CELL STRUCTURE AND FUNCTION 28: 41–48 (2003)

Suppression of Cofilin Phosphorylation in Insulin-Stimulated Ruffling Membrane Formation in KB Cells

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ABSTRACT. **Various cellular events such as cell motility and division are directed by the actin cytoskeleton under the control of its regulatory system. Cofilin is a low molecular weight actin-modulating protein that severs and depolymerizes F-actin and is shown to enhance actin filament dynamics. The activity of cofilin is negatively regulated by phosphorylation at Ser-3. In human epidermoid carcinoma KB cells, insulin treatment induces characteristic ruffling membranes, and it was reported that LIMK1, a cofilin kinase, was activated in these cells treated with insulin. Since cofilin is a key protein responsible for establishing the rapid turnover of actin filaments, it appears to be contradictory that cofilin is phosphorylated (inactivated) by a stimulus that is known to induce the highly dynamic actin structure, ruffling membranes. Therefore, we examined the phosphorylation state of endogenous cofilin in KB cells treated with insulin. The dephosphorylated form of cofilin increased with insulin treatment, as analyzed by nonequilibrium pH gradient gel electrophoresis (NEpHGE)-immunoblotting. Cell labeling with ^{32}P orthophosphate indicated that cofilin was being continuously phosphorylated and dephosphorylated, and that the apparent insulin-induced dephosphorylation was due to suppression of continuous phosphorylation and not to enhanced dephosphorylation. Further, we examined the localization of the phosphorylated form of cofilin using phospho-specific antibody raised against phosphorylated cofilin. Surprisingly, phosphorylated cofilin was concentrated in the ruffling membranes induced by insulin. These results suggest that the examination of the kinetics and spatial regulation of phosphorylation is critical for the elucidation of the role of cofilin and upstream kinases in actin reorganization.**

Key words: cofilin/actin/ruffling membrane/phosphorylation/insulin

Various cellular events such as cell motility and division are directed by the actin cytoskeleton under the control of its regulatory system. Cofilin is a low molecular weight actin-modulating protein of the cofilin/actin depolymerizing factor (ADF; also known as destrin) family that was originally isolated from porcine brain (Nishida *et al.*, 1984) and is ubiquitously distributed in eucaryotes from budding yeast to mammals (Aizawa *et al.*, 1995; Iida *et al.*, 1993; Moon *et al.*, 1993). Cofilin binds to actin in both monomeric and polymerized forms in a 1:1 molar ratio and depolymerizes F-actin in a pH dependent manner (Nishida *et al.*, 1984; Yonezawa *et al.*, 1985). Recently, cofilin was reported to enhance actin filament dynamics (Theriot, 1997). It was shown that cofilin is a critical protein for the turnover of actin filament (Rosenblatt *et al.*, 1997, Lappalainen and Drubin, 1997). The effect of cofilin on actin filament dynamics could be attributable either to the severing of filamentous actin (Moriyama and Yahara, 1999) or to the acceleration of actin treadmilling (Carlier *et al.*, 1997) or to both.

Confocal microscopic dissection of *Dictyostelium discoideum* cells immunofluorescently labeled with anti-cofilin antibodies revealed that cofilin is codistributed with actin on ruffling membranes but not on adhesion plaques (Aizawa *et al.*, 1995). Overexpression of cofilin in these cells stimulates membrane ruffling and cell motility (Aizawa *et al.*, 1996). The condensation of cofilin in ruffling areas was also observed in other cell types (Heyworth *et al.*, 1997;

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Abbreviations: LIMK, LIM kinase; NEpHGE, non-equilibrium pH gradient gel electrophoresis; TESK, testicular protein kinase; DMEM, Dulbecco's modified Eagle medium; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A.

Nagaoka *et al.*, 1996). These observations, together with the effect of cofilin on actin filament dynamics, suggested that cofilin might be involved in the formation of dynamic, rather than static, actin-based structures like ruffling membranes. The activity of cofilin is regulated by phosphorylation at Ser-3 (Agnew *et al.*, 1995; Moriyama *et al.*, 1996). Phosphorylation of cofilin abolishes essentially all its activities towards actin. Cofilin is rapidly dephosphorylated upon stimulation in various cell types including platelets, neutrophils, thyroid cells, T-cells, parotid gland cells, astrocytes and oocytes (Davidson and Haslam, 1994; Djafarzadeh and Niggli, 1997; Heyworth *et al.*, 1997; Suzuki *et al.*, 1995; Okada *et al.*, 1996; Saito *et al.*, 1994; Samstag *et al.*, 1994; Kanamori *et al*, 1995; Takuma *et al.*, 1996; Baorto *et al.*, 1992; Abe *et al.*, 1996). These activation processes are well correlated with changes in cytoskeletal organization and assembly.

The kinases responsible for phosphorylating cofilin reported so far are LIM kinases, LIMK1 and LIMK2, closely related serine/threonine kinases (Lawler 1999) and testicular protein kinases (TESKs), TESK1 and TESK2, which have a kinase domain related to those of LIMKs (Toshima *et al.*, 2001a, Toshima *et al.*, 2001b). Arber *et al.* and Yang *et al.* (Arber *et al.*, 1998, Yang *et al.*, 1998) first reported that LIMK1 specifically phosphorylated cofilin under the control of Rac, a Rho family small GTPase that is central regulator of formation of ruffling membranes/lamellipodia. These reports suggest that activation of LIMK1 by Rac results in the phosphorylation and inactivation of cofilin, leading to stabilization of actin filaments and formation of ruffling membranes. This model of formation of ruffling membranes is not consistent with the fact that such membranes are not static structures; rather, actin filaments are being rapidly turned over in these ruffling areas. Since cofilin is a key protein for establishing highly dynamic actin cytoskeleton and cofilin localizes in ruffling areas in many cell types, it is likely that cofilin activity is required for the formation of ruffling membranes. Cofilin might be activated in the cells stimulated by ruffling-inducing stimuli. We therefore attempted to examine the state of cofilin phosphorylation and phosphorylation/dephosphorylation kinetics in cells treated with ruffling-inducing agents. We also examined the localization of phophorylated cofilin in cells that were induced to form ruffling membranes.

Materials and Methods

Cell Culture

Human epidermoid carcinoma KB cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 60 mg/l kanamycin sulfate (Banyu Pharmaceutical Co., Ltd.) in 5% CO_2.

Non-equilibrium pH gradient electrophoresis (NEpHGE) and immunoblotting

KB cells were plated at a density of 1.5×10^5 cells per 60 mm culture dish. The day following plating, the medium was changed to serum-free DMEM and cells were maintained in this medium for 36–44 h (serum-starved condition). The cells were stimulated with 5 μg/ml insulin (Sigma Chemical Co.) and harvested at various time points. The cells were washed twice with ice-cold PBS and lysed in 300 μl of lysis buffer (20 mM Tris-HCl (pH 7.6), 0.5% TritonX-100, 10 mM NaF, 10 μg/ml aprotinin, 1 mM PMSF). The lysate was centrifuged for 15 min at 15,000 rpm at 4°C. Proteins were precipitated with 10% trichloroacetic acid (TCA) and washed twice with ice-cold acetone. Protein pellets were dissolved in NEpHGE sample buffer (8.5 M urea, 5% (w/v) CHAPS, 2% (v/v) ampholine, 5% (v/v) 2-mercaptoethanol). The composition of NEpHGE was 4% (w/v) acrylamide (4% T, 5.4% C), 8.5 M urea, 2% (w/v) CHAPS, and 2% (v/v) ampholine. Ten microliters of the sample was loaded and the same volume of overlay solution (5 M urea, 1% (v/v) ampholine) was laid over the sample. The lower and upper reservoirs were filled with 10 mM NaOH and 40 mM H_3PO_4, respectively. Electrophoresis was conducted initially at 200 V for 10 min, and at 400 V for 75 min. To remove urea from the gel and to bind SDS to the proteins in the gel, the gel was washed for 50 min with buffer (5 mM Tris-HCl (pH 8.0), 1% (w/v) SDS, 50% (w/w) methanol) during which the buffer was changed every 10 min and the gel was left in this buffer for an additional 70 min. Prior to transfer to PVDF membrane, the gel was equilibrated with transfer buffer (25 mM Tris, 250 mM glycine, 10% (v/v) methanol) for 20 min, and electrotransfer was then conducted at 8 V/cm for 2 h. The membrane was blocked with 5% (w/v) skimmed milk in TBS overnight at 4°C, and incubated for 1 h at room temperature with primary antibody (rabbit polyclonal anti-cofilin antibody (Moriyama *et al.*, 1996)) at 1:250 dilution in antibody diluent (1% (v/v) FCS in TBS). Secondary antibody (sheep anti-rabbit Ig; Amersham Pharmacia Biotech AB, Uppsala, Sweden) was used at 1:1000 dilution (40 min at room temperature). The immunoblot was visualized with ECL system (Amersham Pharmacia Biotech AB).

Determination of kinetics of phosphorylation and dephosphorylation of cofilin

Changes in the phosphorylation rate of cofilin in response to insulin were determined as follows. KB cells were seeded and serum-starved as described above. They were then washed three times with phosphate-free DMEM, and incubated in 1–1.5 ml of the same medium containing ^{32}P orthophosphate (NEN Life Science Products, 7.4 MBq/ml) for 5 min before stimulation. Insulin was added at 5 μg/ml, and control cells were treated with HBS (20 mM HEPES-NaOH (pH 7.4), 150 mM NaCl). At various time points, cells were washed twice with ice-cold HBS, and lysed with 300 μl of lysis buffer and the lysates were stored frozen at –80°C until immunoprecipitation. The dephosphorylation rate of cofilin was determined as follows. KB cells were seeded and serum starved as

described above. After serum-starvation for 36–44 h, the medium was changed to phosphate-free DMEM, in which 3.7 MBq/ml ^{32}P orthophosphate was added. Cells were labeled in this medium for 2 hours, washed three times with DMEM containing non-radioactive H_3PO_4 at the same concentration. The cells were maintained in 1 ml of this medium for 10 min and then stimulated with insulin. For controls, cells were treated with HBS.

For immunoprecipitation, the cell lysates were thawed and centrifuged for 15 min at 15,000 rpm at 4°C. Protein concentrations of the supernatants were determined, after which they were adjusted to the same concentrations among samples. Protein A-Sepharose (Pharmacia) was added to the supernatants and rocked for 1 h in ice. After pelleting the beads, anti-cofilin antibody (Moriyama *et al.*, 1996) was added to the pretreated supernatants and rocked for 1 h in ice. The mixtures then received fresh protein A-Sepharose and were further rocked for 1 h in ice. Immune complex was washed 4 times with lysis buffer, and suspended in 2× SDS-PAGE sample buffer. The samples were boiled for 5 min, and subjected to SDS-PAGE. The gel was silver-stained and exposed to an imaging plate overnight. The image of the gel was captured with optical scanner and the radioactivity of each band was analyzed using a BAS-2500 system (Fujifilm, Tokyo, Japan). Radioactivity was normalized to the optical density of silver-stained bands as analyzed by NIH-Image.

Immunofluorescence

KB cells were plated on 14 mm coverslips coated with poly-L-lysine at a density of 5×10^3 cells per 22 mm well. After 24 h, the medium was changed to serum-free DMEM and left in this medium for a further 36–48 h. The cells were stimulated with 5 μg/ml insulin. After 5 min, cells were fixed with 3.7% formaldehyde-PBS for 15 min, washed with PBS, and then permeabilized with 0.2% TritonX-100-PBS for 15 min. The coverslips were double-stained with anti-cofilin monoclonal antibody (MAB22; a generous gift from Drs. T. Obinata and H. Abe, Chiba University; Abe *et al.*, 1989) and anti-phosphorylated cofilin polyclonal antibody (Aizawa *et al.*, 2001). Secondary antibodies were goat anti-mouse IgG-Cy3 (Amersham) and goat anti-rabbit IgG-FITC (Jackson ImmunoLaboratory). The fluorescent image was enhanced using Photoshop software to clearly visualize the ruffling membrane areas. Therefore, the fluorescent intensity may not be proportional to the amount of specific antigen in these images.

Results

Phosphorylation state of cofilin in insulin-treated KB cells

Human epidermoid carcinoma KB cells were reported to show characteristic ruffling membranes in response to various stimuli (Kadowaki *et al.*, 1986). Insulin induces ruffling membranes in these cells and it was reported that the kinase activity of endogenous LIMK1 immunoprecipitated from insulin-treated KB cells was about 1.4 fold higher than resting cells. Since phosphorylation level of cofilin may not be determined solely by LIMK1 kinase activity, we attempted to determine the phosphorylation state and the phosphorylation/dephosphorylation kinetics of endogenous cofilin *in vivo* using KB cells.

First, we investigated the net phosphorylation level of cofilin in insulin-treated KB cells using NEpHGE followed by immunoblotting (Fig. 1A). As shown in Figure. 1B, about 40% of cofilin is the phosphorylated (inactive) form and about 60% is the dephosphorylated (active) form in serum-starved KB cells. Insulin treatment decreased the phosphorylated form to 15% after 5 min of treatment, after which the phosphorylated form gradually increased. Insulin

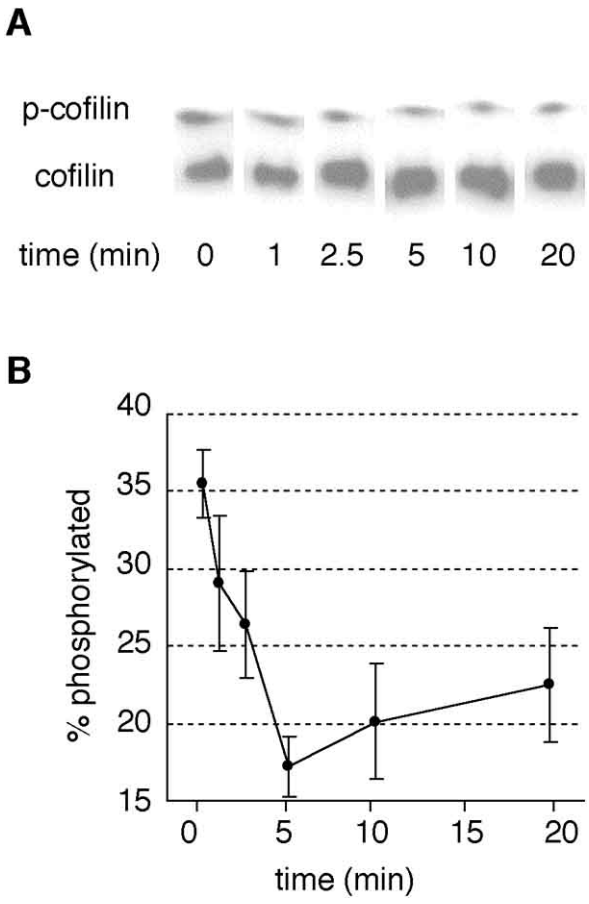

Fig. 1. Changes of phosphorylation level of cofilin in insulin-stimulated KB cells. A. Time-course changes of phosphorylation level of cofilin in KB cells treated with insulin. KB cells were treated with 5 μg/ml insulin and the cells were harvested at indicated times. Cellular proteins were resolved with non-equilibrium pH gradient gel electrophoresis and subjected to immunoblot using anti-cofilin antibody. B. The upper and lower bands represent phosphorylated and dephosphorylated cofilin, respectively. p-cofilin denotes phosphorylated cofilin. The optical density of the bands in A was quantified and the ratio of upper band to total was calculated. The mean value and the standard deviation of three independent experiments were plotted.

treatment transiently increased the active, dephosphorylated form, and decreased the inactive, phosphorylated form of cofilin. It should be noted that the dephosphorylation was transient and the ratio of phosphorylated form gradually recovered in the prolonged insulin exposure. In contrast to the activation of kinase activity of LIMK1 as reported by Yang *et al.*, the net phosphorylation level of endogenous cofilin decreased in insulin-treated KB cells. As the net phosphorylation state of cofilin must be determined from the functions of two antagonizing biochemical events, the phosphorylation and dephosphorylation of the protein, these two events were determined separately using ^{32}P-labeling.

Since a significant portion of cofilin in resting cells is phosphorylated, prolonged prelabeling with ^{32}P-orthophosphate before insulin stimulation may increase the background of radiolabeled cofilin. Thus, KB cells were briefly prelabeled with 7.4 MBq/ml ^{32}P orthophosphate for 5 min before stimulation and then stimulated with insulin. After 5 and 10 min, the cells were lysed and cofilin in the lysates was immunoprecipitated. Incorporation of ^{32}P into cofilin increased in untreated control cells as a function of time (Fig. 2A, B). In contrast, ^{32}P incorporation in insulin-treated cells was significantly less than that in untreated cells after 5 min ($P<0.05$). Incorporation of radiolabel into cofilin at 10 min after stimulation was not significantly different. These findings indicate that insulin treatment of KB cells transiently inhibited cofilin phosphorylation. As it was possible that the lack of increase in cofilin phosphorylation in insulin-stimulated cells was a result of enhanced dephosphorylation, the dephosphorylation kinetics of cofilin was determined.

Cells were prelabeled with 3.7 MBq/ml ^{32}P orthophosphate for a more prolonged period (2 hours) to equilibrate phosphate turnover of phosphorylated cofilin. The medium was then shifted to DMEM without radioactive orthophosphate. The cells were maintained in this medium for an additional 10 min and then treated with insulin or left untreated. Cofilin in the lysates was immunoprecipitated. The results clearly showed that insulin treatment had almost no effect on the dephosphorylation kinetics of cofilin (Fig. 3A, B).

From these findings, it is concluded that cofilin is continuously being phosphorylated and dephosphorylated even in resting cells, and that insulin treatment suppresses cofilin phosphorylation but has little effect on cofilin dephosphorylation. As a result, the net phosphorylation level of cofilin decreases transiently after insulin treatment in KB cells.

Localization of phosphorylated cofilin in insulin-stimulated KB cells

Next, the localization of the phosphorylated form of cofilin in KB cells was examined. Anti-phosphorylated cofilin-specific antibody was produced (Aizawa *et al.*, 2001) and checked for the specificity with immunoblot after both SDS-PAGE and NEpHGE. As shown in Figure 4A, this antibody specifically recognizes phosphorylated cofilin in KB cell extract. Using this antibody, the localization of

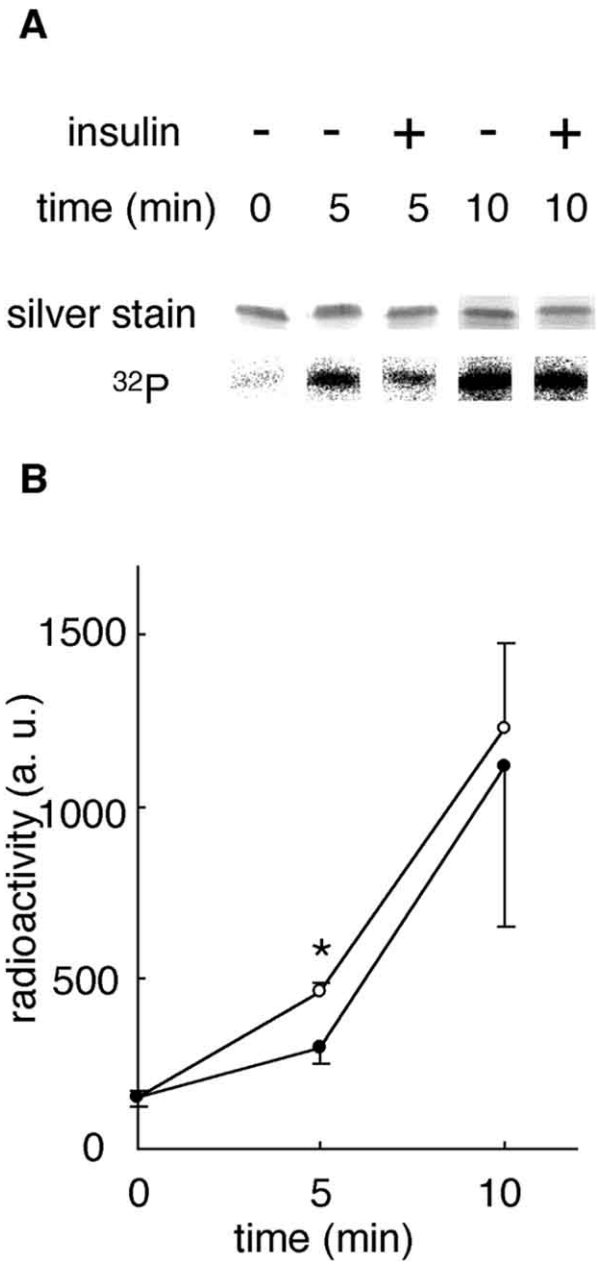

Fig. 2. Phosphorylation kinetics of cofilin in KB cells treated with insulin. A. KB cells were labeled with ^{32}P-orthophosphate and treated with insulin or untreated (control). Cofilin was immunoprecipitated from cell lysate, subjected to SDS-PAGE, followed by silver-staining and radioactivity was analyzed with BAS-2500 (^{32}P). B. Radioactivity of bands was normalized to optical density of silver-stained bands. Mean values and standard deviation of three independent experiments were plotted for insulin-treated (*closed circles*) and control cells (*open circles*). Asterisk denotes significant difference ($P<0.05$) between control and insulin treated cells.

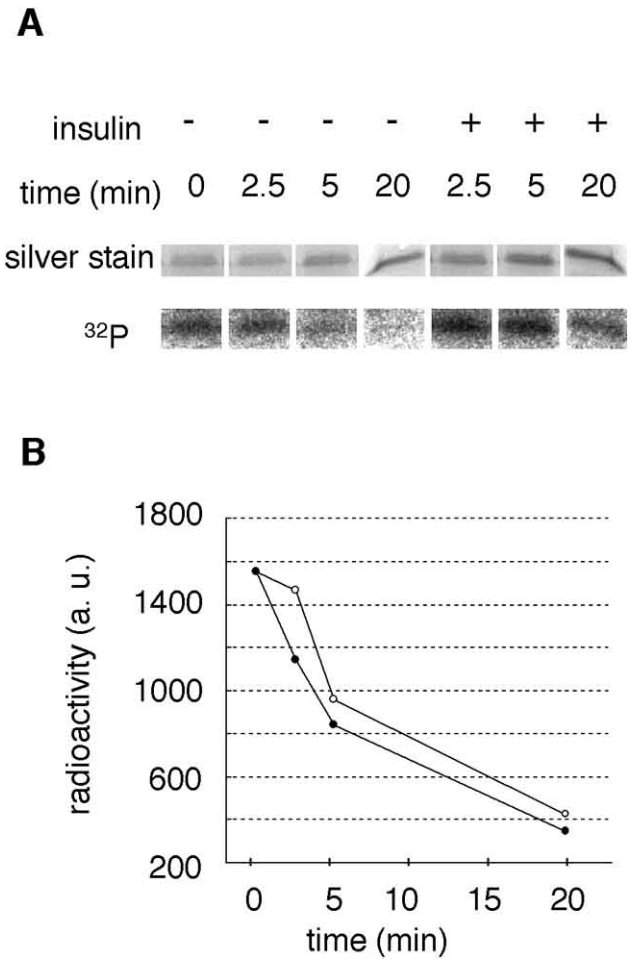

Fig. 3. Dephosphorylation kinetics of cofilin in KB cells treated with insulin. A. KB cells were prelabeled with ^{32}P-orthophosphate, then medium was changed to that not containing radiolabel. After 10 min, cells were treated with insulin or untreated (control). Cofilin was immunoprecipitated from cell lysate, subjected to SDS-PAGE, followed by silver-staining and radioactivity was analyzed with BAS-2500 (^{32}P). B. Radioactivity of bands was normalized to optical density of silver-stained bands and plotted as a function of time and plotted for insulin-treated (*closed circles*) and control cells (*open circles*).

phosphorylated cofilin in KB cells treated with insulin was examined. For double-staining, anti-cofilin monoclonal antibody (MAB22; Abe *et al.*, 1989) was used together with anti-phosphorylated cofilin polyclonal antibody. Phosphorylated cofilin in non-stimulated cells was found to be diffusely distributed in the cytoplasm just as in the case of total cofilin (Fig. 4B b, c). When stimulated with insulin, the phosphorylated form of cofilin was also concentrated in the ruffling membranes as total cofilin (Fig. 4B e, f).

Discussion

The kinases responsible for phosphorylating cofilin reported are LIMKs, LIMK1 and LIMK2, closely related serine/threonine kinases (Lawler, 1999), and testicular protein kinases, TESK1 and TESK2, which have a kinase domain related to those of LIMKs (Toshima *et al.*, 2001a, Toshima *et al.*, 2001b). Since the finding that LIMK1 phosphorylates cofilin downstream of Rac, the regulation of LIMKs has been extensively studied, especially with regard to Rho family GTPases (Lawler 1999). Rho family GTPases centrally regulate actin cytoskeleton and each family members induces specific actin structures: Rho induces stress fibers and focal adhesions; Rac induces membrane rufflings/lamellipodia; Cdc42 induces filopodia; and both Rac and Cdc42 induce focal complexes (Nobes and Hall, 1995, Ridley and Hall, 1992, Ridley *et al.*, 1992). Edwards *et al.* reported Rac activates LIMK1, partly through PAK activation (Edwards *et al.*, 1999). Rho and Cdc42 activate LIMK2 (Sumi *et al.*, 1999), and in the case of Rho, this activation is partly through ROCK activation (Maekawa *et al.*, 1999). These results suggest cofilin is equally inactivated in any actin reorganization process, whatever the upstream GTPases involved. In fact, cofilin activation (dephosphorylation) was observed in many cell types after cell stimulation that is accompanied by cytoskeletal rearrangement. This apparent discrepancy may come from the differences in the dynamic nature of the actin structures in question. The actin structures typically induced by each Rho family member are distinct with regard to actin cytoskeleton dynamics. In contrast to the stress fibers that are relatively static, ruffling membranes/lamellipodia are highly dynamic structures, which may require cofilin activity to form. Our finding clearly shows that cofilin is dephosphorylated (activated) in the formation of these structures. In support of this, Zebda *et al.* showed that inhibition of ADF/cofilin function by expression of kinase domain of LIMK1 did not stimulate, but rather inhibited EGF-induced barbed end formation and subsequent lamellipod extension (Zebda *et al.*, 2000).

The finding that LIMK1 was activated in KB cells by insulin treatment was obtained using *in vitro* kinase assay (Yang *et al.*, 1998). This assay was performed *in vitro* with immunoprecipitated LIMK1 from insulin-treated KB cells using $(His)_6$-cofilin as a substrate. The kinetics of LIMK1 activation after insulin treatment showed that its peak resides at 5 min after treatment. Our present study shows that net phosphorylation level decreased rapidly until 5 min treatment and after that, the phosphorylation level gradually increased. This indicates that cofilin dephosphorylation was restricted to the very short period at the beginning of the insulin action and that cofilin phosphorylation actually took place at prolonged insulin exposure. Although the identity of the kinase(s) which continuously phosphorylate cofilin and is suppressed shortly after insulin treatment remains to be elucidated, it is also possible that subsequent phosphorylation was responsible for LIMKs. Since dominant-negative LIMK1 was shown to inhibit insulin-induced membrane ruffling in KB cells, cofilin inactivation may also be essential in the somewhat later phase for ruffle formation, proba-

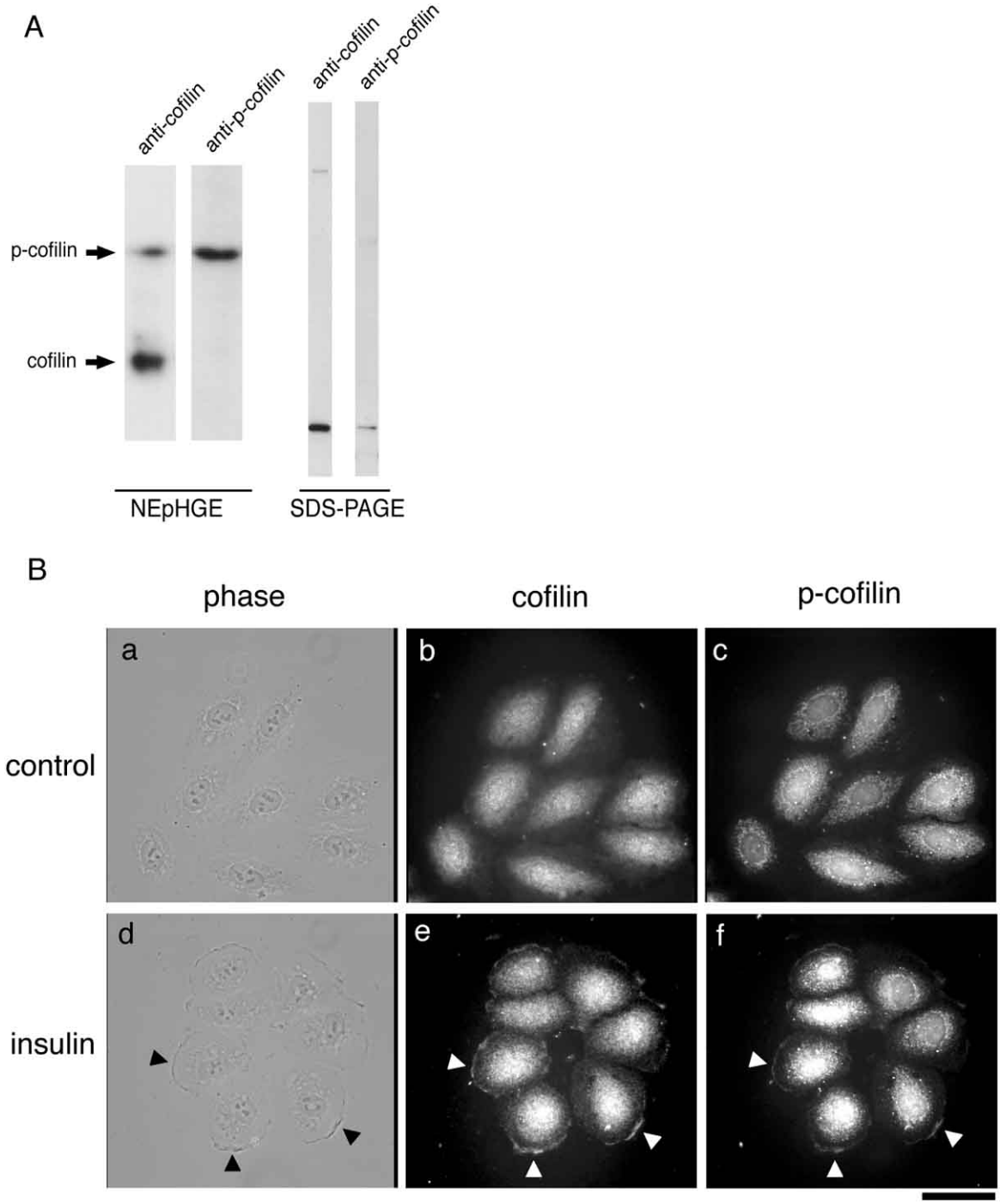

Fig. 4. Localization of total cofilin and phosphorylated cofilin in KB cells. A. Characterization of anti-phosphorylated cofilin antibody used for immunostaining. KB cell extract was resolved both by NEpHGE and SDS-PAGE, followed by immunoblotting using anti-cofilin antibody (Moriyama *et al.*, 1996) and anti-phosphorylated cofilin antibody (Aizawa *et al.*, 2001). The anti-phosphorylated cofilin antibody specifically recognizes phosphorylated form of cofilin. p-cofilin and anti-p-cofilin denote phosphorylated cofilin and anti-phosphorylated cofilin, respectively. B. Localization of phosphorylated cofilin in insulin-stimulated KB cells. Control KB cells (a, b, and c) and insulin-treated cells (d, e, and f) were observed with phase contrast microscopy (a and d), and double-stained with anti-cofilin antibody (MAB22; b and e) and with anti-phosphorylated-cofilin antibody (c and f). Insulin treatment induces characteristic ruffling membranes at 5 min (*arrowheads*). Total cofilin as well as phosphorylated form was concentrated at ruffling membrane areas. The images were processed so that ruffling membrane areas were clearly visible. Therefore, the fluorescent intensity may not be proportional to the amount of a specific antigen in these images. Bar represents 20 μm.

bly through the stabilization of actin filaments that should be static in ruffle maintenance. Analyses of temporal regulation of cofilin phosphorylation thus may be crucial for the elucidation of regulation by upstream kinases.

The phosphatase(s) for continuous dephosphorylation need to be determined. Ambach *et al.* reported that Protein Phosphatase 1 (PP1) and Protein Phosphatase 2A (PP2A) are physically associated with cofilin in T-cells and that PP1 and PP2A dephosphorylated phosphorylated cofilin *in vitro* (Ambach *et al.*, 2000). In a preliminary experiment to clarify the identity of the phosphatase(s) involved in the continuous dephosphorylation of cofilin, we used several phosphatase inhibitors, okadaic acid, calyculin A (both for PP1 and PP2A), and cyclosporin A (for PP2B). In contrast to the results of Ambach *et al.*, we did not observe inhibition of cofilin dephosphorylation in KB cells with any of the phosphatase inhibitors we have used. Thus, the identity of the phosphatase(s) also remains to be elucidated.

The phosphate turnover observed on cofilin in resting cells, *i.e.*, cofilin was being continuously phosphorylated and dephosphorylated, constitutes the so-called futile cycle. Continuous phosphorylation/dephosphorylation of cofilin does not produce any apparent effects on actin cytoskeleton and as a result, ATP in the cells is being continuously and vacantly consumed. Insulin treatment suppressed only the phosphorylation reaction of this cycle. Many reports describing cofilin dephosphorylation in response to cell stimulation assume the activation of phosphatase(s), but this may not always be the case. If the phosphate turnover on a given protein is relatively high, then the suppression of phosphorylation reaction may result in rapid dephosphorylation. The energy consumed by the continuous phosphorylation/dephosphorylation cycle may be used to maintain the high reactivity of the actin cytoskeleton to environmental cues. Repeated cycles of phosphorylation and dephosphorylation may also provide cells with ample chances to regulate each reaction separately and sophisticatedly.

Phosphorylated cofilin was localized to ruffling membranes in KB cells treated with insulin. The fluorescent signal in ruffling membranes using phospho-specific antibody may just be residual phosphorylated cofilin waiting to be dephosphorylated, which may be consistent with the labeling experiments in this study. Alternatively, the ratio of phosphorylated cofilin to total cofilin may actually remain unchanged in the ruffling areas, which opens up two possibilities: 1) phosphorylated cofilin is being recruited to the ruffling areas, and 2) cofilin is being phosphorylated *de novo* in the ruffling areas. The first possibility suggests additional factor(s) that brings cofilin to the ruffling areas, for which phosphoinositides are a prime candidate. Cofilin binds to both F/G-actin and phosphoinositides, and when phosphorylated, binds only to phosphoinositides. Thus, if a mechanism exists that affects the distribution of phosphoinositides, it may also affect the distribution of cofilin. The second possibility is more consistent with the models previously suggested from the actin-binding property of cofilin (Moriyama *et al.*, 1996, Meberg *et al.*, 1998). Since dephosphorylated cofilin may bind to either F- or G-actin, cofilin must be reactivated in the actin-free form before severing F-actin. This reactivation may be achieved from the cycle of phosphorylation and dephosphorylation. In other words, the phosphate turnover on cofilin may actually be enhanced in ruffling areas. Further analyses of the distribution of cofilin and the assessment of the localization of phosphorylation reaction may be crucial for elucidating the roles of cofilin in actin reorganizaion.

Acknowledgments. We thank Drs. I. Yahara and K. Moriyama (Tokyo Metropolitan Institute of Medical Sciences) for their support and comments on this work. We also thank Drs. T. Obinata and H. Abe (Chiba University) for providing us with anti-cofilin monoclonal antibody (MAB22). This work was supported by research grants from the Ministry of Education, Science, Sports, and Culture of Japan.

References

Abe, H., Obinata, T., Minamide, L.S., and Bamburg, J.R. 1996. *Xenopus laevis* actin-depolymerizing factor/cofilin: A phosphorylation-regulated protein essential for development. *J. Cell Biol.*, **132**: 871–885.

Abe, H., Oshima, S., and Obinata, T. 1989. A cofilin-like protein is involved in the regulation of actin assembly in developing skeletal muscle. *J. Biochem.*, **106**: 696–702.

Agnew, B.J., Minamide, L.S., and Bamburg, J.R. 1995. Reactivation phosphorylated actin depolymerizing factor and identification of the regulatory site. *J. Biol. Chem.*, **270**: 17582–17587.

Aizawa, H., Sutoh, K., Tsubuki, S., Kawashima, S., Ishii, A., and Yahara, I. 1995. Identification, characterization and intracellular distribution of cofilin in *D. discoideum*. *J. Biol. Chem.*, **270**: 6381–6388.

Aizawa, H., Sutoh, K., and Yahara, I. 1996. Overexpression of cofilin stimulates bundling of actin filaments, membrane ruffling and cell movement in *Dictyostelium*. *J. Cell Biol.*, **132**: 335–344.

Aizawa, H., Wakatsuki, S., Ishii, A., Moriyama, K., Sasaki, Y., Ohashi, K., Sekine-Aizawa, Y., Sehara-Fujisawa, A., Mizuno, K., Goshima, Y., and Yahara, I. 2001. Phosphorylation of cofilin by LIM-kinase is necessary for semaphorin 3A-induced growth cone collapse. *Nature Neurosci.*, **4**: 367–373.

Ambach, A., Saunus, J., Konstandin, M., Wesselborg, S., Meuer, S.C., and Samstag, Y. 2000. The serine phosphatase PP1 and PP2A associate with and activate the actin-binding protein cofilin in human T lymphocytes. *Eur. J. Immunol.*, **30**: 3422–3431.

Arber, S., Barbayannis, F.A., Hanser, H., Schneider, C., Stanyon, C.A., Bernard, O., and Caroni, P. 1998. Regulation of actin dynamics through phosphorylation of cofilin by LIM-kinase. *Nature*, **393**: 805–809.

Baorto, D.M., Mellado, W., and Shelanski, M.L. 1992. Astrocyte process growth induction by actin breakdown. *J. Cell Biol.*, **117**: 357–367.

Carlier, M.-F., Laurent, V., Santolini, J., Melki, R., Didry, D., Xia, G.-X., Hong, Y., Chua, N.-H., and Pantaloni, D. 1997. Actin depolymerizing factor (ADF/cofilin) enhances the rate of filament turnover: implication in actin-based motility. *J. Cell Biol.*, **136**: 1307–1323.

Davidson, M.M. and Haslam, R.J. 1994. Dephosphorylation of cofilin in stimulated platelets: roles for a GTP-binding protein and Ca^{2+} . *Biochem. J.*, **301**: 288–290.

Djafarzadeh, S. and Niggli, V. 1997. Signaling pathways involved in dephosphorylation and localization of the actin-binding protein cofilin in stimulated human neutrophils. *Exp. Cell Res.*, **236**: 427–435.

Edwards, D.C., Sanders, L.C., Bokoch, G.M., and Gill, G.N. 1999. Activa-

tion of LIM-kinase by Pak1 couples Rac/Cdc42 GTPase signalling to actin cytoskeletal dynamics. *Nature Cell Biol.*, **1**: 253–259.

Heyworth, P.G., Robinson, J.M., Ding, J., Ellis, B.A., and Badwey, J.A. 1997. Cofilin undergoes rapid dephosphorylation in stimulated neutrophils and translocates to ruffled membranes enriched in products of the NADPH oxidase complex. Evidence for a novel cycle of phosphorylation and dephosphorylation. *Histochem. Cell Biol.*, **108**: 221–233.

Iida, K., Moriyama, K., Matsumoto, S., Kawasaki, H., Nishida, E., and Yahara, I. 1993. Isolation of a yeast essential gene, *COF1*, that encodes a homologue of mammalian cofilin, a low-Mr actin-binding and depolymerizing protein. *Gene*, **124**: 115–120.

Kadowaki, T., Koyasu, S., Nishida, E., Sakai, H., Takaku, F., Yahara, I., and Kasuga, M. 1986. Insulin-like growth factors, insulin, and epidermal growth factor cause rapid cytoskeletal reorganization in KB cells: clarification of the roles of type I insulin-like growth factor receptors and insulin receptors. *J. Biol. Chem.*, **261**: 16141–16147.

Kanamori, T., Hayakawa, T., Suzuki, M., and Titani, K. 1995. Identification of two 17-kDa rat parotid gland phophoproteins, subjects for dephosphorylation upon beta-adrenaergic stimulation, as destrin and cofilin-like proteins. *J. Biol. Chem.*, **270**: 8061–8067.

Lappalainen, P. and Drubin, D.G. 1997. Cofilin promotes rapid actin filament turnover in vivo. *Nature*, **388**: 78–82.

Lawler, S. 1999. Regulation of actin dynamics: The LIM kinase connection. *Curr. Biol.*, **9**: R800–802.

Maekawa, M., Ishizaki, T., Boku, S., Watanabe, N., Fujita, A., Iwamatsu, A., Obinata, T., Ohashi, K., Mizuno, K., and Narumiya, S. 1999. Signaling from Rho to the actin cytoskeleton through protein kinases ROCK and LIM-kinase. *Science*, **285**: 895–898.

Meberg, P.J., Ono, S., Minamide, L.S., Takahashi, M., and Bamburg, J.R. 1998. Actin depolymerizing factor and cofilin phosphorylation dynamics: response to signals that regulate neurite extension. *Cell Motil. Cytoskel.*, **39**: 172–190.

Moon, A.L., Janmey, P.A., Loute, K.A., and Drubin, D.G. 1993. Cofilin is an essential component of the yeast cortical cytoskeleton. *J. Cell Biol.*, **120**: 421–435.

Moriyama, K., Iida, K., and Yahara, I. 1996. Phosphorylation of Ser-3 of cofilin regulates its essential function on actin. *Genes to Cells*, **1**: 73–86.

Moriyama, K. and Yahara, I. 1999. Two activities of cofilin, severing and accelerating directional depolymerization of actin filaments, are affected by mutations around the actin-binding helix. *EMBO J.*, **18**: 6752–6761.

Nagaoka, R., Abe, H., and Obinata, T. 1996. Site-directed mutagenesis of the phosphorylation site of cofilin: its role in cofilin-actin interaction and cytoplasmic localization. *Cell Motil. Cytoskel.*, **35**: 200–209.

Nishida, E., Maekawa, S., and Sakai, H. 1984. Cofilin, a protein in porcine brain that binds to actin filaments and inhibits their interaction with myosin and tropomyosin. *Biochemistry*, **23**: 5307–5313.

Nobes, C.D. and Hall, A. 1995. Rho, Rac, and Cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell*, **81**: 53–62.

Okada, K., Takano-Ohmuro, H., Obinata, T., and Abe, H. 1996. Dephosphorylation of cofilin in polymorphonuclear leukocytes derived from peripheral blood. *Exp. Cell Res.*, **227**: 116–122.

Ridley, A.J. and Hall, A. 1992. The small GTP-binding protein rho regulates the assembly of focal adhesion and actin stress fibers in response to growth factors. *Cell*, **70**: 389–399.

Ridley, A.J., Paterson, H.F., Johnston, C.L., Diekmann, D., and Hall, A. 1992. The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell*, **70**: 401–410.

Rosenblatt, J., Agnew, B.J., Abe, H., Bamburg, J.R., and Mitchison, T.J. 1997. *Xenopus* actin depolymerizing factor/cofilin (XAC) is responsible for the turnover of actin filaments in *Listeria monocytogenes* tails. *J. Cell Biol.*, **136**: 1323–1332.

Saito, T., Lamy, F., Roger, P.P., Lecocq, R., and Dumont, J.E. 1994. Characterization and identification as cofilin and destrin of the thyrotropin- and phorbol ester-regulated phosphoproteins in thyroid cell. *Exp. Cell Res.*, **212**: 49–61.

Samstag, Y., Eckerskorn, C., Wesselborg, S., Henning, S., Wallich, R., and Meuer, S.C. 1994. Costimulatory signals for human T-cell activation induce nuclear translocation of pp19/cofilin. *Proc. Natl. Acad. Sci. USA*, **91**: 4494–4498.

Sumi, T., Matsumoto, K., Takai, Y., and Nakamura, T. 1999. Cofilin phosphorylation and actin cytoskeletal dynamics regulated by Rho- and Cdc42-activated LIM-kinase 2. *J. Cell Biol.*, **147**: 1519–1532.

Suzuki, K., Yamaguchi, T., Tanaka, T., Kawanishi, T., Nishimaki-Mogami, T., Yamamoto, K., Tsuji, T., Irimura, T., Hayakawa, T., and Takahashi, A. 1995. Activation induces dephosphorylation of cofilin and its translocation to plasma membranes in neutrophil-like differentiated HL-60 cells. *J. Biol. Chem.*, **270**: 19551–19556.

Takuma, T., Ichida, T., Yokoyama, N., Tamura, S., and Obinata, T. 1996. Dephosphorylation of cofilin in parotid acinar cells. *J. Biochem.*, **120**: 35–41.

Theriot, J.A. 1997. Accelerating on a treadmill: ADF/cofilin promotes rapid actin filament turnover in the dynamic cytoskeleton. *J. Cell Biol.*, **136**: 1165–1168.

Toshima, J., Toshima, J. Y., Takeuchi, K., Mori, R. and Mizuno, K. 2001a. Cofilin phosphorylation and actin reorganization activities of testicular protein kinase 2 and its predominant expression in testicular Sertoli cells. *J. Biol. Chem.*, **276**: 31449–31458.

Toshima, J., Toshima, J. Y., Amano, T., Yang, N., Narumiya, S., and Mizuno, K. 2001b. Cofilin phosphorylation by protein kinase testicular protein kinase 1 and its role in integrin-mediated actin reorganization and focal adhesion formation. *Mol. Biol. Cell*, **12**: 1131–1145.

Yang, N., Higuchi, O., Ohashi, K., Nagata, K., Wada, A., Kangawa, K., Nishida, E., and Mizuno, K. 1998. Cofilin phosphorylation by LIM-kinase 1 and its role in Rac-mediated actin reorganization. *Nature*, **393**: 809–812.

Yonezawa, N., Nishida, E., and Sakai, H. 1985. pH control of actin polymerization by cofilin. *J. Biol. Chem.*, **260**: 14410–14412.

Zebda, N., Bernard, O., Bailly, M., Welti, S., Lawrence, D.S., and Condeelis, J.S. 2000. Phosphorylation of ADF/cofilin abolishes EGF-induced actin nucleation at the leading edge and subsequent lamellipod extension. *J. Cell Biol.*, **151**: 1119–1127.

(*Received for publication, September 26, 2002*
and in revised form, December 24, 2002)