Dephosphorylation of Cofilin Accompanies Heat Shock-induced Nuclear Accumulation of Cofilin*

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Cofilin is a widely distributed 21-kDa actin-modulating protein that forms intranuclear actin/cofilin rods in cultured fibroblastic cells exposed to heat shock or 10% dimethyl sulfoxide. In this study, cofilin was shown to be phosphorylated on a serine residue in cultured rat fibroblastic 3Y1 cells. Two-dimensional gel electrophoresis revealed that about 50% of the cofilin was phosphorylated in 3Y1 cells at 37 °C. Exposure of the cells to heat shock at 43 °C induced dephosphorylation of cofilin. The dephosphorylation of cofilin was detected about 30 min after the temperature shift and was completed within 120 min. Moreover, treatment of cells with 10% dimethyl sulfoxide also caused the dephosphorylation of cofilin. However, incubation of the cells with an isotonic NaCl solution, which induced cytoplasmic actin/cofilin rods, did not induce dephosphorylation of cofilin. Other cellular stress agents such as 6% ethanol or 50 μM sodium arsenite, which caused some heat shock responses in cells, did not induce dephosphorylation of cofilin. Thus, cofilin dephosphorylation was closely correlated with its nuclear accumulation. Incubation of the enucleated 3Y1 cells at 43 °C still induced dephosphorylation of cofilin, suggesting that the dephosphorylation occurred mostly in the cytoplasm in intact cells. It is likely that cofilin is dephosphorylated in the cytoplasm prior to its nuclear accumulation.

Cofilin was isolated as a protein that binds to G- and F-actin stoichiometrically and inhibits actin-myosin interactions in vitro (1). It is a monomeric globular protein in solution with an apparent molecular mass of 21 kDa (1-3). Cofilin is identified in a variety of mammalian tissues (4, 5). The mode of its interaction with actin has been characterized in detail. One of its unique properties is that cofilin is capable of reversibly controlling actin polymerization and depolymerization in a pH-sensitive manner (6).

Recently, cofilin has been shown to be a major component of intranuclear and cytoplasmic actin rods (actin/cofilin rods), which are formed in cultured fibroblastic cells exposed to a variety of extracellular stimuli (7). For example, heat shock or 10% dimethyl sulfoxide treatment induces intranuclear actin/cofilin rods, and incubation in salt buffers induces cytoplasmic rods. We do not know at present how localization of actin/cofilin rods is determined. In this regard, it is notable that cofilin molecules are almost completely translocated into nuclei in cells exposed to heat shock or 10% dimethyl sulfoxide (7). The recent study on cloning and characterization of cofilin cDNA has revealed that cofilin contains an amino acid sequence nearly homologous to the nuclear location signal sequence of SV40 large T antigen (8). We inferred that the nuclear location signal in cofilin could be masked somehow under normal conditions when cofilin exists uniformly in cytoplasm and nuclei; and upon heat shock or exposure to 10% dimethyl sulfoxide, the signal sequence in cofilin might become exposed and work as a nuclear location signal (8). It is likely, therefore, that the cofilin molecule itself is modified according to various extracellular stimuli.

As changes in the phosphorylation state of specific cellular proteins have been shown to play a key role in a variety of stimulus-response coupling systems (9, 10), we examined the possibility of changes in the phosphorylation state of cofilin in cultured fibroblastic cells. Here, we show that cofilin is phosphorylated under normal conditions and that dephosphorylation of cofilin is induced by stimuli that cause nuclear accumulation of cofilin.

EXPERIMENTAL PROCEDURES

Materials—Fetal calf serum was obtained from Flow Laboratories, Eagle’s minimum essential medium (MEM) from Nissui, [32P]orthophosphate from ICN Radiochemicals, [35S]methionine from Amersham Corp., α-chymotrypsin from Sigma, and sodium arsenite from Nacalai Tesque. Cofilin was purified from porcine brain cytosol as described previously (11). The affinity-purified anti-cofilin antibody has been described (5, 7).

Cells—3Y1 cells derived from Fisher rat embryo fibroblasts were cultured in Eagle’s minimum essential medium with 10% FCS at 37 °C.

Labeling of Cells with [32P]Orthophosphate or [35S]Methionine and Immunoprecipitation with Anti-cofilin Antibodies—Cells were grown in plastic tissue culture dishes (3.5-cm diameter) containing 2 ml of the medium supplemented with 10% FCS. At growing phase, the cells were labeled for 18 h in 1.0 ml of phosphate-free RPMI 1640 medium containing 30 mM Hepes/NaOH (pH 7.3), 10% dialyzed FCS, and carrier-free [32P]orthophosphate (0.2 mCi/ml). For labeling with [35S]methionine, the cells were incubated for 18 h in 1.0 ml of methionine-free RPMI 1640 medium containing 30 mM Hepes/NaOH (pH 7.3), 10% Eagle’s minimum essential medium, 10% dialyzed FCS, and [35S]methionine (50 μCi/ml). Cells were incubated at 43 °C for various time periods or at 37 °C with or without the indicated concentrations of test agents such as ethanol, sodium arsenite, dimethyl sulfoxide, and isotonic NaCl buffer (150 mM NaCl, 10 mM Mes/Tris (pH 6.5), 1 mM CaCl2, and 1 mM MgCl2). After the incubation medium was aspirated, the cell monolayers were quickly washed with saline and frozen with liquid nitrogen. The monolayers were thawed and solubilized at 0 °C with 0.15 ml of a solution containing 50 mM Tris/1 The abbreviations used are: MEM, Eagle’s minimum essential medium; FCS, fetal calf serum; NEF/HOE, nonequilibrium pH gradient electrophoresis; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; Mes, 4-morpholinoolanesulfonic acid; PBS, phosphate-buffered saline. 

1 The abbreviations used are: MEM, Eagle’s minimum essential medium; FCS, fetal calf serum; NEF/HOE, nonequilibrium pH gradient electrophoresis; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; Mes, 4-morpholinoolanesulfonic acid; PBS, phosphate-buffered saline.

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Heat Shock-induced Dephosphorylation of Cofilin

HCl (pH 7.4), 0.5 M NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM sodium orthovanadate, 30 mM sodium pyrophosphate, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and 2% aprotinin. The whole cell extract was prepared by scraping the cells from the dishes and sedimenting the insoluble materials by centrifugation at 15,000 x g for 20 min. To one portion of the cell extract (120 µl) was added 1.0 ml of distilled water, 20 µl of 1 mg/ml bovine serum albumin, and 10 µl of 2.0% sodium deoxycholate. After 15 min at room temperature, 0.34 ml of 24% trichloroacetic acid was added to the solution and centrifuged at 15,000 x g for 30 min. The precipitates were washed twice with ether, dried up, and the acid was added to the solution containing 50% (v/v) protein A-Sepharose, 50 mM Tris/HCl (pH 7.4), 0.5 M NaCl, 1.0% Triton X-100, 1 mM EDTA, 1 mM sodium orthovanadate, 30 mM sodium pyrophosphate, 50 µg NaF, 1 mM phenylmethylsulfonyl fluoride, and 2% aprotinin. The immobilized antibodies on protein A-Sepharose were washed with a solution containing 50 mM Tris/HCl (pH 7.4), 0.5 M NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM sodium orthovanadate, 30 mM sodium pyrophosphate, and 50 mM NaF. Immunoprecipitates were eluted from protein A-Sepharose by adding 60 µl of the SDS sample buffer and boiling for 2 min. The samples were centrifuged at 15,000 x g for 1 min. The supernatants were collected, and the eluted phosphoproteins were analyzed by SDS-PAGE. The gels were stained with Coomasie Brilliant Blue, dried, and exposed to Kodak X-Omat film for autoradiography for [32P]-labeled protein bands or for fluorography (EN3HANCE, Du Pont-New England Nuclear) for [35S]-labeled proteins. The relative [32P] incorporation into each band was determined by direct liquid scintillation counting of the excised band.

Two-dimensional Gel Electrophoresis (NEPHGE/SDS-PAGE)—The immunoprecipitation was carried out as described above except that the [32P]- or [35S]-labeled cell monolayers were solubilized with 0.15 ml of a solution containing 50 mM Tris/HCl (pH 7.4), 0.5 M NaCl, 1.0% Triton X-100, 1 mM EDTA, 1 mM sodium orthovanadate, 30 mM sodium pyrophosphate, 50 µg NaF, 1 mM phenylmethylsulfonyl fluoride, and 2% aprotinin. The immobilized antibodies on protein A-Sepharose were washed with a solution containing 50 mM Tris/HCl (pH 7.4), 0.5 M NaCl, 1.0% Triton X-100, 1 mM EDTA, 1 mM sodium orthovanadate, 30 mM sodium pyrophosphate, 50 µg NaF, and 50 mM NaF. Immunoprecipitates were eluted from protein A-Sepharose by adding 55 µg of urea and 100 µl of the lysis buffer containing 9.5 M urea, 2% LKB ampholytes (pH 3.5–10), and 5% (v/v) 2-mercaptoethanol. After incubation at room temperature for 1 h, the samples were centrifuged at 15,000 x g for 1 min. The supernatants were collected, and the eluted proteins were analyzed by NEPHGE/SDS-PAGE. The [32P]- or [35S]-labeled protein bands were analyzed as described above.

Preparation of Cytokasts and Karyoplasts from 3Y1 Cells—3Y1 cells were enucleated according to the method described by Wigler and Weinstein (12). Cells were grown in plastic tissue culture dishes (10-cm diameter) containing 10 ml of the medium supplemented with 10% FCS. After the culture had reached confluence, cell sheets were washed twice with 10 ml of phosphate-buffered saline (PBS) and incubated with 2 ml of 0.5 mM EDTA in PBS for 15 min at 37°C. Cells were scraped by a rubber policeman followed by vigorous pipetting and sedimented by centrifugation at 1000 rpm for 5 min. The pellet was suspended in 12.5% Ficoll in MEM containing 10 µg/ml cytochalasin B and 0.5% dimethyl sulfoxide to a final concentration of 0.8 x 10^6 cells/ml. Three ml of cell suspensions were then layered onto Ficoll density gradients (2.5 ml of 25%, 2.5 ml of 17%, 0.7 ml of 16%, 0.7 ml of 15%, and 2.5 ml of 12.5% Ficoll, all in MEM containing 10 µg/ml cytochalasin B and 0.5% dimethyl sulfoxide) and overlaid with 5 ml of MEM containing 10 µg/ml cytochalasin B and 0.5% dimethyl sulfoxide. Gradients were then centrifuged in a Hitachi SRP28/3A-261 swinging bucket rotor in a Hitachi 55P-7 ultracentrifuge for 60 min at 25,000 rpm at 31°C. After centrifugation, cytokasts (15–17% Ficoll region) and karyoplasts (17–25% Ficoll interface) were recovered separately with 10% FCS for further use. The gradient was centrifuged at 15,000 rpm for 5 min. The pellets were suspended in phosphate-free RPMI 1640 medium containing 30 mM HEPES/NaOH (pH 7.3) and 10% dialyzed FCS for heat shock experiment. For heat shock experiments, freshly prepared cytokasts were replicated in plastic tissue culture dishes (3.5-cm diameter) and labeled for 18 h in 1.0 ml of phosphate-free RPMI 1640 medium containing 30 mM HEPES/NaOH (pH 7.3), 10% dialyzed FCS, and carrier-free [32P]orthophosphate (0.2 mCi/ml). The [32P]-labeled cytokasts were incubated at 43°C for 120 min, and the immunoprecipitation with the anti-cofilin antibodies was carried out as described above.

Hematoxylin-eosin Staining of Cytokasts and Karyoplasts—Freshly prepared cytokasts or karyoplasts were suspended in MEM containing 10% FCS and plated in plastic culture dishes (3.5-cm diameter) with a 22×24-mm glass coverslip per dish and incubated at 37°C in a CO2 incubator for 18 h. Then the samples were washed twice with 2 ml of PBS and fixed with 2 ml of 10% formaldehyde in PBS for 10 min. The samples were washed three times in PBS and incubated with 2 ml of hematoxylin solution for 5 min. After extensive washes in water, samples were incubated with 2 ml of eosin solution for 5 min. Then the samples were washed briefly with water and incubated with 70% ethanol for 10 s and 100% ethanol for 10 s. The coverslips were mounted in 50% glycerol/PBS. All the procedures were carried out at room temperature. The samples were observed and photographed with an Olympus BH5U microscope. Images were recorded on Kodak Tri-X film.

Other Methods—Peptide mapping and phosphoamino acid analysis were carried out as described previously (13). SDS-PAGE was performed in 15% acrylamide by the method of Laemmli (14). NEPHGE was carried out with the ampholine (pH range, 3.5–10) by the method of O'Farrell et al. (15). Protein was determined with bovine serum albumin as standard by the method of Bradford (16).

RESULTS

Cofilin as a Phosphoprotein in 3Y1 Cells—Rat fibroblastic 3Y1 cells in the growing phase were labeled with [32P]orthophosphate at 37°C for 18 h. Immunoprecipitation with the anti-cofilin antibodies showed that cofilin exists as a phosphoprotein in intact 3Y1 cells (Fig. 1A). The phosphoamino acid analysis of cofilin revealed that serine was the only amino acid phosphorylated during the growing phase (Fig. 1B), suggesting that a serine protein kinase is responsible for the phosphorylation of cofilin in vivo.

The immunoprecipitated cofilin was further analyzed by [32P]orthophosphate-labeled 3Y1 cells with anti-cofilin antibodies. 3Y1 cells were labeled for 18 h in phosphate-free RPMI 1640 medium containing [32P]orthophosphate (0.2 mCi/ml). A, the cell extracts were immunoprecipitated with preimmune serum (lanes a, b), serum to cofilin (lane c), or the affinity-purified anti-cofilin antibody (lane d). Phosphoproteins were analyzed by SDS-PAGE followed by autoradiography. An arrow indicates the electrophoretic position of cofilin. B, phosphoamino acid analysis of cofilin. After SDS-PAGE and autoradiography, the band of phosphorylated cofilin (lane c of A) was cut out and incubated with trypsin (100 µg/ml) and chymotrypsin (50 µg/ml) at 37°C for 24 h. The phosphoamino acids of the eluted proteins were determined as described under "Experimental Procedures." P-Ser, phosphoserine; P-Thr, phosphothreonine; P-Tyr, phosphotyrosine.
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Two-dimensional gel electrophoresis (Fig. 2A). The phosphorlated cofilin appears as a major spot with a pI of around 5.5. A minor spot was also detected on the acidic side of the major spot (Fig. 2A, lane b). 3Y1 cells labeled with [35S]methionine were prepared and immunoprecipitated with the affinity-purified anti-cofilin antibody. The immunoprecipitate was combined with purified cofilin from porcine brain and resolved by NEPHGE/SDS-PAGE. The [35S]methionine-labeled cofilin appears as two major spots at pI values of 6.5 (spot 4) and 5.5 (spot 2) (Fig. 2A, lane c). Spot 2 was completely superimposed on the [32P]-labeled major cofilin spot (Fig. 2A, lane b). The molar ratio of spot 4 to spot 2 is about 1:1. Therefore, about 50% of cofilin is phosphorylated in 3Y1 cells at 37°C. The [35S]methionine-labeled spot 4 (pI 6.5) comigrates with the major portion of purified cofilin from porcine brain (Fig. 2A, lane a). Two minor [35S]methionine-labeled spots were observed at pI values of about 6.0 (spot 3) and 5.0 (spot 1). These spots were also detected in purified cofilin as very minor spots (Fig. 2A, lane a), and spot 1 corresponded to the minor spot of phosphorylated cofilin (Fig. 2A, lane b).

One-dimensional peptide mapping was carried out on [35S]methionine-labeled major cofilin spots (Fig. 2A, spots 2, 3, and 4 in lane c and spots 2 and 4 in lane d). The patterns of proteolytic fragments were identical among phosphorylated and unphosphorylated spots and moreover were the same as those of the purified cofilin from porcine brain (Fig. 2B). This verified that every spot is cofilin.

Heat Shock Induces Dephosphorylation of Cofilin—When cultured cells were exposed to elevated temperature, a variety of cellular responses is observed including the synthesis of heat shock proteins (17). Cofilin forms intranuclear actin/cofilin rods in cultured fibroblastic cells exposed to heat shock (7). To see the possible involvement of phosphorylation of cofilin in the heat shock response, 3Y1 cells labeled with [32P]orthophosphate during the growing phase were incubated at 43°C, and the immunoprecipitation was carried out with the affinity-purified anti-cofilin antibody. The decrease in phosphorylation of cofilin was detected about 30 min after the temperature shift and was almost completed within 120 min (Fig. 3, A and C). On the other hand, the radioactivity of cofilin labeled with [35S]methionine remained constant during this time interval (Fig. 3, B and C).

The resolution of [35S]methionine-labeled cofilin by NEPHGE/SDS-PAGE showed that after the temperature shift the phosphorylated cofilin (spot 2) disappeared, whereas the amount of unphosphorylated cofilin (spot 4) increased (Fig. 3D). Thus, the decline in phosphorylation of cofilin by heat shock is due to dephosphorylation rather than proteolytic degradation or a decrease in the biosynthesis of cofilin. Although the phosphorylation of cofilin increased slightly up to 30 min after the temperature shift, the [35S]methionine-labeled cofilin also increased to the same extent (130% of control) as that of phosphorylation of cofilin (Fig. 3C), suggesting that the increase in phosphorylation of cofilin reflects the increase in the synthesis of cofilin up to 30 min after the temperature shift.

Effects of Various Agents on the Phosphorylation of Cofilin—We next examined the effects of other cellular stress agents and incubation conditions on the state of cofilin phosphorylation in 3Y1 cells (Fig. 4A). Among the agents tested, dimethyl sulfoxide (10%, v/v) induced the dephosphorylation of cofilin. The time course of dephosphorylation induced by 10% dimethyl sulfoxide is shown in Fig. 4B. We have shown previously that cofilin forms intranuclear actin/cofilin rods in cultured fibroblastic cells exposed to heat shock or 10% dimethyl sulfoxide, whereas incubation in salt buffers induces cytoplasmic actin/cofilin rods (7). The incubation of 3Y1 cells in a buffered isonicotic NaCl solution (150 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, 10 mM Mes/Tris (pH 6.5), 90 min), which optimally induced cytoplasmic rods, did not affect the phosphorylation state of cofilin at all (Fig. 4A). Treatment of cells with ethanol (6%, v/v, 1 h) or sodium arsenite (50 μM, 1 h) has been shown to induce several heat shock proteins (17). Both of these agents had no effect on the phosphorylation of cofilin (Fig. 4A).

Cofilin Is Dephosphorylated in the Enucleated Cells by Heat Shock—To see whether dephosphorylation of cofilin by heat shock takes place in the cytoplasm prior to accumulation into the nucleus or in the nucleus as a consequence of transport, we prepared enucleated 3Y1 cells (cytoplasts) and examined

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**Fig. 2.** Two-dimensional gel electrophoresis (NEPHGE/SDS-PAGE) analysis of immunoprecipitated cofilin and onedimensional peptide mapping of cofilin from 3Y1 cells. 3Y1 cells prelabeled with [32P]orthophosphate or [35S]methionine were immunoprecipitated with the affinity-purified anti-cofilin antibody. The immunoprecipitates were combined with 3 μg of purified cofilin from porcine brain and subjected to two-dimensional gel electrophoresis (NEPHGE/SDS-PAGE) and autoradiography. A, Coomassie Brilliant Blue staining of the purified cofilin from porcine brain (lane a), an autoradiograph of [32P]-labeled immunoprecipitate (lane b), a fluorograph of [35S]methionine-labeled immunoprecipitate (lane c), and a fluorograph of [35S]methionine-labeled cell lysate (lane d). A portion of the gel is shown here with the acidic end at left, B, one-dimensional peptide maps of cofilin. Spots 2, 3, and 4 of [35S]methionine-labeled immunoprecipitates (lanes a, b, and c, respectively) and spots 2 and 4 of the [32P]methionine-labeled total lysate (lanes d and e, respectively) in A were located by fluorography, cut from the dried gels, and then subjected to limited proteolysis with chymotrypsin (2 μg/lane) and SDS-PAGE in 20% acrylamide followed by fluorography. The Coomassie Brilliant Blue staining pattern of the peptide map of purified cofilin from porcine brain is shown in lane f.
Heat Shock-induced Dephosphorylation of Cofilin

A number of "actin-binding proteins" have been purified and characterized, and several regulators of these proteins have been proposed (18-20). One of the possible regulatory mechanisms for functions of actin-binding proteins is protein modification by phosphorylation. A variety of actin-binding proteins has been shown to be phosphorylated in vivo (21-31). With the exception of vinculin (28), synapsin I (29), and myosin (30), however, little is known about their phosphorylation state in vitro, and hence the relation to stimulus-response coupling has not been explored. The present study has clearly shown that cofilin is phosphorylated in intact 3Y1 cells and that dephosphorylation of cofilin is accompanied by the transport of cofilin into the nucleus by heat shock or 10% dimethyl sulfoxide treatment.

The time course of dephosphorylation of cofilin by heat shock correlated well with that of the accumulation of cofilin in the nucleus, suggesting that the dephosphorylation of cofilin is involved in its nuclear accumulation (Fig. 3). Since the dephosphorylation of cofilin also occurred in the nucleated cells by heat shock (Fig. 5), cofilin may be dephosphorylated mainly in the cytoplasm in intact 3Y1 cells as a result of the inactivation of specific protein kinase(s) or the activation of specific protein phosphatase(s).

Among the cellular stress agents and incubation conditions tested, 10% dimethyl sulfoxide specifically induced the dephosphorylation of cofilin. The incubation of 3Y1 cells in a...
buffered isotonic NaCl solution had no effect on the phosphorylation state of cofilin. We have shown previously that heat shock or 10% dimethyl sulfoxide treatment, the nuclear transport signal in cofilin might become exposed and induce the accumulation of cofilin in the nucleus. It is possible that phosphorylation of cofilin masks the signal sequence and that dephosphorylation exposes it. Determination of the phosphorylated site(s) in cofilin and in vitro mutagenesis studies will be useful to evaluate the functional significance of the dephosphorylation of cofilin on its nuclear transport.

Since the major portion of cofilin purified from porcine brain comigrates with the dephosphorylated form of cofilin from 3Y1 cells on two-dimensional gel electrophoresis (Fig. 2A), brain cofilin is either not phosphorylated or is dephosphorylated during purification. Therefore, the previous in vitro studies on the mode of interaction of cofilin with actin have presumably been done mainly with the dephosphorylated form of cofilin. We are currently investigating effects of in vitro phosphorylation of purified brain cofilin on its actin-binding properties.

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