Down-regulation of the Filamentous Actin Cross-linking Activity of Cortactin by Src-mediated Tyrosine Phosphorylation*

(Received for publication, January 16, 1997, and in revised form, February 28, 1997)

Cai Huang‡, Yansong Ni‡, Tony Wang§, Yamei Gao‡, Christian C. Haudenschild‡, and Xi Zhan‡

From the ‡Department of Experimental Pathology, The Holland Laboratory, American Red Cross, Rockville, Maryland 20855; the §Division of Biology, Glaxo and Welcome Research Institute, Research Triangle Park, North Carolina 27709, and the Departments of ¶Pathology and §Anatomy and Cell Biology, The George Washington University, Washington, D. C. 20037

Cortactin, a prominent substrate for pp60c-src, is a filamentous actin (F-actin) binding protein. We show here that cortactin can promote sedimentation of F-actin at centrifugation forces under which F-actin is otherwise not able to be precipitated. Electron microscopic analysis after negative staining further revealed that actin filaments in the presence of cortactin are cross-linked into bundles of various degrees of thickness. Hence, cortactin is also an F-actin cross-linking protein. We also demonstrate that the optimal F-actin cross-linking activity of cortactin requires a physiological pH in a range of 7.3–7.5. Furthermore, pp60c-src phosphorylates cortactin in vitro, resulting in a dramatic reduction of its F-actin cross-linking activity in a manner depending on levels of tyrosine phosphorylation. In addition, pp60c-src moderately inhibits the F-actin binding activity of cortactin. This study presents the first evidence that pp60c-src can directly regulate the activity of its substrate toward the cytoskeleton and implies a role of cortactin as an F-actin modulator in tyrosine kinase-regulated cytoskeleton reorganization.

Cortactin (p80/p85) was initially discovered as a major phosphotyrosine-containing protein in v-src-transformed chicken embryo fibroblasts (1). The murine homologue was independently isolated as a signaling molecule involved in the transition from G0 to G1 phase in response to fibroblast growth factor (2, 3), and the human cortactin was found as an oncogene that is frequently amplified in subsets of tumors and tumor cell lines (4, 5). A strong association of cortactin with F-actin has been described (14). Consistent with its F-actin binding activity, cortactin primarily localizes within peripheral cell structures such as lamellipodia, pseudopodia, and membrane ruffles (9, 14), which are enriched for cytoskeletal proteins. However, unlike many other F-actin-binding proteins, the protein sequence of cortactin features a unique structure characterized by six and a half 37-amino acid tandem repeats and a Src homology 3 (SH3) domain at the carboxyl terminus. Between the SH3 and the repeat domains are an α-helix domain and a sequence region rich in proline residues.

Recent evidence has indicated that cortactin is a prominent substrate for Src-related protein-tyrosine kinases (1, 6–8). Furthermore, cortactin is implicated in signaling mediated by multiple extracellular stimuli including fibroblast growth factor (3), epidermal growth factor (9), thrombin (10), integrin (11), bacteria-mediated cell invasion (12), and mechanical strain (13). While tyrosine phosphorylation of cortactin is a profound phenomenon in response to many extracellular stimuli, the biological function of cortactin and the physiological role of tyrosine phosphorylation are not clear.

In an attempt to elucidate the function of cortactin, we have examined biochemical properties of cortactin. The study presented here demonstrated that cortactin is a potent F-actin cross-linking protein. Most importantly, the F-actin cross-linking activity is down-regulated upon phosphorylation mediated by pp60c-src. Thus, cortactin may act as an important mediator for intracellular tyrosine kinases in regulating the cytoskeleton reorganization in vivo.

EXPERIMENTAL PROCEDURES

Preparation of Recombinant Cortactin—I Murine cortactin was expressed in Escherichia coli as a glutathione S-transferase fusion protein in pGEX-2T plasmid and purified by affinity chromatography using glutathione-Sepharose (Pharmacia Biotech Inc.) as described previously (15). The glutathione S-transferase part of the fusion protein was removed by cleavage with bovine thrombin (ICN) in a digestion buffer (50 mM Tris-HCl, pH 8.2, containing 100 mM NaCl and 1 mM CaCl2) for 3–4 h at room temperature. The digested materials were loaded onto a DEAE-Sepharose FF (Pharmacia) column and eluted with 200 ml of elution buffer (20 mM Tris-HCl, pH 7.6, containing 1 mM MgCl2, 1 mM dithiothreitol, and 1 mM EGTA, and KCl with a gradient concentration from 20 to 600 mM). The fractions containing cortactin were pooled, and undigested fusion proteins were removed by additional chromatography using glutathione-Sepharose. The concentration of purified cortactin was determined by the D280 protein assay (Bio-Rad) according to the manufacturer’s instructions.

Preparation of Actin—Actin was purified from an acetone powder of rabbit skeletal muscle according to Pardee and Spudich (16). Pyrene-labeled actin was prepared as described by Kouyama and Mihashi (17). The labeled actin was further purified by chromatography using Sephadex G-150 (Pharmacia). Globular actin was polymerized into filaments by adding KCl, MgCl2, and ATP to the final concentrations of 134, 1, and 1 mM, respectively, and incubated for at least 4 h at room temperature.

Phosphorylation of Cortactin by pp60c-src—Recombinant human pp60c-src (18) was preactivated and maintained in a buffer containing 40 μM ATP, 0.8 mM MgCl2, and 1 mg/ml bovine serum albumin at 0 °C. To prepare tyrosine-phosphorylated cortactin, purified proteins were incubated with various amounts of preactivated pp60c-src in 20 μl of kinase buffer (50 mM Tris-HCl, pH 7.4, containing 5 mM MgCl2, 5 mM ATP, and 2 μCi of [γ-32P]ATP, 6000 c/mm) at room temperature for 1 h. For F-actin cross-linking analysis, phosphorylated cortactin proteins were diluted to a final volume of 50 μl of which the final concentrations of KCl and MgCl2 were readjusted to 134 and 2 mM, respectively. To
FIG. 1. Sedimentation of cortactin-cross-linked F-actin. A, pyrene-labeled filaments at a concentration of 4 μM in the presence (shaded bar) or absence (open bar) of cortactin (400 nM) were incubated for 30 min at room temperature and subsequently subjected to centrifugation at different forces as indicated. Fluorescence in the supernatant was measured, and its decrease was used as an indication of the precipitation of F-actin. B, cortactin at different concentrations was incubated with pyrene-labeled F-actin for 30 min followed by centrifugation at 26,300 × g for 10 min. The F-actin cross-linking activity was determined by fluorescence measurement as described above. The value of each point represents the mean of four independent experiments. C, the pellets from experiment B were resolved in SDS sample buffer, and aliquots of each sample were applied to a SDS-PAGE gel. Lane 1, without cortactin; lanes 2–5, with cortactin at concentrations of 26, 53, 106, and 212 nM, respectively. The bands corresponding to cortactin and actin are indicated. D, pyrene-labeled F-actin at a concentration of 4 μM was incubated with cortactin (200 nM) for the times indicated and subsequently centrifuged at 84,000 × g for 2 min. F-actin cross-linking was determined as described above.

FIG. 2. Electron microscopy of cross-linked F-actin in the presence of cortactin. F-actin or a mixture of F-actin and cortactin were stained with phosphotungstic acid as described under “Experimental Procedures.” A and C, filaments only (4 μM); B and D, filaments plus cortactin (4 μM and 107 nM, respectively). A and B have a magnification of × 1,800; C and D have a magnification of × 20,000.
confirm and quantitate phosphorylated cortactin, aliquots of the reaction were combined with an equal volume of 2× SDS sample buffer (19) and fractionated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The gel was stained with Coomassie Blue, and the phosphorylated proteins were visualized by autoradiography.

**F-actin Cross-linking Assay**—Purified cortactin at the indicated concentrations in 50 μl of TKM buffer (50 mM Tris-Cl, pH 7.4, containing 134 mM KCl and 1 mM MgCl₂) was mixed with an equal volume of 8 μM pyrene-labeled F-actin and incubated for 30 min at room temperature. The mixture was then immediately centrifuged at 26,300×g for 10 min at room temperature in a Beckman TL-100 centrifuge. The supernatant was carefully transferred to a new tube and mixed with 300 μl of TKM buffer containing 2 μM phalloidin (Sigma). The fluorescence in the supernatant was recorded on an LS50B luminescence spectrometer (Perkin-Elmer) at an excitation wavelength of 470 nm with a slit of 2.5 nm and an emission wavelength of 510 nm with a slit of 6 nm, respectively. The decrease in the supernatant fluorescence reflects the precipitation of F-actin due to cross-linking. In some experiments, the precipitated pellets were directly analyzed by SDS-PAGE.

**Electron Microscopic Analysis**—F-actin or the mixture of F-actin and cortactin was incubated for 2 min and then the supernatant was removed by centrifugation. The samples were fixed in 2.5% glutaraldehyde in 0.1 M potassium phosphate buffer, pH 7.4. The fixed samples were postfixed in 1% osmium tetroxide and 0.5% potassium ferrocyanide in 0.1 M potassium phosphate buffer, pH 7.4. The samples were dehydrated in an acetone series and then transferred to a 0.25% Formvar 15/95E resin- (Sigma) coated film on a gold grid. The grid was then air-dried overnight. Transmission electron micrographs were taken with a Philips M12 microscope.

**RESULTS AND DISCUSSION**

In an effort to investigate the function of cortactin, the murine cortactin was expressed in *E. coli* as a glutathione S-transferase fusion protein. The glutathione S-transferase-free cortactin was used for the evaluation of its F-actin cross-linking activity by virtue of a co-sedimentation assay (20). To determine optimal conditions for the assay, cortactin was incubated with F-actin for 30 min and subjected to centrifugation at different conditions for 13,000 to 30,000×g for 10 min. Under these conditions, neither filaments nor cortactin was detected in the absence of cortactin (Fig. 1A) nor cortactin alone could be precipitated (data not shown). However, the presence of both cortactin and F-actin resulted in a dramatic increase in the amount of F-actin associated with pellets in a dose-dependent manner (Fig. 1B). The half-maximum effect requires an approximately 1:100 molecular ratio of cortactin to actin, and further cross-linking of actin was not observed at the ratio of 1:36 ratio of cortactin to actin. The sedimentation of F-actin induced by cortactin is also correlated with the association of cortactin with F-actin, as shown by their co-sedimentation at a ratio of 1 cortactin molecule to approximately 15 actin subunits (Fig. 1C), which is in agreement with the stoichiometry for the binding of cortactin to actin (14). The two bands of 85 and 90 kDa shown on the gel most likely reflect different conformations of cortactin, since only one band of 85 kDa was visualized when SDS-PAGE was performed in the presence of 5 M urea (data not shown). A kinetic study further demonstrated that cortactin-mediated F-actin precipitation is a rapid process (Fig. 1D). Incubation with cortactin for 2 min resulted in a sedimentation of more than 60% of F-actin, although the maximum sedimentation (80% of total F-actin) only occurred at 30 min after interaction. The cross-linked F-actin in the presence of cortactin was further examined by electron microscopy after negative staining. Filaments prepared in the absence of cortactin displayed individual single strands (Fig. 2, A and C). In the presence of cortactin, however, most F-actin strands became thicker and formed a bundle-like structure (Fig. 2, B and D).

Since activities of many F-actin cross-linking proteins are dependent on Ca²⁺ and pH, we examined the effects of Ca²⁺ and pH on the activity of cortactin. In the presence of either EGTA or various concentrations of Ca²⁺, the F-actin cross-linking activity of cortactin was not affected (data not shown). However, when the cross-linking assay was performed at different pH values, the optimal sedimentation of F-actin was observed in a range from pH 7.3 to 7.5. The F-actin sedimentation induced by cortactin at pH 6.9 and 8.2 is only approximately 30% of that at pH 7.4 (Fig. 3). The apparent effect of pH on the F-actin cross-linking is not due to its potential effect on actin polymerization, since pH has little influence on the stable polymerization of actin (Fig. 3).

The purified cortactin can be efficiently phosphorylated by pp60src exclusively at tyrosine residues in a manner depending on time and the amount of Src. By incubating cortactin with 500 nM pp60src at room temperature for 1 h, a maximum phosphorylation of cortactin was reached (Fig. 4A). When the phosphorylated cortactin was used in the co-sedimentation assay, a dramatic inhibition for the F-actin cross-linking was observed (Fig. 4B). The treatment of cortactin with 62.5 nM pp60 src reduced the efficiency of F-actin sedimentation from near 60% to approximately 38%, and that with 500 nM pp60 src reduced further the sedimentation of F-actin to 10%. In a control experiment where cortactin was treated with the buffer only but in the absence of pp60 src, no reduction of the F-actin cross-linking was detected (Fig. 4B).

The apparent decrease in the F-actin cross-linking was not due to a possible inhibitory activity of pp60 src, since a solution in the presence of pp60 src itself did not have any detectable effect on F-actin cross-linking (data not shown). Furthermore, we examined whether tyrosine phosphorylation is essential for the inhibition of cortactin’s F-actin cross-linking activity. We carried out the phosphorylation of cortactin in a kinase buffer in the absence of Mg⁰, on which the kinase activity of pp60 src is dependent (21). As shown in Fig. 5A, the Src-mediated tyrosine phosphorylation of cortactin was abolished in the absence of Mg⁰. When the Src-treated cortactin in the absence of Mg⁰ was mixed with F-actin and subsequently subjected to the co-sedimentation analysis, a significant amount of F-actin was detected in the pellet (Fig. 5B, column 4). Although the level of sedimentation of F-actin induced by cortactin and pp60 src in the absence of Mg⁰ was about 33% lower than that by non-Src-treated cortactin (Fig. 5B, compare columns 2 and 4), the lower efficiency could be the result of a trace amount of Mg⁰ present in the F-actin buffer, which may have partially restored the kinase activity of pp60 src. Indeed, when Src-treated cortactin in the absence of Mg⁰ was further incubated with F-actin, cortactin was able to be phosphorylated to the extent of approximately 25% of that in the regular kinase buffer (Fig. 5A,
Taken together, these data demonstrate that the inhibition of the F-actin cross-linking activity of cortactin by pp60<sup>src</sup> is dependent on tyrosine phosphorylation. Cortactin has been previously described as a potent F-actin-binding protein (14). Therefore, we also examined the effect of pp60<sup>src</sup> on its F-actin binding activity by co-sedimentation at a high centrifugation force of 366,000 <sup>3</sup>g, at which actin filaments are able to be precipitated. As shown in Fig. 6, the Src treatment resulted in an inhibition of the F-actin binding activity of cortactin. However, the efficiency of the inhibition is apparently less than that for the F-actin cross-linking. At the concentration of 62.5 nM pp60<sup>src</sup>, the co-precipitated cortactin with F-actin was reduced only from 95 to 85%; at 500 nM pp60<sup>src</sup>, 42% of cortactin was still bound to F-actin. The moderate inhibition by pp60<sup>src</sup> may be due to the existence of multiple F-actin binding sites in cortactin, which are involved in the F-actin cross-linking activity (30). The lower sensitivity to pp60<sup>src</sup> could also be the reason for a failure to observe the inhibition of the F-actin binding activity of cortactin in a system using lysates from v-Src-transformed cells (14). However, we cannot rule out the possibility that different phosphorylation sites or additional kinase(s) may be involved in that system.

The dependence on neutral pH for the optimal cross-linking activity of cortactin is uncommon. For example, α-actinin has the highest cross-linking activity at pH 6.8 (22). Talin shows a reduced actin cross-linking activity when pH is increased from 6.5 to 7.3, whereas its optimal activity is at pH 6.5 (23). EF1α

**Fig. 4.** Effects of the amount of pp60<sup>src</sup> on the F-actin cross-linking activity of cortactin. A, cortactin was incubated with different amounts of pp60<sup>src</sup> for 1 h in the presence of [γ-<sup>32</sup>P]ATP. The phosphorylated cortactin was analyzed by SDS-PAGE and visualized by autoradiography (top). The intensity of each band was quantitated by densitometry analysis (bottom). B, phosphorylated cortactin proteins using different concentrations of pp60<sup>src</sup> (●) were analyzed for F-actin cross-linking. As a negative control (■), cortactin was treated with the same amounts of the buffer used for pp60<sup>src</sup> (see “Experimental Procedures”).

**Fig. 5.** Src-mediated inhibition of the F-actin cross-linking activity of cortactin is dependent on tyrosine phosphorylation. A, cortactin (400 nM) was incubated with pp60<sup>src</sup> in the presence of [γ-<sup>32</sup>P]ATP in either a regular kinase buffer (with Mg<sup>2+</sup>, open bar) or an inhibitory buffer (without Mg<sup>2+</sup>, striated bar). Half volume of the reactions were terminated by adding an equal amount of 2 × SDS sample buffer (columns 1 and 2), and the other half were further incubated with F-actin for an additional 30 min (columns 3 and 4). All reactions were analyzed by SDS-PAGE followed by autoradiography. The relative levels of tyrosine phosphorylation of cortactin were determined by densitometry analysis. B, cortactin treated with pp60<sup>src</sup> in either the presence (column 3) or absence (column 4) of Mg<sup>2+</sup> was subjected to F-actin cross-linking analysis. Column 1, no cortactin; column 2, cortactin without pp60<sup>src</sup> treatment.
has high F-actin-cross-linking activity at low pH (6.2–6.5) (24). It has been well recognized that pH is involved in the regulation of the actin cytoskeleton (24) and cell motility (25, 26). A recent study also indicates that the induction of Rho on stress actin filaments is dependent on Na+/H+ exchange (27). Since both intracellular pH and tyrosine kinase activities are regulated by a variety of extracellular signals (28, 29), cortactin could act as an important mediator for ligands to regulate the cytoskeleton reorganization.

Acknowledgment—We thank Nick Greco for critical reading of the manuscript and Diana Norman for expert photographic assistance.

REFERENCES
1. Wu, H., Reynolds, A. B., Kanner, S. B., Vines, R. R., and Parsons, J. T. (1991) Mol. Cell. Biol. 11, 5113–5124
2. Zhan, X., Hu, X., Friesel, R., and Maciag, T. (1993) J. Biol. Chem. 268, 9611–9620
3. Zhan, X., Hu, X., Hampton, B., Burgess, W. H., Friesel, R., and Maciag, T. (1993) J. Biol. Chem. 268, 24427–24431
4. Schuuring, E. D., Verhoeven, E., Litvinov, S., and Michalides, R. J. A. M. (1993) Mol. Cell. Biol. 13, 2891–2898
5. Bringuier, P. P., Tamimi, Y., Schuuring, E., and Schalken, J. (1996) Oncogene 12, 1747–1753
6. Nada, S., Okada, M., Aizawa, S., and Nakagawa, H. (1994) Oncogene 9, 3571–3578
7. Obisal, Y., Nada, S., Okada, M., and Nakagawa, H. (1994) Jpn. J. Cancer Res. 85, 1023–1028
8. Thomas, S. M., Soriano, P., and Imamoto, A. (1995) Nature 376, 267–271
9. Maas, M., Wilson, L. K., Meyers, J. S., Vines, R. R., Parsons, J. T., and Parsons, S. J. (1992) Oncogene 7, 2429–2438
10. Wong, S., Reynolds, A. B., and Papkoff, J. (1992) Oncogene 7, 2407–2415
11. Kim, K. H., and Ruoslahti, E. (1995) J. Biol. Chem. 270, 22263–22266
12. Debow, C., Prevost, M. C., and Sansonetti, P. J. (1995) EMBO J. 14, 2471–2482
13. Liu, H., Qin, Y., Tangwell, A. K., and Post, M. (1996) J. Biol. Chem. 271, 7066–7071
14. Wu, H., and Parsons, J. T. (1993) J. Cell Biol. 120, 1417–1426
15. Zhan, X., Plourde, C., Hu, X., Friesel, R., and Maciag, T. (1994) J. Biol. Chem. 269, 20221–20224
16. Pardee, J. D., and Spudich, J. A. (1982) Methods Enzymol. 85, 164–181
17. Kueyama, T., and Sotani, N. (1981) Eur. J. Biochem. 114, 33–38
18. Barch, S. C., Kaul, D. B., Weigl, D., Huang, X., Luther, M. A., and Knight, W. B. (1995) Biochemistry 34, 14834–14851
19. Laemmli, U. K. (1970) Nature 227, 680–685
20. Brtsch, E. A., Hartwig, J. H., and Stossel, T. P. (1978) J. Biol. Chem. 253, 8988–8993
21. Richert, N. D., Blitthe, D. L., and Pastan, I. (1982) J. Biol. Chem. 257, 7143–7150
22. Condeelis, J., and Wahey, M. (1982) J. Cell Biol. 94, 466–471
23. Zhang, J., Rohou, R. M., Schmidt, J. M., and Stenmer, M. H. (1996) Biochem. Biophys. Res. Commun. 218, 520–527
24. Edmonds, B. T., Murray, J., and Condeelis, J. (1995) J. Biol. Chem. 270, 15222–15230
25. Shimchowitz, L., and Crague, E. J., Jr. (1986) J. Biol. Chem. 261, 6492–6500
26. Dzion, B. V., and Inouye, K. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 4951–4955
27. Vexler, Z. S., Verhoeven, E., Litvinov, S., and Michalides, R. J. A. M. (1993) Mol. Cell. Biol. 13, 2891–2898
28. Grinstein, S., Rotin, D., and Mason, M. J. (1989) Biochim. Biophys. Acta 988, 73–97
29. Erpel, T., and Courtneidge, S. A. (1995) J. Biol. Chem. 271, 176–182
30. Matsudaira, P. (1991) Trends Biochem. Sci. 16, 87–92

FIG. 6. Inhibition of the F-actin binding activity of cortactin by Src-mediated phosphorylation. Recombinant cortactin (200 nM) was incubated with pp60c-src at different concentrations in 20 μl of 50 mM Tris-HCl, pH 7.4, containing 5 mM MgCl2 and 5 mM ATP for 1 h at room temperature. The reactions were diluted to a final volume of 50 μl, mixed with 50 μl of 4 μM F-actin, and incubated for 30 min on ice. The mixtures were then centrifuged at 336,000 × g for 15 min. The generated supernatant and pellets were analyzed by immunoblotting analysis with 4F11. The relative amounts of cortactin bound to F-actin were determined by densitometry analysis (upper panel). The value for each point represents the mean of three independent experiments. One representative photograph of immunoblot analysis was also shown (lower panel).
Down-regulation of the Filamentous Actin Cross-linking Activity of Cortactin by Src-mediated Tyrosine Phosphorylation

Cai Huang, Yansong Ni, Tony Wang, Yamei Gao, Christian C. Haudenschild and Xi Zhan

*J. Biol. Chem.* 1997, 272:13911-13915.
doi: 10.1074/jbc.272.21.13911

Access the most updated version of this article at [http://www.jbc.org/content/272/21/13911](http://www.jbc.org/content/272/21/13911)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 30 references, 15 of which can be accessed free at [http://www.jbc.org/content/272/21/13911.full.html#ref-list-1](http://www.jbc.org/content/272/21/13911.full.html#ref-list-1)