for residues cross-linked to actin in Ala103-Met115 of cofilin are Lys112 and/or Lys114 (Fig. 3, marked with O).

Homology of the Sequence of the Dodecapeptide with Other Actin-binding Proteins—A dodecapeptide corresponding to the sequence of Trp14-Met150 of cofilin was synthesized to examine the function of this region (Fig. 3, top row). Interestingly, the prediction of the secondary structure according to the method of Chou and Fasman (33) showed that only this region and the C-terminal short segment displayed random coil potential. As shown in Fig. 3, a sequence very similar to the dodecapeptide is found in destrin (9), ADF (14, 15), and depactin (16), mammalian, chicken, and echinodermatous actin-depolymerizing proteins, respectively. It should be noted that only this region in the depactin sequence exhibits close similarity with the corresponding sequence of cofilin, destrin, or ADF. Moreover, other actin-binding proteins, severin (residues 257–268) (34), dystrophin (residues 2975–2989) (35), villin (residues 485–496) (36), and α-spectrin (residues 218–229) (37), have a portion weakly homologous to the dodecapeptide sequence.

Inhibition of the Actin-Cofilin Cross-linking by the Dodecapeptide—The synthetic dodecapeptide inhibited the cross-linking of cofilin to actin by EDC in a dose-dependent manner (Fig. 4, a and b), and instead of cofilin the dodecapeptide itself was cross-linked to actin (Fig. 4b, lane 2). On the other hand, a synthetic peptide corresponding to the C-terminal portion (residues 150–166) of cofilin did not inhibit the cross-linking of cofilin to actin (Fig. 4a, O; and Fig. 4b, lane 3). These results suggest that the dodecapeptide specifically competes with cofilin for binding to actin.

Inhibition of Actin Polymerization by the Dodecapeptide—The pelleting assay revealed that the dodecapeptide did not inhibit the binding of cofilin to F-actin at all (Table I). The amount of F-actin was, however, decreased in the presence of the dodecapeptide irrespective of the presence or absence of cofilin (data not shown). We examined the effect of the dodecapeptide on the time course of actin polymerization by using pyrene-labeled actin. Fig. 5a shows that the dodecapeptide increased the lag time for actin polymerization, slowed down the speed of polymerization, and decreased the final extent of polymerization. Actin polymerization was almost completely inhibited in the presence of 100 dodecapeptides/actin (Fig. 5a). The pelleting assay confirmed that the decrease in the fluorescence intensity of pyrene actually reflects the decrease in the amount of F-actin (data not shown). The inhibitory activity of the peptide for actin polymerization was not dependent on changes in pH of the medium between pH 7.0 and 8.3 (data not shown). The dodecapeptide had the ability to depolymerize F-actin (Fig. 5b). Almost complete depolymerization occurred in the presence of 100 dodecapeptides/actin. The other synthetic peptide, corresponding to the C-terminal portion (residues 150–166) of cofilin, had no effect on actin polymerization and did not depolymerize F-actin at all (Fig. 5, a and b, broken lines). The F-actin depolymerization induced by the dodecapeptide was much slower than that induced by cofilin at pH 8.3 (3). This suggests that the dodecapeptide depolymerizes F-actin by sequestering G-actin equilibrated with F-actin. However, the possibility cannot be excluded that the dodecapeptide might also have the ability to attack F-actin directly because the initial rate of F-actin depolymerization increased slightly with increasing amounts of dodecapeptide (Fig. 5b). The apparent dissociation constant for the interaction of the dodecapeptide with actin was roughly estimated to be 20–60 μM, based on the dodecapeptide concentration required for complete inhibition of actin polymerization (Fig. 5a) and the critical concentration of actin under the ionic conditions used. Thus, the affinity of the dodecapeptide for actin is 2–3 orders of magnitude lower than that of cofilin (0.1–0.2 μM). It should be noted that such a short peptide with a potent inhibitory activity for actin polymerization has not been reported previously.

Thus, determination of an actin-cross-linking site on cofilin led us to identify a dodecapeptide capable of inhibiting actin polymerization strongly. As a sequence very similar to the dodecapeptide exists in a family of actin-depolymerizing proteins, this sequence region may constitute the domain responsible for actin-depolymerizing activity.

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, 1410–1411
4. Yonezawa, N., Nishida, E., Iida, K., Yahara, I., and Sakai, H. (1990) J. Biol. Chem. 265, 8382–8386
5. Nishida, E., Iida, K., Yonezawa, N., Koyasu, S., Yahara, I., and Sakai, H. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 5262–5266
6. Matsuizaki, F., Matsumoto, S., Yahara, I., Yonezawa, N., Nishida, E., and Sakai, H. (1989) J. Biol. Chem. 264, 11564–11568
7. Nishida, E., Maekawa, S., Muneyuki, E., and Sakai, H. (1984) J. Biochem. (Tokyo) 95, 378–398
8. Nishida, E., Muneyuki, E., Maekawa, S., Ohta, Y., and Sakai, H. (1985) Biochemistry 24, 6624–6630
9. Moriyama, K., Nishida, E., Yonezawa, N., Sakai, H., Matsumoto, S., Iida, K., and Yahara, I. (1990) J. Biol. Chem. 265, 5771–5773
10. Bamberg, J. R., Harris, H. E., and Weeds, A. G. (1980) FEBS Lett. 121, 178–182
11. Giuliani, K. A., Khatib, F. A., Hayden, S. M., Daoud, E. W. R., Adams, M. E., Amorose, D. A., Bernstein, B. W., and Bamberg, J. R. (1988) Biochemistry 27, 8931–8938
12. Mabuchi, I. (1983) J. Biol. Cell. 97, 1612–1621
13. Cooper, J. A., Blum, J. D., Williams, R. C. Jr., and Pollard, T. D. (1986) J. Biol. Chem. 261, 477–485
14. Adams, M. E., Minamide, L. S., Dwek, G., and Bamberg, J. R. (1990) Biochemistry 29, 7414–7420
15. Abe, H., Endo, T., Yamamoto, K., and Obinata, T. (1990) Biochemistry 29, 7420–7425
16. Takagi, T., Konishi, K., and Mabuchi, I. (1988) J. Biol. Chem. 263, 3097–3102
17. Sutoh, K. (1982) Biochemistry 21, 5059–5068
18. Sutoh, K., and Mabuchi, I. (1984) Biochemistry 23, 6757–6761
19. Sutoh, K., and Mabuchi, I. (1986) Biochemistry 25, 6186–6192
20. Sutoh, K., and Hatano, S. (1986) Biochemistry 25, 435–440
21. Sutoh, K., and Yin, H. L. (1989) Biochemistry 28, 5269–5275
22. Bartegi, A., Fattoum, A., and Kassab, R. (1990) J. Biol. Chem. 265, 2231–2237
23. Vandenberghove, S. J., Kaiser, D. A., and Pollard, T. D. (1989) J. Cell Biol. 109, 619–626
24. Muneyuki, E., Nishida, E., Sutoh, K., and Sakai, H. (1985) J. Biol. Chem. (Tokyo) 97, 563–568
25. Bresnick, A. R., Warren, V., and Condeelis, J. (1990) J. Biol. Chem. 265, 9236–9240
26. Yonezawa, N., Nishida, E., Ohba, M., Seki, M., Kumagai, H., and Sakai, H. (1989) Eur. J. Biochem. 183, 235–238
27. Yonezawa, N., Nishida, E., Maekawa, S., and Sakai, H. (1988) Biochem. 25, 121–127
28. Spudich, J. A., and Watt, S. (1971) J. Biol. Chem. 246, 4866–4871
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29. Laemmli, U. K. (1970) *Nature* **227**, 680–685
30. Sutoh, K., and Mabuchi, I. (1989) *Biochemistry* **28**, 102–106
31. Yonezawa, N., Nishida, E., Koyasu, S., Maekawa, S., Ohta, Y., Yahara, I., and Sakai, H. (1987) *Cell Struct. Funct.* **12**, 443–452
32. Brenner, S. L., and Korn, E. D. (1983) *J. Biol. Chem.* **258**, 5013–5020
33. Chou, P. W., and Fasman, G. D. (1978) *Annu. Rev. Biochem.* **47**, 251–276
34. André, E., Lottspeich, F., Schleicher, M., and Noegel, A. (1988) *J. Biol. Chem.* **263**, 722–727
35. Lemaire, C., Heilig, R., and Mandel, J. L. (1988) *EMBO J.* **7**, 4157–4162
36. Bazari, W. L., Matsudaire, P., Wallek, M., Smeal, T., Jakes, R., and Ahmed, Y. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 4986–4990
37. Wasenius, V.-M., Saraste, M., Salven, P., Eramaa, M., Holm, L., and Lehto, V.-P. (1989) *J. Cell. Biol.* **108**, 79–93