The Role of Tyrosine Phosphorylation of Cortactin in the Locomotion of Endothelial Cells*

(Received for publication, June 1, 1998, and in revised form, July 27, 1998)

Cai Huang‡, Jiali Liu‡, Christian C. Haudenschild‡§, and Xi Zhan‡¶

From the ‡Department of Experimental Pathology, The Holland Laboratory, American Red Cross, Rockville, Maryland 20855 and the Departments of §Anatomy and Cell Biology and ¶Pathology, The George Washington University, Washington, D.C. 20037

Cortactin, a filamentous actin cross-linking protein and a substrate of Src protein tyrosine kinase, is phosphorylated at tyrosine residues upon stimulation by extracellular signals. We have previously demonstrated that the filamentous actin cross-linking activity of cortactin is attenuated by Src (Huang, C., Ni, Y., Gao, Y., Haudenschild, C. C., and Zhan, X. (1997) J. Biol. Chem. 272, 13911–13915). In vitro, tyrosine phosphorylation of cortactin occurs specifically within the region between the proline-rich sequence and the Src homology 3 domain. Among the nine tyrosine residues in this region, mutations at Tyr421, Tyr466, and Tyr482 significantly reduced Src-mediated tyrosine phosphorylation both in vitro and in vivo. Ectopic expression of wild-type cortactin in ECV304, a spontaneously transformed human umbilical endothelial cell line, resulted in an enhanced cell migration. In contrast, overexpression of a cortactin mutant deficient in tyrosine phosphorylation impaired the migration of endothelial cells. These findings reveal an intracellular signaling mechanism whereby the motility of endothelial cells is regulated by a Src-mediated tyrosine phosphorylation of cortactin.

Endothelial cells, which line the lumen of blood vessels, are versatile in morphology and undergo a rapid remodeling during vascularization. Whereas the mechanism for remodeling endothelial cells is not fully understood, the stimulation of the cytoskeletal reorganization that begins underneath the plasma membrane by extracellular signals, especially angiogenic factors, is likely to play an important role in physiological and pathological functions of endothelial cells such as angiogenesis.

In a previous effort to characterize signaling molecules of fibroblast growth factor 1 (FGF-1),¹ a potent endothelial cell growth factor, we identified a phosphotyrosyl protein as cortactin, a substrate of Src protein tyrosine kinase (2). Tyrosine phosphorylation of cortactin induced by FGF-1 is elevated during the late G1 phase (4–6 h) in Balb 3T3 fibroblasts. The stimulation with FGF-1 also results in a transient association of Src with cortactin as well as in an association of FGF receptor 1 with Src (3), suggesting that the activation of Src is a mechanism for the tyrosine phosphorylation of cortactin induced by FGF-1. In addition to FGF-1, various other extracellular stimuli, which often result in a cell motility response, also stimulate the tyrosine phosphorylation of cortactin. These signals include epidermal growth factor (4), thrombin (5), collagen (6), integrin activation (7, 8), phagocytosis (9), and mechanical strain (10).

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-helical structure and a sequence rich in proline residues. This structural feature containing a repeat domain and a carboxyl-terminal SH3 domain resembles neufectin, a F-actin-associated protein (11). Indeed, cortactin localizes within F-actin-enriched peripheral structures such as lamellipodia and membrane ruffles (12) and is found as a potent F-actin-binding protein in vitro (13). Whereas these data suggest that cortactin may function as a modulator for the actin cytoskeleton, its biological significance for the cellular response to growth factors and the role of tyrosine phosphorylation of cortactin in this process remains unclear.

In a recent study, we described that cortactin acts as a F-actin cross-linker. Importantly, the F-actin cross-linking activity of cortactin is attenuated in vitro upon tyrosine phosphorylation mediated by Src (14). In this study, we characterize the tyrosine residues targeted by Src and demonstrate that these residues are important for the Src-mediated attenuation of the F-actin cross-linking activity of cortactin. In addition, we examined the role of tyrosine phosphorylation of cortactin within endothelial cells by analyzing endothelial cell transfecants expressing a cortactin mutant deficient in tyrosine phosphorylation. We show here that overexpression of wild-type cortactin results in a significant increase in endothelial cell migration. In contrast, expression of the cortactin mutant impairs cell migration. Our data provide evidence for the involvement of tyrosine phosphorylation of cortactin in the migration of endothelial cell.

EXPERIMENTAL PROCEDURES

Antibodies—Homemade polyclonal antibody (C001) against murine cortactin has been described previously (2). Monoclonal anti-phosphotyrosine antibody (4G10) and monoclonal anti-cortactin antibody 4F11 were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Monoclonal anti-tyc antibody 9E10 was kindly provided by Tom Maciag (Maine Medical Center, Portland, ME).

Cell Culture—Human endothelial ECV304 cells (a gift of Tom Maciag) were maintained in M199 medium supplemented with 10% fetal bovine serum and antibiotics. NIH 3T3 cells (American Type Culture Collection, Rockville, MD) and 3T3/v-Src, a cell line with constitutive expression of the v-src oncogene (kindly provided by J. Brugge, ARIAD Pharmaceuticals, Inc., Cambridge, MA), were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) calf

---

*(Revised) 1. The abbreviations used are: FGF, fibroblast growth factor; F-actin, filamentous actin; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SH3, Src homology 3; GST, glutathione S-transferase.
Cell Migration Requires the Tyrosine Phosphorylation of Cortactin

Preparation of Recombinant Cortactin Proteins—GST fusion proteins were prepared, digested with thrombin, and purified as GST-free proteins as described previously (14). The concentrations of proteins were determined by De Protein Assay kit (Bio-Rad) according to the manufacturer's instructions.

Actin Preparation—Actin was purified from an acetone powder of rabbit skeletal muscle using the protocol described by Spudich and Watt (15). Pyrene-labeled actin was prepared as described by Kouyama and Mihashi (16). The labeled actin was further purified using Sephadex G-150 as described previously (14).

Preparation of Recombinant Cortactin Proteins—GST fusion proteins were prepared, digested with thrombin, and purified as GST-free proteins as described previously (14). The concentrations of proteins were determined by De Protein Assay kit (Bio-Rad) according to the manufacturer's instructions.

Analysis of Tyrosine Phosphorylation of Cortactin and Its Mutants—Equal amounts (3 μg) of purified cortactin and its mutant proteins were incubated at room temperature for 30 min in a kinase reaction buffer containing recombinant Src (from Tony Wang, Glaxo Wellcome, La Jolla, CA) according to the manufacturer's instructions.

Phosphoamino Acids Analysis—Phosphoamino acid analysis was carried out based on the method described previously (18). Briefly, the phosphorylated cortactin mutants were excised and purified as GST-free proteins as described previously (14). The samples were analyzed by SDS-PAGE and visualized by autoradiography.

Phosphopeptide Analysis of Cortactin—Phosphopeptide analysis was carried out in a volume of 20 l. The kinase reaction was terminated by adding an equal volume of 2× SDS sample buffer (17) and further analyzed by SDS-PAGE and visualized by autoradiography.

Actin Preparation—Actin was purified from an acetone powder of rabbit skeletal muscle using the protocol described by Spudich and Watt (15). Pyrene-labeled actin was prepared as described by Kouyama and Mihashi (16). The labeled actin was further purified using Sephadex G-150 as described previously (14). The samples were added with 30 μl of Sepharose 4B beads (50% v/v) conjugated with cortactin antibody 4F11 as described previously (14).

Immunofluorescence Microscopic Analysis—Cells grown at log phase were trypsinized and plated on glass coverslips precoated with human fibronectin (50 μg/ml). After 2 days of plating, cells were fixed in phosphate-buffered saline containing 4% paraformaldehyde for 10 min and permeabilized in 0.5% Triton X-100 in phosphate-buffered saline.
for 5 min. The permeabilized cells were then incubated with anti-cortactin antibody C001 at a concentration of 2 μg/ml in phosphate-buffered saline containing 5% bovine serum albumin for 1 h and further incubated for an additional hour with rhodamine-conjugated goat anti-rabbit IgG antibody (Pierce) at a concentration of 5 μg/ml and fluorescein isothiocyanate-labeled phalloidin (Sigma) at a concentration of 1 μM. Between each step, three washes with phosphate-buffered saline were applied. After the final wash, the cells were mounted on a glass slide and inspected under a laser confocal scanning microscope (Noran Instrument, Inc., Middleton, WI) equipped with the Image-1 analysis program (Universal Imaging Co., West Chester, PA). The resulting digital images were further analyzed by Adobe Photoshop.

RESULTS AND DISCUSSION

Murine cortactin contains a total of 27 tyrosine residues that localize mostly in the six repeats and the region between the repeat and the SH3 domains. To obtain a location of tyrosine residues targeted by Src, we initially analyzed several cortactin deletion and truncation mutants in vitro. As shown in Fig. 1, mutants CortD375–496 and CortD496–546 could not be phosphorylated by Src in vitro. However, cortactin fragment Cort323–496, which contains the region between the repeat and the SH3 domains, and deletion mutant CortD495–546, which lacks the SH3 domain, were efficiently phosphorylated by Src exclusively at tyrosine residues, as determined by phosphoamino acid analysis (Fig. 1). Whereas the SH3 domain also contains two tyrosine residues, a peptide fragment containing the SH3 domain alone is unable to be phosphorylated by Src (data not shown). Therefore, these data indicate that tyrosine residues located between the repeat and the SH3 domains are responsible for the Src-mediated phosphorylation.

The exact residues targeted by Src were determined by site-directed mutagenesis at the nine tyrosine residues located between the repeat and the SH3 domains (Fig. 2A). Whereas substitution of tyrosine with phenylalanine at Tyr334, Tyr433, Tyr442, Tyr460, and Tyr475 did not significantly impair the Src-mediated tyrosine phosphorylation (data not shown), mutations at single amino acids Tyr421 or Tyr466 reduced the levels of tyrosine phosphorylation by 40 and 20%, respectively (Fig. 2B). However, a mutant at both Tyr421 and Tyr466 did not to-
Cell Migration Requires the Tyrosine Phosphorylation of Cortactin

Fig. 3. Inhibition of F-actin cross-linking activity requires the presence of tyrosine residues targeted by Src. Purified recombinant cortactin and its mutants (3 μg) were incubated with or without recombinant Src (250 nM) in kinase buffer for 1 h. The phosphorylated proteins were analyzed for the association with pyrene-labeled F-actin. The F-actin cross-linking activity was determined by co-sedimentation analysis as described under “Experimental Procedures.” A, the F-actin cross-linking activity (efficiency of sedimentation) was plotted for each mutant. The data represent three independent experiments. B, the relative inhibitory activity of Src on each cortactin mutant. The percentage of inhibition by Src was calculated by the ratio of the difference of the F-actin cross-linking activity measured in the presence and absence of Src to that measured in the absence of Src.

tally abolish the response to Src, with the remaining levels of tyrosine phosphorylation about 50% of that of wild-type cortactin (Fig. 2B). This indicated the presence of additional tyrosine residue(s) that can be phosphorylated by Src. Indeed, a mutant at Tyr406 and Tyr462 showed only 33% tyrosine phosphorylation levels of wild-type cortactin (data not shown). A mutant with triple mutations at Tyr421, Tyr466, and Tyr482 had a poorer response to Src compared with wild-type cortactin (14%), whereas the mutant at Tyr421, Tyr466, Tyr482, and Tyr485 reduced tyrosine phosphorylation slightly further (Fig. 2B). To further verify those phosphotyrosine residues, wild-type cortactin and mutants by Src were subjected to phosphopeptide analysis by proteolysis with V8 Glu-C endoproteinase followed by fractionation with reverse-phase high-performance liquid chromatography. As shown in Fig. 2C, a total of three phosphopeptide-containing elutes were detected from wild-type cortactin, whereas less phosphopeptide elutes were found from mutants. Mutant CortF421 generated two phosphopeptide elutes, mutant CortF421F466 generated only one phosphopeptide elute, and mutant CortF421F466F482 did not generate any apparent elute containing phosphopeptides.

To examine whether Tyr421, Tyr466, and Tyr482 can be also targeted by Src in vitro, the corresponding mutants were tagged by a myc epitope (19) in pMEXneo, a mammalian expression vector (20), and transfected into 3T3/v-Src. The lysates from the resulting transient transfectants were immunoprecipitated and analyzed by phosphotyrosine immunoblotting analysis using monoclonal 4G10 antibody. As shown in Fig. 2D, the myc-tagged wild-type cortactin (myc-cortactin), shown as a 90-kDa band, was efficiently phosphorylated. In contrast, the levels of tyrosine phosphorylation of myc-CortF421 and myc-CortF421F466 mutants were dramatically reduced, and no tyrosine phosphorylation was detected with myc-CortF421F466 and myc-CortF421F466F482 mutants. The assay also displayed a band of 85 kDa, which was likely due to contamination from the endogenous cortactin during the purification procedure, as indicated by its co-migration with the cortactin band shown in the control sample (Fig. 2D, lane 1). These results indicate that Tyr421, Tyr466, and Tyr482 are the major sites phosphorylated by Src both in vitro and in vivo. Indeed, there are several acidic residues in the proximity of these residues that agree with the consensus sequences (I/VYEE) for Src-related tyrosine kinases (21). Furthermore, all of these three sites are conserved among chicken, mouse, and human. It is noteworthy that Tyr421 matches the consensus sequence (Tyr + two acidic amino acids) for the association with SH2 domains of Src tyrosine kinases (22) and may be responsible for its interaction with Src upon phosphorylation (3).

Our previous study has demonstrated that Src inhibits the F-actin cross-linking activity of cortactin in vitro (14). To verify that this inhibition requires phosphorylation of these characterized tyrosine residues, we examined the effects of Src on the F-actin cross-linking activities of various mutants with substitution at those tyrosine residues. The inhibitory activity of Src on the mutants apparently depended on the numbers of tyrosine residues in the region between the repeat and the SH3 domains (Fig. 3). Whereas Src was able to inhibit the cross-linking activity of wild-type cortactin by 80%, as reported previously (14), it only inhibited CortF421F466F482 and CortF421F466F482F485 mutants by 36 and 18%, respectively. Thus, tyrosine phosphorylation of these tyrosine residues is necessary for the Src-mediated attenuation of the activity of cortactin. Furthermore, the result confirms our previous conclusion that it is tyrosine phosphorylation that is responsible for the inhibition of the F-actin cross-linking activity (14).

To analyze the role of tyrosine phosphorylation of cortactin within cells, we transfected wild-type cortactin and mutant CortF421F466F482 as myc-tagged fusion proteins into ECV304 cells, a spontaneously transformed human umbilical endothelial cell line (23). Several stable transfectants were isolated. The expression of myc-cortactin proteins in the resulting stable transfectants was verified by immunoblotting analysis, and a typical result is shown in Fig. 5A. The slightly lower molecular weight of CortF421F466F482 mutant (lane 1) as compared with that of myc-cortactin (lane 2) was expected because of the change from tyrosine to phenylalanine. The subcellular distribution of cortactin derivatives in the transfectants was examined by immunofluorescent staining using the polyclonal cortactin antibody C001, which specifically recognizes the murine cortactin, as demonstrated by its exclusive staining of trans-
fected cells in a transient transfection assay (Fig. 4, a and b). Both wild-type (Fig. 4c) and mutant proteins (Fig. 4e) are found within the cytoplasm and perinuclear area in a pattern similar to that of the endogenous cortactin of Swiss 3T3 cells (Fig. 4g). In addition, both proteins co-localize with F-actin along the cell periphery, showing punctate patterns (Fig. 4, d and f). Thus, tyrosine phosphorylation is required neither for the distribution of cortactin into membrane structures nor for F-actin association. However, the overall morphology of cells expressing wild-type cortactin is different from that of cells expressing the mutant. Cells expressing myc-cortactin tend to be spindle-shaped and have elongated membrane protrusions at the edge of the cell (Fig. 4, c and d). In contrast, the cells expressing the mutant have a more circular shape and display visible stress fibers within the cytoplasm (Fig. 4, e and f).

There has been evidence that tyrosine phosphorylation of cortactin within endothelial cells can be promoted by the activation of cell motility-regulatory molecules such as integrins or adhesion molecules (8, 24). A recent report also described a correlation of the impairment of FGF-1-induced migratory response in senescent human umbilical vein endothelial cells with the inert Src tyrosine kinase and reduced tyrosine phos-

**Fig. 4. Immunofluorescent analysis of ECV304 transfectants overexpressing myc-cortactin and cortactin mutant.** ECV304 transfectants were plated on fibronectin-coated coverslips and double immunostained with cortactin antibody C001 (a, c, e, and g) and phalloidin (b, d, f, and h). The cells were inspected with a confocal scanning microscope. a and b, transient ECV304 cells expressing myc-cortactin. Whereas all cells were stained for F-actin (b), only transfected cells showed cortactin staining, indicating that the staining is specific. c and d, stable ECV304 cells expressing myc-cortactin and displaying a pattern similar to that of transient cells; e and f, stable ECV304 cells expressing myc-Cort$_{F421F466F48}$; g and h, quiescent nontransfected Swiss 3T3 cells shown as controls for the location of the endogenous cortactin and F-actin.
phorylation of cortactin (25). Thus, we suspected that ectopic expression of cortactin mutants may affect the locomotion of endothelial cells. The motility of ECV304 transfectants was evaluated using a wound healing assay. Within 20 h, ECV304 cells overexpressing myc-cortactin healed nearly 100% of a wound of 0.75 mm (Fig. 5B, b). In contrast, cells expressing the mutant filled less than 50% of the wound, and nontransfected cells filled nearly 60% of the wound. The difference in the motility of these cells was more apparent when the assay was performed on a larger wound of 2 mm. In this case, cells expressing the mutant showed much less migration into the wound compared with that of either wild-type transfectants or control cells, even after 4 days (Fig. 5C). Inhibition of cell migration by overexpression of the mutant CortF421F466F482 indicates that the mutant acts in a dominant negative manner. Indeed, the inhibitory activity is also correlated with the levels of expression of the mutant, because transfectants expressing lower levels of the mutant protein did not show significant deficiency in cell migration (data not shown).

The differential motility exhibited by ECV304 cells overexpressing the wild-type cortactin and its mutant is not likely to be due to their effects on cell proliferation because neither of the transfectants showed altered growth rates compared with that of control cells (data not shown). Because cortactin does not affect the formation of actin filaments in vitro, it is unlikely that the regulation of actin assembly is the direct cause for the observed increase in cell motility manifested by the overexpression of myc-cortactin. On the other hand, the dominant negative cortactin mutant that lacks tyrosine phosphorylation sites is unable to be phosphorylated by Src, which attenuates the F-actin cross-linking activity of wild-type cortactin. Thus, we propose that endothelial cell motility can be either up- or down-regulated by a novel mechanism that alters the F-actin cross-linking activity of cortactin through Src-mediated phosphorylation.

Tyrosine phosphorylation that occurs with the region between the repeat and the SH3 domain seems to be conservative in the cortactin family. It has been reported that HS1, a protein closely related to cortactin that is specifically expressed in hematopoietic cells (26), utilizes the same domain as the target for Src-related protein tyrosine kinases (27, 28). Tyrosine phosphorylation of the counterpart of HS1 is important for B-cell antigen receptor-mediated apoptosis and its nuclear localization (28). It has been shown that tyrosine phosphorylation of Tyr378 and Tyr397 of HS1 involves a sequential phosphorylation event, initially requiring Syk and later requiring Lyn or Fgr (29). Whereas the association of cortactin with Syk in phorbol ester-stimulated K562 leukemic cells has been described previously (30), cortactin is expressed mainly in adherent cells (31), where Syk is less abundant. Therefore, it is less likely that Syk is the primary kinase for cortactin in endothelial cells. However, it is still of interest to determine whether cortactin also undergoes a similar sequential phosphorylation by other intracellular kinases that function in concert with Src. In addition, the existence of three primary sites for tyrosine phosphorylation suggests that there may be also cooperative interactions among these residues.

---

C. Huang, unpublished observations.
Acknowledgment—We acknowledge Diana Norman for help with the photography and manuscript preparation.

REFERENCES

1. Inger, D. E., Prusty, D., Sun, Z., Betensky, H., and Wang, N. (1995) J. Biomech. 28, 1471–1484
2. Zhan, X., Hu, X., Hampton, B., Burgess, W. H., Friesel, R., and Maciag, T. (1995) J. Biol. Chem. 268, 24427–24431
3. Zhan, X., Plourde, C., Hu, X., Friesel, R., and Maciag, T. (1994) J. Biol. Chem. 269, 20221–20224
4. Maa, M., Wilson, L. K., Moyers, J. S., Vines, R. R., Parsons, J. T., and Parsons, S. J. (1992) Oncogene 7, 2429–2438
5. Wang, K., Knipfer, M., Huang, Q. Q., van Heerden, A., Hsu, L. C., Haudenschild, C. C., and Zhan, X. (1997) Blood 89, 457–464