Actin depolymerization-induced tyrosine phosphorylation of cortactin: the role of Fer kinase

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The F-actin-binding protein cortactin is an important regulator of cytoskeletal dynamics, and a prominent target of various tyrosine kinases. Tyrosine phosphorylation of cortactin has been suggested to reduce its F-actin cross-linking capability. In the present study, we investigated whether a reciprocal relationship exists, i.e. whether the polymerization state of actin impacts on the cortactin tyrosine phosphorylation. Actin depolymerization by LB (latrunculin B) induced robust phosphorylation of C-terminal tyrosine residues of cortactin. In contrast, F-actin stabilization by jasplakinolide, which redistributed cortactin to F-actin-containing patches, prevented cortactin phosphorylation triggered by hypertonic stress or LB. Using cell lines deficient in candidate tyrosine kinases, we found that the F-actin depolymerization-induced cortactin phosphorylation was mediated by the Fyn/Fer kinase pathway, independent of Src and c-Abl. LB caused modest Fer activation and strongly facilitated the association between Fer and cortactin. Interestingly, the F-actin-binding region within the cortactin N-terminus was essential for the efficient phospho-

rylation of C-terminal tyrosine residues. Investigating the structural requirements for the Fer–cortactin association, we found that (i) phosphorylation-incompetent cortactin still bound to Fer; (ii) the isolated N-terminus associated with Fer; and (iii) the C-terminus alone was insufficient for binding. Thus the cortactin N-terminus participates in the Fer–cortactin interaction, which cannot be fully due to the binding of the Fer Src homology 2 domain to C-terminal tyrosine residues of cortactin. Taken together, F-actin stabilization prevents cortactin tyrosine phosphorylation, whereas depolymerization promotes it. Depolymerization-induced phosphorylation is mediated by Fer, and requires the actin-binding domain of cortactin. These results define a novel F-actin-dependent pathway that may serve as a feedback mechanism during cytoskeleton remodelling.

Key words: actin cytoskeleton, depolymerization cortactin, Fer kinase, latrunculin B, tyrosine phosphorylation.

INTRODUCTION

Dynamic reorganization of the F-actin cytoskeleton is central to a variety of cellular functions, including motility, invasiveness, resistance against osmotic and mechanical stress and membrane trafficking. The F-actin-binding protein cortactin has emerged as an important regulator of each of these functions [1–7], presumably via its effects on F-actin remodelling. Cortactin is composed of an N-terminal half, harbouring an NTA (N-terminal acidic region) that serves as the binding site for the Arp2/3 (actin-related protein 2/3) complex [8,9], and six-and-a-half tandem repeats of a 37-amino-acid motif, involved in F-actin binding [10]. These domains have been shown to promote F-actin nucleation, *de novo* actin polymerization and Arp2/3-mediated actin branching and stability [9,11,12]. The C-terminal half of cortactin contains an α -helical domain followed by a Pro–Ser–Tyr-rich sequence, which is targeted by various protein kinases [1], and an SH3 (Src homology 3) domain, which can complex with different proteins, including the endocytic GTPase dynamin [13], and the tight junction component, Zonula occludens-1 [14]. Cortactin was originally identified as a major substrate of v-Src [15,16], and subsequently has been shown to be phosphorylated by various tyrosine kinases within the C-terminus, including c-Src [17], the Fyn/Fer pathway [18–20], c-Met [21] and Syk [22,23]. The kinase

involved depends on the cell type and the particular stimulus, such as various growth factors [18,23,24], osmotic stress [19,20], thromboxane A₂ [23] or overexpression of oncogenic kinases [25,26].

Whereas recent studies have resulted in a breakthrough in our knowledge regarding the function of the cortactin N-terminus in regulating F-actin assembly and cytoskeletal dynamics, the exact significance and regulation of the C-terminal tyrosine phosphorylation remains much less understood. The major importance of this process is underlined by the fact that the level of cortactin tyrosine phosphorylation shows strong positive correlation with cell motility and tumour invasiveness [3,26], and inhibition of phosphorylation or overexpression of phosphorylation-incompetent (tyrosine to phenylalanine) mutants hinder cell migration and metastasis formation [3,4]. The phosphorylated tyrosine residues may serve as docking sites for SH2-domain-containing proteins and, therefore, phospho-cortactin may act as an adaptor protein. Indeed, on the basis of *in vitro* studies, phosphotyrosine–SH2 interactions have been proposed between cortactin and Src or Fer kinase [18,25]. Another intriguing possibility is that tyrosine phosphorylation may negatively regulate the F-actin cross-linking/bundling capability of cortactin, as suggested by Huang et al. [17]. Our previous results are consistent with the idea that tyrosine phosphorylation may promote the disassembly of

Abbreviations used: Arp2/3, actin-related protein 2/3; CHO, Chinese-hamster ovary; FL, full-length; GFP, green fluorescence protein; JK, jasplakinolide; LB, latrunculin B; NTA, N-terminal acidic region; PP2, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine; SH, Src homology; WT, wild-type.

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actin polymerization-related complexes. Specifically, we found that inhibition of cortactin tyrosine phosphorylation increased the osmotic stress-induced association of cortactin with Arp2/3 [5].

The notion that tyrosine phosphorylation of cortactin regulates F-actin organization prompted us to ask whether a reciprocal relationship also exists, i.e. the organization or polymerization state of actin impacts on cortactin phosphorylation. The dependence of F-actin organization on cortactin phosphorylation and cortactin phosphorylation on F-actin polymerization may be an important mechanism during F-actin remodelling. Indeed, the recent recognition of the direct regulation of c-Abl tyrosine kinase by F-actin provides a compelling example for the relevance of such reciprocal regulation. Furthermore, consistent with our assumption, alteration of the cytoskeleton by cytochalasin B has been reported to affect cortactin phosphorylation [27]. However, the responsible kinase(s), the underlying mechanisms and the cortactin structural requirements have not been clarified in any detail.

To address the role of the polymerization state of actin in the regulation of cortactin tyrosine phosphorylation, we used LB (latrunculin B), a potent monomer-sequestering and F-actin-depolymerizing drug [28]. We show that LB induces robust tyrosine phosphorylation of cortactin via the Fyn/Fer kinase pathway, and this effect requires the actin-binding domain of cortactin. The actin-depolymerization-induced Fer-mediated cortactin phosphorylation represents a novel F-actin-dependent signalling pathway that may play an important role in the regulation of actin dynamics.

MATERIALS AND METHODS

Materials

[Val⁵]Angiotensin II, ATP and the monoclonal anti-FLAG antibody were purchased from Sigma. Protease inhibitor cocktail, containing 0.8 mg/ml benzamidine/HCl, 0.5 mg/ml aprotinin, 0.5 mg/ml leupeptin, 0.5 mg/ml pepstatin A and 50 mM PMSF in pure ethanol, was obtained from Pharmingen (San Diego, CA, U.S.A.). Protein G-Sepharose beads were obtained from Amersham Biosciences or Pierce. LB, JK (jasplakinolide) and PP2 [4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-*d*]pyrimidine] were purchased from Calbiochem and rhodamine-labelled phalloidin was from Molecular Probes. Monoclonal anti-cortactin (4F11), anti-p130Cas, anti-phosphotyrosine (4G10) and peroxidase-conjugated streptavidin were purchased from Upstate Biotechnology. Anti-GFP (green fluorescence protein) was from Santa Cruz Biotechnology. Polyclonal antibodies against Fer, Fer4 [29] and FerLA [30], and phospho-421 (anti-pY421) and phospho-466 (anti-pY466) cortactin [31] have been described previously. The monoclonal antibody against c-Myc (9E10) and its biotinylated form were purchased from Santa Cruz Biotechnology and Covance Research Products (Denver, PA, U.S.A.) respectively. Peroxidase-conjugated anti-mouse and anti-rabbit IgG, the ECL[®] kit and [γ -³²P]ATP (3000 Ci/mmol) were from Amersham Biosciences. FITC and Cy3-labelled mouse and rabbit anti-IgG were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, U.S.A.).

Cells

Unless otherwise indicated, experiments were performed on confluent, serum-starved CHO (Chinese-hamster ovary) cells, grown in α -minimal essential medium, containing 25 mM NaHCO₃ and supplemented with 10 % (v/v) foetal calf serum and 1 % antibiotic suspension (penicillin and streptomycin; Sigma),

under humidified atmosphere of air/CO₂ (19:1) at 37 °C. Wild-type (WT), Fyn^{-/-} and Src^{-/-} fibroblasts were kindly provided by Dr S. M. Thomas (Fred Hutchinson Cancer Center, Seattle, U.S.A.) [19,32]. WT (Fer^{+/+}) and Fer-deficient (Fer^{-/-}) cells have been described previously [33]. Abl^{+/+} and Abl^{-/-} fibroblasts [34] were kindly provided by Dr C. Mastick and Dr A. R. Sanguinetti (University of Nevada, Reno, NV, U.S.A.). All fibroblast cell lines were maintained in Dulbecco's modified Eagle's medium.

Constructs and cell transfection

The N-terminally Myc-tagged constructs encoding WT cortactin (Myc-WT-cort) or a mutant in which Tyr-421, Tyr-466 and Tyr-482 were replaced by phenylalanine (Myc-F-cort) were described previously [3] and used in our studies [20]. Constructs encoding for the C-terminally Myc-tagged full-length (FL, amino acids 1–546) and truncation mutants of cortactin, i.e. N-term (N-terminal half of the protein; amino acids 1–326), Δ NTA (lacking the first 77 amino acids), C-term (C-terminal half of the protein; amino acids 336–546) and Δ repeat (lacking amino acids 85–327), were generated by PCR. For the latter construct, first the NTA and C-term were obtained using the following primer pairs: primer 1/primer 2 (GCGAATTCGAAAGCCTCTGCAGGCC)/(GGCCTCAATGGGGACAGTCTTATAGCCGTGGGAAGCC-TTGGGTCCCG) and primer 3/primer 4 (CCCAAGGCTTCCC-ACGGCTATAAGACTGTCCCCATTGAGGCCG)/(GCCTCGA-GCTACTGCCGCACTCCAC). Then, Δ repeat was generated by overlapping PCR using NTA and C-term, and amplified further by adding primer 1/primer 4 to the system. For each construct, the forward (5') primer contained an *Eco*RI or *Hind*III site, whereas the reverse (3') primer contained an *Xho*I site. The amplified products were digested with the corresponding enzymes and subcloned into pCDNA3.1/Myc/His vector (Invitrogen). The FL and mutant FLAG-tagged cortactin constructs [8], the GFP-WT, GFP-N-term and GFP-C-term cortactin [5] as well as plasmids for kinase-active GFP-Fer and Myc-Fer [35,36] have been described previously. Transient transfection was performed on CHO cells using the FuGene6[™] reagent (Roche Molecular Biochemicals) according to the manufacturer's instructions. Further details regarding transfection and co-transfection are described in the Figure legends.

Preparation of cell extracts

Before the experiments, confluent cultures were serum-starved overnight. Cells were then preincubated in isotonic control medium (125 mM NaCl/3 mM KCl/1 mM MgCl₂/1 mM CaCl₂/5 mM glucose/20 mM Hepes, pH 7.4) for 10 min and subjected to various treatments as indicated. When used, 10 μ M LB was applied for 30 min. The medium was then aspirated, and the cells were scraped into ice-cold lysis buffer (100 mM NaCl/30 mM Hepes/20 mM NaF/1 mM EGTA/1 % Triton X-100, pH 7.5), supplemented with 1 mM Na₃VO₄, 1 mM PMSF and 20 μ l/ml protease inhibitor mixture. To obtain cells in suspension, confluent monolayers were treated with Ca²⁺- and Mg²⁺-free isotonic medium, supplemented with 3 mM Na₂-EDTA (5 ml/10 cm dish). After 10 min, the detached cells were vigorously pipetted until a single cell suspension was attained. The cell suspension was gently rotated at 37 °C and treated as indicated. Subsequently, the cells were sedimented and dissolved in cold lysis buffer.

Western blotting and immunoprecipitation

Lysates containing equal amounts of protein were clarified by centrifuging at 12 000 *g* for 10 min. Supernatants were precleared for

40 min using 40 μ l of 50 % suspension of Protein G–Sepharose beads, and then incubated with the corresponding antibodies for at least 1 h. Immunocomplexes were captured using 40 μ l of Protein G–Sepharose beads, which were washed four times with lysis buffer containing 1 mM Na_3VO_4 . Immunoprecipitated proteins were diluted with Laemmli sample buffer, boiled for 5 min and subjected to SDS/PAGE (8 or 10 % gels). The separated proteins were transferred on to nitrocellulose membrane using a Bio-Rad mini Protean III apparatus. Blots were blocked in Tris-buffered saline containing 5 % BSA and incubated with the corresponding primary antibody. The bound antibody was visualized by the relevant peroxidase-coupled secondary antibodies using the enhanced chemiluminescence method. Quantification of the bands was performed using a Bio-Rad GS-690 Imaging Densitometer and the Molecular Analyst program as described in [5].

In vitro kinase assays

The activity of Fer was determined essentially as described, using [Val⁵]angiotensin II as substrate [20]. Briefly, cell lysates obtained from control, LB- or hypertonically treated cells, containing equal amounts of protein (800–1000 μ g), were subjected to immunoprecipitation (see above) using anti-Fer LA. The precipitates were washed with kinase buffer (50 mM Tris/HCl/10 mM MgCl_2 , pH 7.7) supplemented with 0.25 mM Na_3VO_4 . The beads were then incubated for 10 min at 30 °C in 25 μ l of kinase buffer containing 5 mM [Val⁵]angiotensin II, 5 μ M ATP (potassium salt) and 10 μ Ci of [γ -³²P]ATP/sample. The reaction was terminated by adding 35 μ l of stop solution [20 % (w/v) trichloroacetic acid and 5 mM ATP]. This mixture (25 μ l) was layered on to P81 phosphocellulose squares (Upstate Biotechnology), and after extensive washing with 0.85 % phosphoric acid, radioactivity bound to the filter was measured by scintillation counting. The background, detected in samples that had been incubated without the substrate, was determined in each experiment and subtracted from the total. Results were normalized to the amount of total protein in the lysate.

F-actin quantification and flow cytometry

CHO cells kept in suspension were left untreated or challenged with 10 μ M LB for 30 min and then fixed with 3.7 % (w/v) paraformaldehyde for 15 min. Cells were then pelleted, washed and resuspended in isotonic PBS, containing 0.2 % Triton-X 100 and 0.33 μ M rhodamine phalloidin for 15 min. After extensive washings with PBS, rhodamine fluorescence was quantified using a Beckman Coulter flow cytometer (Epics XL/XC), as described in [37]. The mean channel fluorescence of the control was taken as 100 %. Three separate experiments were performed, measuring 5000 cells for each condition.

Immunofluorescence microscopy

Confluent cultures grown on coverslips were preincubated for 10 min in the isotonic medium and treated as specified in the Figure legends. Cells were fixed with 3.7 % paraformaldehyde for 30 min and washed with PBS. After quenching the formaldehyde with 100 mM glycine in PBS, the cells were permeabilized with 0.1 % Triton-X 100 for 20 min at room temperature (22–24 °C) and blocked with 3 % (w/v) albumin for 1 h. The samples were incubated with anti-cortactin and anti-p421Y cortactin in 1:100 dilution for 1 h, followed by washing and incubation with FITC-labelled anti-mouse and Cy-3-labelled anti-rabbit IgG. Coverslips were washed and mounted on glass slides using fluorescence mounting medium (Dako, Carpinteria, CA, U.S.A.). The staining

was visualized using a Nikon Eclipse TE 200 immunofluorescence microscope equipped with a Hamamatsu-cooled charge-coupled device camera, controlled by the Simple PCI software [38].

Other methods

Protein concentration was determined by the bicinchoninic acid assay (Pierce) using BSA as standard. Results are presented as means $\pm$ S.E.M. for the number of experiments indicated (*n*) or as representative immunoblots for at least three similar experiments.

RESULTS

F-actin depolymerization induces cortactin tyrosine phosphorylation

To address whether the polymerization state of actin affects cortactin tyrosine phosphorylation, we induced F-actin depolymerization with LB, a potent actin monomer-sequestering drug [28]. Cortactin was immunoprecipitated from lysates of control and LB-treated cells, and the precipitates were probed with an antiphosphotyrosine antibody. LB induced a significant phosphotyrosine accumulation in cortactin without affecting the total amount of the precipitated protein (Figure 1A). This effect was abolished by the Src family inhibitor PP2, indicating the involvement of Src-type kinases (Figure 1A). Previously, we have shown that hyperosmolarity, which reduces cell volume and alters cell shape, causes strong tyrosine phosphorylation of cortactin. Since LB induces cell rounding, it was conceivable that this shape change rather than actin depolymerization might be the direct trigger for cortactin phosphorylation. To examine this possibility, we induced cell rounding by detaching the cells using EDTA (Figure 1A). This treatment did not lead to cortactin tyrosine phosphorylation. Moreover, rounded cells in suspension remained responsive to LB: the drug provoked a sizable tyrosine phosphorylation in cortactin, and this effect was entirely prevented by PP2. Although the response was somewhat smaller in suspended cells when compared with that in attached cells, these experiments clearly indicated that the effect of LB cannot be solely attributed to the shape change.

Next, we quantified the effect of LB on F-actin using rhodamine phalloidin. Flow-cytometric analysis of suspended CHO cells showed that a 30 min treatment with LB resulted in a 50 % reduction in their F-actin content (Figure 1B). To substantiate that the LB-induced actin depolymerization is the predominant factor in cortactin phosphorylation, we pretreated the cells with JK, an F-actin stabilizing sponge toxin. JK strongly reduced the LB-induced cortactin tyrosine phosphorylation (Figure 1C), confirming that F-actin disassembly is necessary for the phosphorylation-inducing action of LB.

Next, we examined whether LB induces general phosphotyrosine accumulation in substrates of the Src family, or effect of LB is more restricted, i.e. LB shows selectivity towards cortactin. To this end, we examined the effect of LB treatment on p130Cas, a prominent Src family substrate that does not directly bind F-actin. Interestingly, LB caused a significant reduction in tyrosine phosphorylation of p130Cas, which was opposite to the effect seen on the cortactin. Additionally, LB treatment might cause redistribution of p130Cas, because a much higher amount was immunoprecipitated from the Triton X-100-soluble lysate of LB-treated cells when compared with control cells (Figure 1D). Similar to p130Cas, LB has been shown to induce the tyrosine dephosphorylation of p125 FAK, another Src substrate [39].

Figure 1 F-actin depolymerization induces tyrosine phosphorylation of cortactin

(A) Confluent monolayers of CHO cells were serum deprived, and either preincubated with an isotonic medium on 10 cm dishes (attached cells), or detached using the same medium lacking Ca^{2+} and Mg^{2+} , but supplemented with 3 mM EGTA, and kept in suspension by gentle rotation (suspended cells). Both attached and suspended cells were then incubated in the absence or presence of 10 μM PP2 for 10 min, and then either left untreated (Con) or challenged with 10 μM LB for 30 min, as indicated. Subsequently, the cells were lysed, cortactin was immunoprecipitated and the precipitates were subjected to SDS/PAGE and Western-blot analysis using antiphosphotyrosine (PY). The blot was then stripped and reprobed with anti-cortactin (Cort). (B) LB-induced changes in F-actin were quantified by flow cytometry in suspended cells stained with rhodamine phalloidin, as described in the Materials and methods section. The mean channel fluorescence of control cells (Con) was taken as 100% ($n=3$ independent experiments). (C) JK inhibits the LB-induced cortactin phosphorylation. Where indicated, attached cells were preincubated with 1 μM JK for 30 min, and then left untreated or stimulated with 10 μM LB for an additional 30 min. Cells were then processed as in (A). (D) LB-induced cortactin phosphorylation shows selectivity when compared with Src substrates. Attached cells were treated and processed as in (A), except that p130Cas was immunoprecipitated and probed for tyrosine phosphorylation.

Thus F-actin depolymerization differentially affects the tyrosine phosphorylation status of various Src family substrates, and it appears to enhance selectively phosphotyrosine accumulation in cortactin.

LB-induced cortactin tyrosine phosphorylation is mediated by Fyn and Fer kinases

Increased tyrosine phosphorylation of cortactin after LB treatment might result from increased kinase activity and/or enhanced accessibility of the substrate for its interaction with the kinase. To gain insight into the underlying mechanisms, we conducted

Figure 2 F-actin depolymerization-induced cortactin phosphorylation is mediated by Fyn and Fer, independent of Src and Abl

(A) WT, Fyn-deficient (Fyn^{-/-}) and Src-deficient (Src^{-/-}) embryonic fibroblasts were grown to confluence, serum-starved and then incubated in the absence (Con) or presence of LB (10 μM for 30 min). After cell lysis, cortactin was immunoprecipitated and probed with anti-phosphotyrosine and anti-cortactin antibodies. (B) Similar experiments were performed using control (Fer^{+/+}) and Fer-deficient (Fer^{-/-}) fibroblasts. (C) Fer is a major contributor to the hypertonicity-induced cortactin phosphorylation. Fer^{+/+} and Fer^{-/-} cells were preincubated in isotonic medium. After 10 min, the medium was replaced for 15 min with either the same isotonic solution (Con) or a hypertonic solution (H), composed of the isotonic medium supplemented with 300 mM sucrose. Tyrosine phosphorylation of cortactin was assessed as above. (D) Cortactin phosphorylation was tested in control (Abl^{+/+}) and c-Abl-deficient (Abl^{-/-}) fibroblasts as in (A).

experiments designed to identify the responsible kinase(s) and the cortactin domains required for LB-induced phosphorylation.

Initially, we compared the effect of LB on cortactin tyrosine phosphorylation in WT, Src-deficient (Src^{-/-}) and Fyn-deficient (Fyn^{-/-}) fibroblasts [19,32]. LB treatment induced strong tyrosine phosphorylation of cortactin in both WT and Src^{-/-} cells, whereas it caused a much weaker response in Fyn^{-/-} cells (Figure 2A). Together, these results suggest that Fyn, but not Src, is involved in LB-triggered cortactin tyrosine phosphorylation. We have shown previously that hypertonicity stimulates cortactin phosphorylation through Fyn-mediated activation of Fer kinase [20]. To test whether the Fyn/Fer pathway mediates LB-induced cortactin phosphorylation as well, we used fibroblast cell lines derived from mouse embryos that were either WT (Fer^{+/+}) or homozygous for an Fer kinase-inactivating missense mutation (Fer^{-/-}) [33]. Whereas LB treatment resulted in strong cortactin phosphorylation in the Fer^{+/+} cells, it evoked only a marginal response in the Fer^{-/-} cells (Figure 2B). Similarly, the hypertonicity-induced cortactin phosphorylation was strongly reduced

Figure 3 Effect of LB and hyperosmolarity on Fer kinase activity (A) and cortactin tyrosine phosphorylation (B)

(A) Serum-starved CHO cells were left untreated (Con), or challenged with 10 μ M LB for 30 min, or treated for hyperosmolarity (+300 mM sucrose) for 10 min, and lysed. Fer kinase was immunoprecipitated from the samples using anti-FER-LA antibody, and its activity determined in an *in vitro* kinase assay using [Val⁵]angiotensin as substrate ($n = 5$), as described in the Materials and methods section. Each of the three groups is significantly different ($P < 0.05$) from the others. It is noteworthy that LB did not induce larger Fer activation at earlier time points either (results not shown). (B) Cortactin was immunoprecipitated from aliquots of the corresponding cell lysates and probed for tyrosine phosphorylation and total cortactin.

in Fer^{-/-} cells (Figure 2C). It is worth mentioning that the Fer^{-/-} cells have normal F-actin organization and content [33]. Together, these findings suggest that the Fer kinase is a major mediator of both the LB-induced and the hyperosmotically provoked cortactin tyrosine phosphorylation.

Recently, it was shown that the non-receptor tyrosine kinase c-Abl directly binds to F-actin, and that this interaction inhibits Abl activity. Since LB stimulated c-Abl [40], we wanted to test whether this kinase could be an F-actin-sensitive upstream element of the Fyn/Fer signalling pathway. To address this possibility, LB-induced cortactin phosphorylation was compared in Abl^{+/+} and Abl^{-/-} fibroblasts. The absence of c-Abl did not affect the F-actin depolymerization-induced cortactin phosphorylation (Figure 2D), implying that c-Abl is not involved. These results suggest that the Fyn/Fer signalling pathway represents a novel, Abl-independent, F-actin-regulated kinase cascade.

In principle, LB may exert its effects by stimulating Fer kinase and/or by facilitating the interaction between Fer and cortactin. To address the first possibility, we determined the effect of LB on Fer activity using an *in vitro* kinase assay. To assess the relative magnitude of the effect of LB on Fer activity, we also measured the hypertonicity-induced Fer activation. LB treatment resulted in a modest increase in Fer activity, whereas hypertonicity strongly stimulated the kinase (Figure 3A). In contrast, LB induced more robust cortactin phosphorylation than osmotic stress (Figure 3B). These results suggest that direct Fer kinase activation is probably not the exclusive mechanism responsible for the LB-induced cortactin tyrosine phosphorylation.

Figure 4 F-actin depolymerization-induced phosphorylation targets C-terminal tyrosine residues of cortactin

(A) CHO cells grown on 10 cm dishes were transfected with 2 μ g of cDNA encoding Myc-tagged WT cortactin (Myc-cortactin) or a Myc-tagged mutant in which Tyr-421, Tyr-466 and Tyr-482 were replaced by phenylalanine (Myc-F-cortactin). Cells were placed 48 h later into an isotonic medium, and incubated in the absence (Con) or presence of 10 μ M LB for 30 min. Samples were lysed, and the tagged cortactin constructs were immunoprecipitated using an anti-Myc antibody. The precipitates were then probed with a general antiphosphotyrosine antibody (PY) and, after stripping, reprobed with a pY421-cortactin-specific antibody (PY₄₂₁). Finally, the same blot was probed with anti-Myc to verify equal expression and loading (Myc). Duplicate experiments were performed for each condition. (B) Myc-cortactin was immunoprecipitated from control and LB-treated transfected cells, and probed with pY466-cortactin-specific antibody (PY₄₆₆), followed by stripping and reprobing with anti-Myc.

Identification of tyrosine residues and domain requirements for LB-induced cortactin phosphorylation

To gain insight into the structural basis of the actin depolymerization-induced cortactin phosphorylation, we investigated which tyrosine residues on cortactin are phosphorylated, and which domains of cortactin are required for this effect. We initially compared the phosphorylation of WT, Myc-tagged cortactin with a Myc-tagged mutant (F-cortactin) in which three tyrosine residues (Tyr-421, Tyr-466 and Tyr-482) that are primary targets of Src-type kinases [3,31] and Fer [20], were replaced by phenylalanine residues. Figure 4(A) shows that LB caused strong tyrosine phosphorylation in WT Myc-tagged cortactin but failed to induce phosphorylation in F-cortactin. These results indicate that Tyr-421, Tyr-466 and Tyr-482 are the predominant sites of the actin depolymerization-triggered phosphorylation. Indeed, using tyrosine phosphorylation site-specific cortactin antibodies, we directly confirmed that LB induced a robust increase in the phosphorylation of Tyr-421 and Tyr-466 (Figures 4A and 4B).

The target tyrosine residues are located in the C-terminal half of cortactin, whereas the F-actin-binding domain is found in the N-terminal half of the protein. We therefore examined whether the N-terminal half had any impact on the phosphorylation of the C-terminal tyrosine residues. To this end, cells were transfected

Figure 5 Presence of the N-terminal half of cortactin is a prerequisite for the LB-induced and hypertonicity-triggered phosphorylation of the C-terminal cortactin tyrosine residues

(A) CHO cells were transfected with C-terminally Myc-tagged FL (FL-Myc) or C-terminal cortactin (C-term-Myc) using 4 μ g of the corresponding cDNA per 10 cm dish. Cells were incubated 2 days after transfection in the absence or presence of LB, as in Figure 4. The cortactin constructs were immunoprecipitated through the tag, and the precipitates were Western-blotted first with biotinylated anti-Myc (left), and then with biotinylated antiphosphotyrosine (right). Immunoreactive bands were visualized using streptavidin-conjugated horseradish peroxidase. Direct labelling was necessary because C-term-Myc co-migrated with the heavy chain of the precipitating antibody. (B) Similar experiments were performed as in (A), except that cells were transfected with N-terminally FLAG-tagged FL (FLAG-FL) or C-terminal (FLAG-C-term) cortactin. Where indicated, the cells were challenged with LB or hypertonicity (H), added as 300 mM sucrose. Cortactin constructs were immunoprecipitated with an anti-FLAG antibody, and probed with antiphosphotyrosine and anti-FLAG, as shown.

with cDNA constructs encoding either Myc-tagged FL cortactin, or the C-term, which harbours the target tyrosine residues, but lacks the N-terminal domain (Figure 5A). For these experiments, we generated C-terminally Myc-tagged constructs to ensure that the relationship between the target region and the tag is the same in the FL and truncated proteins. Control or LB-treated cells were lysed and processed for immunoprecipitation 2 days after transfection using an anti-Myc antibody. Since the light chain of the immunoprecipitating antibody migrates very close to the Myc-tagged C-term, we used biotinylated anti-Myc and antiphosphotyrosine primary antibodies for these experiments. Although both the FL and the C-term cortactin proteins were abundantly expressed, there was a significant difference in their response to LB: FL showed marked tyrosine phosphorylation similar to the endogenous protein, whereas no or only marginal phosphotyrosine accumulation was detected in the C-term (Figure 5A). To substantiate this finding further, we performed similar experiments using N-terminally FLAG-tagged FL or C-term cortactin constructs. FLAG-C-term migrates faster than the heavy chain of the precipitating antibody, providing good separation of the specific signal [31]. Both LB and hypertonicity induced strong tyrosine phosphorylation of FLAG-FL but failed to induce any

Figure 6 Repeat region in the N-terminal half is required for the efficient phosphorylation of C-terminal tyrosine residues of cortactin

(A) Cells were transfected with C-terminally Myc-tagged constructs (4 μ g of DNA per 10 cm dish) encoding the FL cortactin (FL) or Δ NTA, a truncation mutant lacking the NTA. Cells were stimulated with hypertonicity or LB as in Figure 5. Myc was immunoprecipitated from the samples, and tyrosine phosphorylation of the constructs as well as their expression was detected as in the previous experiments. Phosphorylation normalized to the total amount of precipitated cortactin constructs in each sample was quantified by densitometry. The bar diagram compares the level of tyrosine phosphorylation after LB-treatment in FL (taken as 100%) and Δ NTA cortactin ($n = 4$). The truncation of NTA did not cause detectable difference in tyrosine phosphorylation. (B) Experiments were performed and quantified exactly as in (A), except that cells were transfected either with FL or with Δ repeat (ΔRep) cortactin, in which the repeat region was deleted, and NTA was coupled with the C-terminal half of cortactin. The bar diagram compares the normalized tyrosine phosphorylation induced by LB in FL and Δrepeat, as in (A). Deletion of the repeat region significantly reduced the phosphorylation of cortactin.

phosphorylation of FLAG-C-term (Figure 5B). Together, these observations suggest that the presence of the cortactin N-terminus (N-term) is required for the efficient phosphorylation of C-terminal target tyrosine residues after F-actin depolymerization.

The N-terminal half of cortactin is composed of two functionally different domains: the NTA, which contains the binding site for the Arp2/3 complex, and the repeat domain, which is responsible for F-actin binding [1]. To analyse the contribution of these domains to the LB-induced phosphorylation of the C-terminal tyrosine residues, we generated two Myc-tagged cortactin mutants: Δ NTA, which lacks the first 77 amino acids of the N-terminus, and Δ repeat from which the repeat region was deleted, resulting in a direct coupling of NTA with the C-terminal half of the protein. Both of these constructs were expressed at high and similar levels in CHO cells. LB treatment induced similar tyrosine phosphorylation in Δ NTA compared with FL (Figure 6A). In contrast, significantly reduced tyrosine phosphorylation was observed in Δ repeat (Figure 6B), implying that this part of the N-terminus is a critical region for C-terminal phosphorylation.

Spatial distribution of tyrosine-phosphorylated cortactin after actin depolymerization

Recently, Head et al. [31] proposed a central mechanism for cortactin phosphorylation that requires Rac-mediated translocation

Figure 7 Distribution of cortactin and pY421 phospho-cortactin in control and LB-treated cells

CHO cells grown to confluence on glass coverslips were serum deprived overnight, and then either left untreated or challenged with 10 μ M LB for 30 min. Subsequently, cells were fixed with paraformaldehyde and co-stained for cortactin and pY421 phospho-cortactin, using a mouse monoclonal anti-cortactin, and a rabbit polyclonal anti-pY421-specific phospho-cortactin antibody. Binding of the antibodies was visualized with FITC-labelled anti-mouse and Cy-3-labelled anti-rabbit IgG respectively. Control and LB-treated samples were viewed using the same illumination and camera settings.

of cortactin and Src to the cell periphery, where cortactin is phosphorylated at Tyr-421 and Tyr-466. Accordingly, these authors showed that cortactin phosphorylated at Tyr-421 accumulated at the cell periphery. To address whether the LB-induced Fyn/Fer-catalysed cortactin phosphorylation shows similar spatial distribution, control or LB-treated cells were co-stained with antibodies against cortactin and Tyr-421 phospho-cortactin (pY421) (Figure 7). Whereas total cortactin was readily visualized, the pY421 cortactin antibody showed only a faint labelling in unstimulated cells. In contrast, LB-treated cells exhibited strong pY421-cortactin staining, in agreement with our Western-blot results (Figure 4). As expected, LB also caused general cell rounding but not cell detachment. Total cortactin staining showed a fairly smooth cytosolic distribution with nuclear exclusion in LB-treated cells, although, we also observed some enrichment at the periphery. Interestingly, no peripheral pY421 cortactin accumulation was observed in LB-treated cells. Instead, pY421 cortactin was distributed in a punctate pattern as fine or coarse aggregates throughout the cytosol. These results suggest that the depolymerization-provoked cortactin phosphorylation may not require peripheral cortactin translocation, and most of the Fyn/Fer-mediated phosphorylation probably occurred in the cytosol.

Association between Fer and cortactin

To assess whether actin depolymerization enhances the association between cortactin and Fer, we performed co-immunoprecipitation studies. Cell lysates were obtained from control and LB-treated cells, and Fer was immunoprecipitated with one of two anti-Fer antibodies (anti-Fer4 or anti-Fer-LA), raised against different regions of the Fer protein. Probing these precipitates with an anti-cortactin antibody revealed that some cortactin was associated with Fer even under resting conditions. Treatment of cells with LB led to a significant (approx. 2-fold) increase in the association of Fer and cortactin (Figure 8). Previously, it was proposed that the interaction between Fer and cortactin was

Figure 8 LB facilitates the association between Fer and cortactin

Cells were incubated in the absence or presence of LB and lysed, as in the previous experiments. Fer kinase was immunoprecipitated from the lysates using either anti-FER4 or anti-FER-LA polyclonal antibodies. To assess the amount of co-precipitating cortactin, the Fer-immunoprecipitates were probed with anti-cortactin antibodies. The same nitrocellulose membranes were stripped and reprobed with the corresponding anti-Fer antibodies to verify that a similar amount of Fer was pulled down from control and LB-treated cells.

mediated by the binding of the Fer SH2 domain to phosphorylated tyrosine residues on cortactin [18]. However, we found that under resting conditions, cortactin was often not (or only marginally) phosphorylated, suggesting that cortactin phosphorylation may not be an absolute prerequisite for the association of these proteins. To address this issue directly, we transfected cells with GFP-Fer along with Myc-tagged WT or non-phosphorylatable F-cortactin, and after immunoprecipitation of Fer with an anti-GFP antibody, the presence of the cortactin constructs in the precipitates was examined using an anti-Myc antibody. Figure 9(A) shows that the overall expression of WT and F-cortactin was comparable, and a similar amount of GFP-Fer was present in the two cell populations. Importantly, GFP-Fer precipitated similar amounts of WT and F-cortactin, indicating that the critical Src family/Fer target tyrosine residues (Tyr-421, Tyr-466 and Tyr-482) are not required for the basal association of these proteins. Similar results were obtained when endogenous Fer was immunoprecipitated from cells expressing WT or F-cortactin (results not shown).

Having established that cortactin tyrosine phosphorylation was not an absolute requirement for the association of Fer with the cortactin, we sought to determine whether the isolated C-terminus alone is sufficient to mediate a stable interaction between the two proteins. Cells co-transfected with Myc-Fer and either FLAG-FL or FLAG-C-term cortactin were lysed, and the cortactin constructs were immunoprecipitated through the tag. Fer was present in the immunoprecipitates from cells expressing FLAG-FL, but it was not detectable in the immunoprecipitates of FLAG-C-term (Figure 9B). This was not due to a difference in the expression level of the two constructs, since similar amounts of FL and C-term were precipitated from the two cell populations (Figure 9B). Whereas a conformation change that hinders the association of the isolated C-term with Fer cannot be entirely excluded, it does not seem probable since we found that Fer, when strongly overexpressed, was capable of mediating the tyrosine phosphorylation of the isolated C-term as well. Nevertheless, we

Figure 9 Structural requirements for the *in situ* association of Fer and cortactin in non-stimulated cells

(A) Phosphorylation of C-terminal Tyr-421, Tyr-466 and Tyr-482 is not a prerequisite for basal association between Fer and cortactin. Cells were co-transfected with GFP-Fer (2 µg/10 cm dish) along with Myc-WT or phosphorylation-incompetent Myc-F-cortactin (2 µg/10 cm dish). After 2 days, the cells were lysed and GFP-Fer was immunoprecipitated with an anti-GFP antibody. The precipitates were subjected to SDS/PAGE and Western-blot analysis using an anti-Myc antibody to detect co-precipitating cortactin proteins (top panel). Note that both WT and F-cortactin are present in the precipitates, and WT migrates slightly slower than the phenylalanine-substituted mutant. Above the cortactin proteins, a non-specific cross-reacting band was also visualized. The membrane was re-probed with anti-GFP to test for the amount of the precipitated Fer (middle panel). Similar expression of WT and F-cortactin was verified by Western blotting of cell lysates containing an equal amount of protein with anti-Myc (bottom panel). (B) Fer kinase co-precipitates with FL but not with C-terminal cortactin. Cells were co-transfected with Myc-Fer and either FLAG-tagged FL (FLAG-FL) or C-terminal (FLAG-C-term) cortactin, using 2 µg of cDNA for both the Fer and cortactin constructs. Lysates obtained from the two cell populations were subjected to immunoprecipitation with an anti-FLAG antibody. The precipitates were probed for the co-transfected Fer using anti-Myc (top panel), and subsequently for the cortactin constructs with anti-FLAG (bottom panel). Equality of Fer expression was verified by immunoblotting total cell lysates from the two cell populations with anti-Myc (middle panel). (C) The isolated N-term can weakly associate with Fer. Cells were transfected with 4 µg of Myc-tagged FL (FL-Myc) or N-terminal (N-term-Myc) cortactin. The cells were lysed 2 days post transfection and endogenous Fer was immunoprecipitated from the lysates with anti-FER-LA. The precipitates were probed for the presence of cortactin constructs by an anti-Myc antibody (top panel) and for Fer with anti-FER-LA (middle panel). Similarity of total expression of the cortactin constructs was verified by Western-blot analysis of the corresponding whole-cell lysates with anti-Myc antibodies (bottom panel).

could not detect association between Fer and phospho-C-term, suggesting that C-terminal tyrosine phosphorylation is neither required for the basal association, nor sufficient in the absence of the N-terminal half of cortactin.

To assess whether the isolated cortactin N-terminus alone is capable of associating with Fer *in situ*, further co-precipitation studies were performed. Cells were transfected with WT or N-term Myc-tagged cortactin and endogenous Fer was immuno-

precipitated. Using this approach, a substantial amount of FL cortactin was found in the Fer precipitates (Figure 9C). Importantly, N-term was also pulled down with Fer, although Fer associated with much less N-term than FL, despite similar expression of the constructs (Figure 9C). Whereas the cortactin N-terminus appears to be capable of binding to Fer, this interaction is much weaker than that with the FL cortactin molecule. We could not determine unambiguously whether the weak interaction between Fer and N-term was affected by actin depolymerization. Taken together, these results suggest that the C-terminal tyrosine residues (Tyr-421, Tyr-466 and Tyr-482) of cortactin are not responsible for the basal Fer-cortactin association, and that the N-terminus is required for both the actin depolymerization-induced tyrosine phosphorylation of the C-terminus and for the efficient association of Fer with cortactin.

Enhanced F-actin polymerization inhibits the osmotic stress-induced phosphorylation of cortactin

Having shown that F-actin depolymerization promotes cortactin phosphorylation, we sought to determine whether enhanced actin polymerization would interfere with stimulus-induced phosphorylation of cortactin. To test if actin remodelling is involved in cortactin phosphorylation, cells were treated with the F-actin polymerizing drug, JK. As shown in Figure 10(A), JK induced the formation of large F-actin clusters that accumulated predominantly but not exclusively close to the plasma membrane. Interestingly, endogenous cortactin showed a striking accumulation in these F-actin clusters. Recruitment of cortactin into JK-induced F-actin patches required the presence of the N-terminal half of cortactin. Whereas the GFP-N-term readily accumulated in clusters, GFP-C-term showed no or only marginal accumulation (Figure 10B). We next tested the effect of JK on the hypertonicity-triggered cortactin phosphorylation. In agreement with our previous reports, hyperosmotic stress induced cortactin tyrosine phosphorylation in control samples. Clustering of F-actin and cortactin by JK had no effect on basal cortactin phosphorylation but it almost entirely prevented the hypertonic response (Figure 10C, left panels). We considered the possibility that JK directly inhibited the responsible tyrosine kinases. To address this issue, we compared the effect of JK and PP2, a tyrosine kinase inhibitor that abolishes hypertonic cortactin phosphorylation. In these experiments, we initially induced cortactin tyrosine phosphorylation by hypertonicity, and only then treated the cells with PP2 or JK. The two compounds had significantly different effects: PP2 induced rapid dephosphorylation of cortactin, indicating that continuous tyrosine kinase activity is required to maintain net phosphorylation. In contrast, once the osmotically induced phosphorylation was attained, JK failed to reverse this effect (Figure 10C, right panels). These results suggest that kinase inhibition is not the underlying mechanism by which JK prevents cortactin phosphorylation. In agreement with this conclusion, JK did not reduce the hyperosmotic activation of Fer (results not shown). Collectively, these results suggest that actin-depolymerization augments, whereas enforced F-actin polymerization and the concomitant actin-cortactin clustering reduces cortactin tyrosine phosphorylation. In addition, dynamic actin remodelling seems to be necessary for stress-induced cortactin phosphorylation.

DISCUSSION

Protein tyrosine phosphorylation is one of the major means whereby cellular signalling is linked to cytoskeleton remodelling. On the other hand, there is increasing evidence to support the

Figure 10 JK-induced F-actin polymerization redistributes cortactin to F-actin-rich patches, and inhibits its tyrosine phosphorylation owing to hyperosmotic stress

(A) CHO cells grown on coverslips were exposed to 1 μ M JK and then co-stained for F-actin and endogenous cortactin, using rhodamine phalloidin and monoclonal anti-cortactin plus FITC-labelled anti-mouse secondary antibody respectively. The arrows indicate identical points on the two images. Note that JK induced the formation of large peripheral F-actin patches, in which cortactin and F-actin showed striking co-localization. (B) The N-terminal half is necessary for the JK-induced redistribution of cortactin. CHO cells were transfected with GFP-tagged cortactin N-terminus (GFP-N-term, upper panels) or C-terminus (GFP-C-term, lower panels), using 2 μ g of DNA from the corresponding constructs. Cells were exposed 2 days later to JK for 30 min as in (A), and stained with rhodamine phalloidin. F-actin and the cortactin mutants were visualized using confocal microscopy [5]. The arrows on the upper panels indicate large aggregates of F-actin, which show co-localization with GFP-N-term (arrows). Similar F-actin aggregates (lower left panel, *) show no co-localization with GFP-C-term. In untreated cells, both GFP-N-term and GFP-C-term displayed an even cytosolic distribution as shown previously [5]. (C) Left panels, JK inhibits the hyperosmotically induced phosphorylation of cortactin. Confluent cells were preincubated in the absence (control) or presence of 1 μ M JK for 30 min, and then either left in the isotonic medium (I) or challenged with hypertonicity (H, 300 mM sucrose) for 10 min. Cells were then lysed, and cortactin-immunoprecipitated and probed for tyrosine phosphorylation as in the previous experiments. Right panels, JK, as opposed to PP2, does not inhibit tyrosine kinases involved in cortactin phosphorylation. Cells were first exposed to hypertonicity for 10 min, and then the hypertonic medium was supplemented with either 1 μ M JK (H $\rightarrow$ JK) or 10 μ M PP2 (H $\rightarrow$ PP2) for 10 min. Subsequently, cortactin was immunoprecipitated and probed as before.

notion that cytoskeletal organization has an important impact on protein tyrosine phosphorylation, thereby constituting a feedback loop that may be crucial for the fine-tuning of actin cytoskeletal arrangement and signal transduction. One of the central findings of the present study is that F-actin depolymerization induces tyrosine phosphorylation of the F-actin-binding protein cortactin

by a pathway involving Fyn and Fer kinases. Recently, the non-receptor tyrosine kinase c-Abl has been shown to be regulated by F-actin, which directly binds to the extreme C-terminus of the kinase, and inhibits its activity [41]. Accordingly, actin depolymerization stimulates c-Abl. Since c-Abl promotes the assembly of F-actin structures and induces microspike formation and branching in neurons [40], its inhibition by F-actin represents reciprocal regulation that may provide a self-limiting mechanism to control actin dynamics. Although cross-talk may exist between c-Abl and Src-type kinases [42], the LB-induced cortactin phosphorylation is independent of c-Abl, as evidenced by our findings that the process remains intact in Abl $^{-/-}$ cells, whereas it is strongly suppressed in Fyn $^{-/-}$ and Fer $^{-/-}$ cells. Moreover, our results indicate that F-actin disassembly and not the accompanying cellular shape change is the critical factor for the LB-triggered cortactin phosphorylation. Collectively, these results imply that the Fyn/Fer-mediated cortactin phosphorylation is an additional and novel mechanism through which the polymerization state of actin can modify protein tyrosine phosphorylation.

Interestingly, c-Src, the prototypic member of the family, does not participate in the F-actin disassembly-mediated cortactin phosphorylation, underscoring the specificity of the pathway. The concomitant dephosphorylation of p130Cas further supports the specific involvement of Fer, since Fer overexpression has been reported to reduce p130Cas phosphorylation [43], presumably through the activation of tyrosine phosphatases [44,45]. The PP2 sensitivity of the LB-induced cortactin phosphorylation suggests that Fyn acts upstream of Fer, similar to in the osmotic signalling pathway [20]. However, it cannot be excluded that both Fyn and Fer could directly contribute to cortactin phosphorylation.

The mechanisms underlying the F-actin-dependent regulation of c-Abl and the Fyn/Fer pathway are bound to differ, since Fyn or Fer, in contrast with c-Abl, does not contain an F-actin-binding site through which their activities could be directly modulated. Indeed, it was not even certain whether their activity is affected by actin depolymerization, or the effect is due to spatial rearrangements allowing a better access of the kinase(s) to their targets. Our results show that the activity of Fer, presumably downstream to Fyn, is slightly increased after LB-induced F-actin depolymerization. This is in agreement with a previous report showing that perturbation of the cytoskeleton by the polymerization inhibitor cytochalasin D caused a modest increase in Src activity, and induced the phosphorylation of the adaptor protein FISH (five SH3 domain protein) [46]. However, several observations suggest that the increase in Fyn/Fer activity is not the only reason for the robust cortactin phosphorylation. First, LB induces more pronounced cortactin phosphorylation than hyperosmolarity despite the fact that hyperosmolarity induces much stronger Fer activation when compared with LB. Secondly, the LB-induced effect shows substrate specificity, inasmuch as cortactin is readily phosphorylated, whereas other prominent Src family substrates (e.g. focal adhesion kinase, p130Cas) are not. Thirdly, LB promotes the association of Fer with cortactin, indicating that the interaction of these proteins is modulated by F-actin. Moreover, pharmacologically enforced actin polymerization results in the redistribution of cortactin into F-actin-containing patches and concomitantly inhibits hypertonic-induced cortactin phosphorylation. Thus, in addition to kinase activation, the other important component appears to be an enhanced accessibility of cortactin for the Fyn/Fer pathway.

Analysis of the structural requirements of F-actin disruption-induced cortactin phosphorylation revealed that the N-terminal half of cortactin, which contains the Arp2/3 and F-actin-binding domains is indispensable for the phosphorylation of the C-terminal tyrosine residues. Additional truncation experiments verified

that the actin-binding repeat region but not the Arp2/3-binding site is required for efficient phosphorylation. This intriguing finding is in agreement with the recent observation of Head et al. [31]. When the present studies were at a preliminary stage, these authors reported that the N-terminal half of cortactin is necessary for the Src-mediated cortactin phosphorylation. They interpreted their finding to mean that the N-terminus is necessary for the Rac-induced cortactin translocation, which is then a prerequisite for the subsequent phosphorylation catalysed by membrane-associated Src. Whereas this mechanism may clearly be important for the Src-mediated cortactin phosphorylation, our previous and current findings suggest that the small GTPase-dependent translocation is not the exclusive mechanism, and that Fer-mediated cortactin phosphorylation is due to an alternative process. This notion is supported by the following observations: (i) inhibition of small GTPases by dominant-negative constructs or *Clostridium difficile* toxin B did not abolish the osmotically induced cortactin phosphorylation [5]; (ii) the NTA domain, which was shown to be required for the Rac-mediated translocation of cortactin [8], is not necessary for the LB-induced or hypertonic phosphorylation; and (iii) in contrast with Rac, which induces peripheral F-actin assembly (and thereby generates near-membrane docking sites for cortactin), LB disrupts the F-actin skeleton. However, it is conceivable that the submembraneous cytoskeleton is more resistant to depolymerization than the central structures and therefore actin-binding proteins might be liberated from the centre and translocate to the periphery. Since both hypertonicity [5] and LB reduce or eliminate stress fibres, such a possibility cannot be entirely discarded. On the other hand, it seems unlikely that this is the only mechanism, because the distribution of pY421-cortactin shows striking differences in cells treated with epidermal growth factor or LB. Whereas in the former case substantial pY421-cortactin accumulation was observed at the periphery [31], the LB-induced pY421-cortactin did not show peripheral distribution, rather it accumulated in patches dispersed in the cytosol.

On the basis of these findings, we propose an alternative but not exclusive mechanism for the LB-induced, Fer-mediated cortactin phosphorylation. Short actin filaments (not visualized as stress fibres) may be bound to cortactin under resting conditions. LB disassembles these fibres, which liberates the cortactin N-terminus. The unbound cortactin is then more easily accessible to protein kinases, especially to Fer. Consistent with this assumption, the N-terminus of cortactin is required, albeit not sufficient, for the tight association between Fer and cortactin. This finding was surprising since it was previously believed that the SH2 domain of Fer mediated binding to cortactin in a phosphotyrosine-dependent manner [18]. However, the mutant cortactin lacking these tyrosine residues also associated with Fer, indicating that, at least under basal conditions, Tyr-421, Tyr-466 and Tyr-482 are not involved in the Fer–cortactin interaction. This does not completely exclude the possibility that Fer binds to cortactin through its SH2 domain, since the cortactin N-terminus also contains tyrosine residues that are subject to phosphorylation [31]. However, these residues are not the major targets and/or cannot be efficiently visualized by the available phosphotyrosine antibodies [31]. Alternatively, the N-terminal half of cortactin may associate with Fer in a tyrosine phosphorylation/SH2-independent manner. Indeed, the cortactin N-terminus has been shown to bind GRB2 (growth factor receptor-bound protein 2) in an SH2-independent way [21], whereas Fer interacts with its substrate p120 catenin, without the involvement of its SH2 domain [18]. Once an initial interaction involving the cortactin N-terminus with Fer occurs, the Fer-mediated phosphorylation of the C-terminal tyrosine residues may be facilitated, and this may serve to stabilize the association of Fer and cortactin.

Our studies raise the possibility of a reciprocal regulation between the association of cortactin with F-actin and its ability to become tyrosine-phosphorylated. Once incorporated into an F-actin meshwork, cortactin may be less accessible to tyrosine kinases. On the other hand, tyrosine-phosphorylated cortactin may have reduced actin bundling [17,26] and Arp2/3-binding [5] capability. Consistent with this possibility, the tyrosine-phosphorylation-dependent interaction of cortactin with myosin light-chain kinase inhibits the Arp2/3 plus cortactin-induced actin polymerization [47].

A variety of stimuli (e.g. growth factors, hypertonicity) induce F-actin depolymerization and stress fibre disassembly in the centre of the cell, an effect that probably contributes to cortactin phosphorylation at these locations. Moreover, cell motility involves a continuous actin polymerization/depolymerization cycle. It is noteworthy that cortactin tyrosine phosphorylation is necessary for cell motility, and evidence is accumulating that, in addition to Src kinase, Fyn [26] and Fer [48] are also involved in the regulation of cell migration. It is conceivable that tyrosine phosphorylation at the leading edge may help liberate cortactin, and thereby allows its re-incorporation (after dephosphorylation) at the rapidly protruding cell boundary. The F-actin–Arp2/3-associated cortactin may be relatively protected from tyrosine kinases, which extends the half-life of the F-actin architecture. In short, the dynamic recycling of cortactin may be regulated by the interplay between F-actin binding and tyrosine phosphorylation. Such a scenario would explain that whereas tyrosine phosphorylation of cortactin is not required for its peripheral translocation [5,49], it is necessary for the continuous maintenance of cell migration [3]. Furthermore, this view may explain why overphosphorylation of cortactin is also associated with the inhibition of cell migration [50]. Future studies are warranted to validate this model.

In summary, our studies on the LB-induced cortactin tyrosine phosphorylation identified novel mechanisms whereby cytoskeletal organization impacts on signalling. The sensitivity of the Fyn/Fer pathway to F-actin may be an important regulatory feature involved in various physiological and pathological functions, such as resistance against osmotic stress and normal or dysregulated cell migration.

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REFERENCES

- Weed, S. A. and Parsons, J. T. (2001) Cortactin: coupling membrane dynamics to cortical actin assembly. *Oncogene* **20**, 6418–6434.
- Patel, A. S., Schechter, G. L., Wasilenko, W. J. and Somers, K. D. (1998) Overexpression of EMS1/cortactin in NIH3T3 fibroblasts causes increased cell motility and invasion *in vitro*. *Oncogene* **16**, 3227–3232.
- Huang, C., Liu, J., Haudenschild, C. C. and Zhan, X. (1998) The role of tyrosine phosphorylation of cortactin in the locomotion of endothelial cells. *J. Biol. Chem.* **273**, 25770–25776.
- Li, Y., Tondravi, M., Liu, J., Smith, E., Haudenschild, C. C., Kaczmarek, M. and Zhan, X. (2001) Cortactin potentiates bone metastasis of breast cancer cells. *Cancer Res.* **61**, 6906–6911.
- Di Ciano, C., Nie, Z., Szaszi, K., Lewis, A., Urano, T., Zhan, X., Rotstein, O. D., Mak, A. and Kapus, A. (2002) Osmotic stress-induced remodeling of the cortical cytoskeleton. *Am. J. Physiol. Cell Physiol.* **283**, C850–C865.

- 6 Birukov, K. G., Birukova, A. A., Dudek, S. M., Verin, A. D., Crow, M. T., Zhan, X., DePaola, N. and Garcia, J. G. (2002) Shear stress-mediated cytoskeletal remodeling and cortactin translocation in pulmonary endothelial cells. *Am. J. Respir. Cell Mol. Biol.* **26**, 453–464
- 7 Cao, H., Orth, J. D., Chen, J., Weller, S. G., Heuser, J. E. and McNiven, M. A. (2003) Cortactin is a component of clathrin-coated pits and participates in receptor-mediated endocytosis. *Mol. Cell. Biol.* **23**, 2162–2170
- 8 Weed, S. A., Karginov, A. V., Schafer, D. A., Weaver, A. M., Kinley, A. W., Cooper, J. A. and Parsons, J. T. (2000) Cortactin localization to sites of actin assembly in lamellipodia requires interactions with F-actin and the Arp2/3 complex. *J. Cell Biol.* **151**, 29–40
- 9 Urano, T., Liu, J., Zhang, P., Fan, Y., Egile, C., Li, R., Mueller, S. C. and Zhan, X. (2001) Activation of Arp2/3 complex-mediated actin polymerization by cortactin. *Nat. Cell Biol.* **3**, 259–266
- 10 Wu, H. and Parsons, J. T. (1993) Cortactin, an 80/85-kilodalton pp60src substrate, is a filamentous actin-binding protein enriched in the cell cortex. *J. Cell Biol.* **120**, 1417–1426
- 11 Weaver, A. M., Karginov, A. V., Kinley, A. W., Weed, S. A., Li, Y., Parsons, J. T. and Cooper, J. A. (2001) Cortactin promotes and stabilizes Arp2/3-induced actin filament network formation. *Curr. Biol.* **11**, 370–374
- 12 Weaver, A. M., Heuser, J. E., Karginov, A. V., Lee, W. L., Parsons, J. T. and Cooper, J. A. (2002) Interaction of cortactin and N-WASP with Arp2/3 complex. *Curr. Biol.* **12**, 1270–1278
- 13 McNiven, M. A., Kim, L., Krueger, E. W., Orth, J. D., Cao, H. and Wong, T. W. (2000) Regulated interactions between dynamin and the actin-binding protein cortactin modulate cell shape. *J. Cell Biol.* **151**, 187–198
- 14 Katsube, T., Takahisa, M., Ueda, R., Hashimoto, N., Kobayashi, M. and Togashi, S. (1998) Cortactin associates with the cell–cell junction protein ZO-1 in both *Drosophila* and mouse. *J. Biol. Chem.* **273**, 29672–29677
- 15 Kanner, S. B., Reynolds, A. B., Vines, R. R. and Parsons, J. T. (1990) Monoclonal antibodies to individual tyrosine-phosphorylated protein substrates of oncogene-encoded tyrosine kinases. *Proc. Natl. Acad. Sci. U.S.A.* **87**, 3328–3332
- 16 Wu, H., Reynolds, A. B., Kanner, S. B., Vines, R. R. and Parsons, J. T. (1991) Identification and characterization of a novel cytoskeleton-associated pp60src substrate. *Mol. Cell. Biol.* **11**, 5113–5124
- 17 Huang, C., Ni, Y., Wang, T., Gao, Y., Haudenschild, C. C. and Zhan, X. (1997) Down-regulation of the filamentous actin cross-linking activity of cortactin by Src-mediated tyrosine phosphorylation. *J. Biol. Chem.* **272**, 13911–13915
- 18 Kim, L. and Wong, T. W. (1998) Growth factor-dependent phosphorylation of the actin-binding protein cortactin is mediated by the cytoplasmic tyrosine kinase FER. *J. Biol. Chem.* **273**, 23542–23548
- 19 Kapus, A., Szasz, K., Sun, J., Rizoli, S. and Rotstein, O. D. (1999) Cell shrinkage regulates Src kinases and induces tyrosine phosphorylation of cortactin, independent of the osmotic regulation of Na⁺/H⁺ exchangers. *J. Biol. Chem.* **274**, 8093–8102
- 20 Kapus, A., Di Ciano, C., Sun, J., Zhan, X., Kim, L., Wong, T. W. and Rotstein, O. D. (2000) Cell volume-dependent phosphorylation of proteins of the cortical cytoskeleton and cell–cell contact sites. The role of Fyn and FER kinases. *J. Biol. Chem.* **275**, 32289–32298
- 21 Crostella, L., Lidder, S., Williams, R. and Skouteris, G. G. (2001) Hepatocyte growth factor/scatter factor-induces phosphorylation of cortactin in A431 cells in a Src kinase-independent manner. *Oncogene* **20**, 3735–3745
- 22 Maruyama, S., Kurosaki, T., Sada, K., Yamanashi, Y., Yamamoto, T. and Yamamura, H. (1996) Physical and functional association of cortactin with Syk in human leukemic cell line K562. *J. Biol. Chem.* **271**, 6631–6635
- 23 Gallet, C., Rosa, J. P., Habib, A., Lebre, M., Levy-Toledano, S. and Maclof, J. (1999) Tyrosine phosphorylation of cortactin associated with Syk accompanies thromboxane analogue-induced platelet shape change. *J. Biol. Chem.* **274**, 23610–23616
- 24 Zhan, X., Hu, X., Hampton, B., Burgess, W. H., Friesel, R. and Maciag, T. (1993) Murine cortactin is phosphorylated in response to fibroblast growth factor-1 on tyrosine residues late in the G1 phase of the BALB/c 3T3 cell cycle. *J. Biol. Chem.* **268**, 24427–24431
- 25 Okamura, H. and Resh, M. D. (1995) p80/85 cortactin associates with the Src SH2 domain and colocalizes with v-Src in transformed cells. *J. Biol. Chem.* **270**, 26613–26618
- 26 Huang, J., Asawa, T., Takato, T. and Sakai, R. (2003) Cooperative roles of Fyn and cortactin in cell migration of metastatic murine melanoma. *J. Biol. Chem.* **278**, 48367–48376
- 27 Abedi, H. and Zachary, I. (1998) Cytochalasin D stimulation of tyrosine phosphorylation and phosphotyrosine-associated kinase activity in vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.* **245**, 646–650
- 28 Spector, I., Shochet, N. R., Blasberger, D. and Kashman, Y. (1989) Latrunculin – novel marine macrolides that disrupt microfilament organization and affect cell growth: I. Comparison with cytochalasin D. *Cell Motil. Cytoskeleton* **13**, 127–144
- 29 Kim, L. and Wong, T. W. (1995) The cytoplasmic tyrosine kinase FER is associated with the catenin-like substrate pp120 and is activated by growth factors. *Mol. Cell. Biol.* **15**, 4553–4561
- 30 Haigh, J., McVeigh, J. and Greer, P. (1996) The fps/fes tyrosine kinase is expressed in myeloid, vascular endothelial, epithelial, and neuronal cells and is localized in the trans-Golgi network. *Cell Growth Differ.* **7**, 931–944
- 31 Head, J. A., Jiang, D., Li, M., Zorn, L. J., Schaefer, E. M., Parsons, J. T. and Weed, S. A. (2003) Cortactin tyrosine phosphorylation requires Rac1 activity and association with the cortical actin cytoskeleton. *Mol. Biol. Cell* **14**, 3216–3229
- 32 Thomas, S. M., Soriano, P. and Imamoto, A. (1995) Specific and redundant roles of Src and Fyn in organizing the cytoskeleton. *Nature (London)* **376**, 267–271
- 33 Craig, A. W., Zirngibl, R., Williams, K., Cole, L. A. and Greer, P. A. (2001) Mice devoid of fer protein–tyrosine kinase activity are viable and fertile but display reduced cortactin phosphorylation. *Mol. Cell. Biol.* **21**, 603–613
- 34 Sanguinetti, A. R. and Mastick, C. C. (2003) c-Abl is required for oxidative stress-induced phosphorylation of caveolin-1 on tyrosine 14. *Cell. Signal.* **15**, 289–298
- 35 Zirngibl, R., Schulze, D., Mirski, S. E., Cole, S. P. and Greer, P. A. (2001) Subcellular localization analysis of the closely related Fps/Fes and Fer protein–tyrosine kinases suggests a distinct role for Fps/Fes in vesicular trafficking. *Exp. Cell Res.* **266**, 87–94
- 36 Craig, A. W., Zirngibl, R. and Greer, P. (1999) Disruption of coiled-coil domains in Fer protein–tyrosine kinase abolishes trimerization but not kinase activation. *J. Biol. Chem.* **274**, 19934–19942
- 37 Rizoli, S. B., Rotstein, O. D., Parodo, J., Phillips, M. J. and Kapus, A. (2000) Hypertonic inhibition of exocytosis in neutrophils: central role for osmotic actin skeleton remodeling. *Am. J. Physiol. Cell Physiol.* **279**, C619–C633
- 38 Masszi, A., Di Ciano, C., Sirokmany, G., Arthur, W. T., Rotstein, O. D., Wang, J., McCulloch, C. A., Rosivall, L., Mucci, I. and Kapus, A. (2003) Central role for Rho in TGF- β 1-induced α -smooth muscle actin expression during epithelial–mesenchymal transition. *Am. J. Physiol. Renal Physiol.* **284**, F911–F924
- 39 Mehta, D., Tiruppathi, C., Sandoval, R., Minshall, R. D., Holinstat, M. and Malik, A. B. (2002) Modulatory role of focal adhesion kinase in regulating human pulmonary arterial endothelial barrier function. *J. Physiol. (Cambridge, U.K.)* **539**, 779–789
- 40 Woodring, P. J., Litwack, E. D., O'Leary, D. D., Lucero, G. R., Wang, J. Y. and Hunter, T. (2002) Modulation of the F-actin cytoskeleton by c-Abl tyrosine kinase in cell spreading and neurite extension. *J. Cell Biol.* **156**, 879–892
- 41 Woodring, P. J., Hunter, T. and Wang, J. Y. (2001) Inhibition of c-Abl tyrosine kinase activity by filamentous actin. *J. Biol. Chem.* **276**, 27104–27110
- 42 Plattner, R., Kadlec, L., DeMali, K. A., Kazlauskas, A. and Pendergast, A. M. (1999) c-Abl is activated by growth factors and Src family kinases and has a role in the cellular response to PDGF. *Genes Dev.* **13**, 2400–2411
- 43 Rosato, R., Veltmaat, J. M., Groffen, J. and Heisterkamp, N. (1998) Involvement of the tyrosine kinase fer in cell adhesion. *Mol. Cell. Biol.* **18**, 5762–5770
- 44 Arregui, C., Pathre, P., Lilien, J. and Balsamo, J. (2000) The nonreceptor tyrosine kinase fer mediates cross-talk between N-cadherin and β 1-integrins. *J. Cell Biol.* **149**, 1263–1274
- 45 Greer, P. (2002) Closing in on the biological functions of Fps/Fes and Fer. *Nat. Rev. Mol. Cell. Biol.* **3**, 278–289
- 46 Lock, P., Abram, C. L., Gibson, T. and Courtneidge, S. A. (1998) A new method for isolating tyrosine kinase substrates used to identify fish, an SH3 and PX domain-containing protein, and Src substrate. *EMBO J.* **17**, 4346–4357
- 47 Dudek, S. M., Birukov, K. G., Zhan, X. and Garcia, J. G. (2002) Novel interaction of cortactin with endothelial cell myosin light chain kinase. *Biochem. Biophys. Res. Commun.* **298**, 511–519
- 48 Craig, A. W. and Greer, P. A. (2002) Fer kinase is required for sustained p38 kinase activation and maximal chemotaxis of activated mast cells. *Mol. Cell. Biol.* **22**, 6363–6374
- 49 Weed, S. A., Du, Y. and Parsons, J. T. (1998) Translocation of cortactin to the cell periphery is mediated by the small GTPase Rac1. *J. Cell Sci.* **111**, 2433–2443
- 50 Landriscina, M., Prudovsky, I., Mouta Carreira, C., Soldi, R., Tarantini, F. and Maciag, T. (2000) Amlexanox reversibly inhibits cell migration and proliferation and induces the Src-dependent disassembly of actin stress fibers *in vitro*. *J. Biol. Chem.* **275**, 32753–32762

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