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Interaction of Cofilin with Triose-phosphate Isomerase Contributes Glycolytic Fuel for Na,K-ATPase via Rho-mediated Signaling Pathway*

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We reported previously that cofilin, an actin-binding protein, interacts with Na,K-ATPase and enhances its activity (Lee, K., Jung, J., Kim, M., and Guidotti, G. (2001) *Biochem. J.* 353, 377–385). To understand the nature of this interaction and the role of cofilin in the regulation of Na,K-ATPase activity, we searched for cofilin-binding proteins in the rat skeletal muscle cDNA library using the yeast two-hybrid system. Several cDNA clones were isolated, some of which coded for triose-phosphate isomerase, a glycolytic enzyme. The interaction of cofilin with triose-phosphate isomerase as well as Na,K-ATPase was confirmed by immunoprecipitation and confocal microscopy in HeLa cells. Cofilin was translocated to the plasma membrane along with triose-phosphate isomerase by the Rho activator lysophosphatidic acid but not by the p160 Rho-associated kinase inhibitor Y-27632, suggesting that the phosphorylated form of cofilin bound to TPI interacts with Na,K-ATPase. Ouabain-sensitive $^{86}Rb^{+}$ uptake showed that Na,K-ATPase activity was increased by the overexpression of cofilin and lysophosphatidic acid treatment, but not by the overexpression of mutant cofilin S3A and Y-27632 treatment. Pretreatment with the glycolytic inhibitor iodoacetic acid caused a remarkable reduction of Na,K-ATPase activity, whereas pretreatment with the oxidative inhibitor carbonyl cyanide *m*-chlorophenylhydrazone caused no detectable changes, suggesting that the phosphorylated cofilin is involved in feeding glycolytic fuel for Na,K-ATPase activity. These findings provide a novel molecular mechanism for the regulation of Na,K-ATPase activity and for the nature of the functional coupling of cellular energy transduction.

Cofilin exists ubiquitously in eukaryotes and is essential for cell viability (2). Cofilin binds to both G- and F-actins and severs and depolymerizes actin filaments. Cofilin is usually localized to the regions that require rapid actin assembly, such as neural growth cones, developing skeletal muscle, cell cortex, ruffling membranes, and cleavage furrow (3–8). In recent studies, it has been shown that human cofilin forms oligomers, which exhibit actin severing activity, and human cyclase-associated protein-1 is a key factor in the recycling of cofilin and actin (9, 10). It has been reported that in some cell types, the dephosphorylation of cofilin occurs in a calcium-dependent or -independent manner and accompanies nuclear uptake (11–18). On the other hand, dephosphorylation without nuclear uptake occurs in thyroid cells, and nuclear accumulation without dephosphorylation occurs in myotubes (19, 20). In all cases, Slingshot, a family of phosphatases, regulates actin reorganization through the dephosphorylation of cofilin (21).

One suggested mechanism for the regulation of cofilin activity is the reversible phosphorylation of a conserved serine 3 residue, the phosphorylation depending on LIM kinase-1 or -2, which are regulated, respectively, by the GTPases Rac and Rho/Cdc42 (22–25). Another possible mechanism is through binding of phosphatidylinositol 4,5-bisphosphate to the actin binding domain of cofilin (26, 27). However, elucidation of the mechanisms for phosphorylation of cofilin and its inactivation has not resulted in an understanding of the physiological role of the inactive form of cofilin.

We reported previously that cofilin interacts with Na,K-ATPase and enhances its activity (1). To understand the functional role of cofilin in the regulation of Na,K-ATPase activity, we screened a rat skeletal muscle cDNA library for cDNAs coding for novel proteins that interact with cofilin, using the yeast two-hybrid system. We found that cofilin interacts with triose-phosphate isomerase (TPI),[1] which catalyzes the interconversion of dihydroxyacetone phosphate and glyceraldehyde 3-phosphate, a process that completes the preparatory phase of glycolysis. Because it has been demonstrated that ATP from glycolysis is required for Na,K-ATPase activity and that glycolytic enzymes are associated with structural proteins and plasma membrane complexes (28–31), we hypothesized that the interaction of cofilin with TPI might serve to feed glycolytic ATP for Na,K-ATPase activity.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screen Assay—The yeast two-hybrid screen assay was performed as described previously (1). Clone 267 of rat cofilin which encodes residues 45–166 was fused to the LexA DNA binding domain and used as a bait to screen the cDNA library from rat skeletal muscle. The yeast strain EGY48/pSH18-34/pEG clone 267 carrying *LexAop-*

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[1] The abbreviations used are: TPI, triose-phosphate isomerase; BFP, blue fluorescence; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; FACS, fluorescence-activated cell sorter; GFP, green fluorescence; HA, hemagglutinin; IAA, iodoacetic acid; LPA, lysophosphatidic acid; PBS, phosphate-buffered saline; ROCK, p160 Rho-associated kinase.

Leu2, *LexAop-LacZ* reporters was transformed with the library, using the high efficiency method. Galactose Ura-His-Trp-Leu-dependent transformants were obtained. Plasmid DNA species were rescued and reintroduced individually into EGY48/pSH18-34, either alone or with the LexA fusion plasmids to eliminate false positives. β-Galactosidase activity was measured as described (1). Plasmids encoding potential cofilin-interacting proteins were identified by DNA sequencing with the primer 5′-TACCCTTATGATGTGCCA-3′, corresponding to the hemagglutinin (HA) tag sequences in the pJG4-5 library vector.

Coimmunoprecipitations—HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 units/ml streptomycin. An N-terminal HA-tagged cofilin construct in pcDNAneo (Invitrogen), C-terminal blue fluorescence protein (BFP)-tagged cofilin, or cofilin S3A (in which Ser-3 is replaced with Ala), and C-terminal BFP-tagged TPI were transiently transfected with the LipofectAMINE PLUS (Invitrogen). After transfection, the HeLa cells were treated with 10 μM lysophosphatidic acid (LPA) (Sigma) or 10 μM Y-27632 (Tocris) for 30 min after serum starvation and incubated with ice-cold lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 2 mM EGTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, NaF, Na_3VO_4, Complete™ protease inhibitor mixture tablets) for 30 min on ice and gently homogenized with Pyrex 7727-07. The cell lysates were centrifuged at 10,000 × *g* at 4 °C for 10 min. The total cell lysates (1 mg/ml) were incubated overnight with 12CA5 monoclonal antibody, rat anti-cofilin polyclonal antiserum, or rabbit affinity-purified anti-GFP polyclonal antibody (Clontech) at 4 °C. Protein A- or G-agarose (Roche Molecular Biochemicals) was then added and the mixture incubated for an additional 4 h at 4 °C. Protein A- or G-agarose-bound immune complexes so obtained were pelletized and washed twice with ice-cold phosphate-buffered saline (PBS). After washing, the immune complexes were eluted with 2× SDS sample buffer and separated on 12% SDS-PAGE. Blots were incubated with 12CA5 monoclonal antibody, rat anti-cofilin polyclonal antiserum, rabbit anti-TPI polyclonal antiserum, rabbit anti-GFP polyclonal antibody, rabbit anti-phosphoserine polyclonal antibody (Zymed Laboratories Inc.), or rabbit anti-Na,K-ATPase polyclonal antibody (Upstate Biotechnology).

Membrane Fractionation—HeLa cells cultured as described above were treated with 10 μM LPA or 10 μM Y-27632 for 30 min, incubated with ice-cold lysis buffer for 30 min on ice, and gently homogenized with Pyrex 7727-07. The homogenates were centrifuged at 2,500 × *g*, at 4 °C for 10 min, and the supernatant was centrifuged again at 40,000 × *g*, at 4 °C for 30 min. After centrifugation, the supernatant containing the cytosolic fraction and the pellet containing crude membrane fraction were separated, and the pellets were then resuspended in lysis buffer (1% Triton X-100). Each fraction was loaded onto a 12% SDS-polyacrylamide gel and identified using anti-cofilin polyclonal antiserum, anti-GFP polyclonal antibody, anti-TPI polyclonal antiserum, and anti-Na,K-ATPase polyclonal antibody.

Immunofluorescence Microscopy—HeLa cells were cultured on an 18-mm ϕ cover glass in the presence or absence of serum and treated with 10 μM LPA or 10 μM Y-27632 after serum starvation. The cells were fixed for 10 min in 3.7% formaldehyde in PBS containing Ca^{2+}/Mg^{2+} and incubated with rat anti-cofilin polyclonal antiserum, rabbit anti-TPI polyclonal antiserum, or mouse Na,K-ATPase monoclonal antibody (Upstate Biotechnology) in PBS containing 0.1% saponin for 1 h at room temperature on a tilting platform. Subsequently, the cells were incubated with anti-rat aminomethylcoumarin, anti-rabbit fluorescein isothiocyanate, or anti-mouse-rhodamine second antibody (Jackson Laboratories), mounted in anti-bleaching agent solution, and analyzed by laser confocal microscopy LSM 510 (Carl Zeiss).

Measurement of Na,K-ATPase Activity—500 μl of HeLa cells ($3.0 \times 10^5 \sim 1 \times 10^6$ cells/ml) was inoculated into 24 wells and transiently transfected with cofilin-BFP or cofilin-BFPS3A plasmids. After 24–36 h, the serum-starved cells were washed with Krebs-Ringer phosphate buffer (140 mM NaCl, 5 mM KCl, 10 mM Na_2HPO_4, 1 mM $MgSO_4$, 1.4 mM $CaCl_2$, 2.5 mM glucose, pH 7.4) and incubated at 37 °C for 15 min with or without 1 mM ouabain in 0.1% bovine serum albumin-containing Krebs-Ringer phosphate buffer. The cells were treated with 10 μM LPA, or 10 μM Y-27632, or 50 μM iodoacetic acid (IAA), or 0.1 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) for 30 min. The $^{86}Rb^+$ was used as a tracer to measure the uptake of K^+. After a 5∼10-min incubation with $^{86}Rb^+$ (2 μCi/ml), cellular $^{86}Rb^+$ uptake was stopped by the addition of ice-chilled Krebs-Ringer phosphate buffer, and the cells were washed twice and lysed by adding 50 mM NaOH. The cell lysates were then counted after mixing with scintillation mixture. The difference between $^{86}Rb^+$ uptake in the presence and absence of ouabain was taken as ouabain-sensitive $^{86}Rb^+$ uptake.

Determination of Intracellular Sodium—500 μl of HeLa cells ($3.0 \times 10^5 \sim 1 \times 10^6$ cells/ml) was inoculated into 24 wells and incubated at 37 °C for 18 h in Dulbecco's modified Eagle's medium. After serum starvation, the cells were treated with 10 μM LPA, 10 μM Y-27632, 50 μM IAA, or 0.1 μM CCCP for 30 min and trypsinized. The cells were then incubated at room temperature for 30 min with a buffer prepared as follows. A stock solution of 2 mM sodium green tetraacetate (Molecular Probes) in dimethyl sulfoxide was mixed with an equal volume of 20% (w/v) pluronic F-127 in dimethyl sulfoxide and then diluted with Ham's F-12 medium (in 2 mM sodium bicarbonate and 10 mM HEPES, pH 7.3, without phenol red and serum) to a final concentration of 8 μM sodium green tetraacetate and less than 0.1% detergent. The cells were then washed with PBS three times. Changes in the fluorescence intensity of sodium green tetraacetate were determined, employing flow cytometry (BD Biosciences). Excitation was performed with a 488 nm argon laser, and fluorescent emission was detected at 530 nm (FL-1). All flow cytometric analyses were accomplished using CellQuest software.

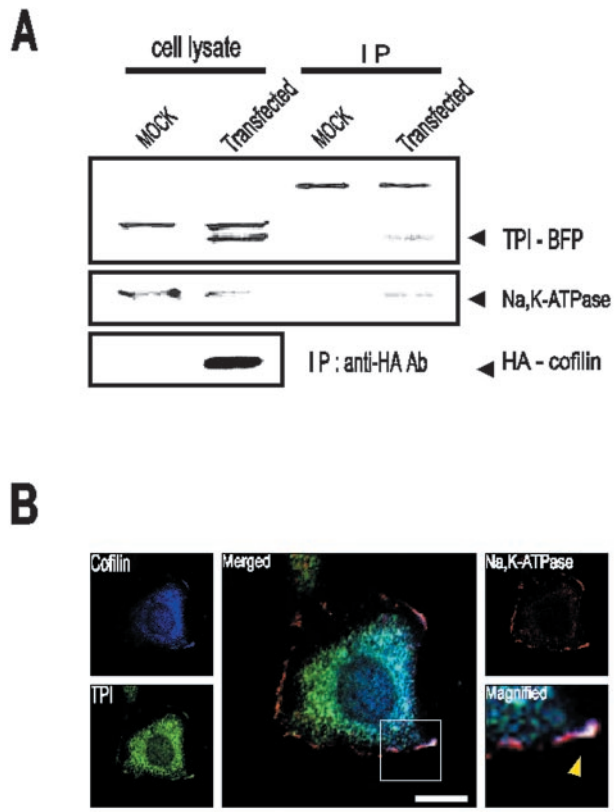

FIG. 1. **Cofilin interacts with TPI and Na,K-ATPase simultaneously.** *A*, HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 100 units/ml penicillin. The HA-tagged cofilin (N terminus) and BFP-tagged TPI (C terminus) were transiently transfected. Immunoprecipitates were prepared from 1 mg/ml HeLa cell extracts with 12CA5 monoclonal antibody. Total cell lysates (50 μg) and immune complexes (*IP*) were blotted sequentially with anti-GFP polyclonal antibody, anti-Na,K-ATPase polyclonal antibody, and anti-HA monoclonal antibody. *B*, HeLa cells were cultured in Dulbecco's modified Eagle's medium and fixed with 3.7% formaldehyde. After fixing, endogenous cofilin (*blue*), TPI (*green*), and Na,K-ATPase (*red*) were stained with rat anti-cofilin polyclonal antiserum, rabbit anti-TPI polyclonal antiserum, and mouse anti-Na,K-ATPase monoclonal antibody, respectively. The merged images of three molecules were magnified and showed colocalization at the plasma membrane (*white*). *Bar*, 20 μm.

RESULTS

Cofilin Interacts with TPI and Na,K-ATPase Simultaneously at the Plasma Membrane—To understand the functional role of cofilin in the regulation of Na,K-ATPase activity, we searched for cofilin-binding proteins using the yeast two-hybrid assay. Using this approach, we were able to isolate 25 clones coding for rat TPI, 3 clones coding for rat proteasome subunit RC5, and several clones coding unknown proteins. Because full-length cofilin clone activates reporter genes without interact-

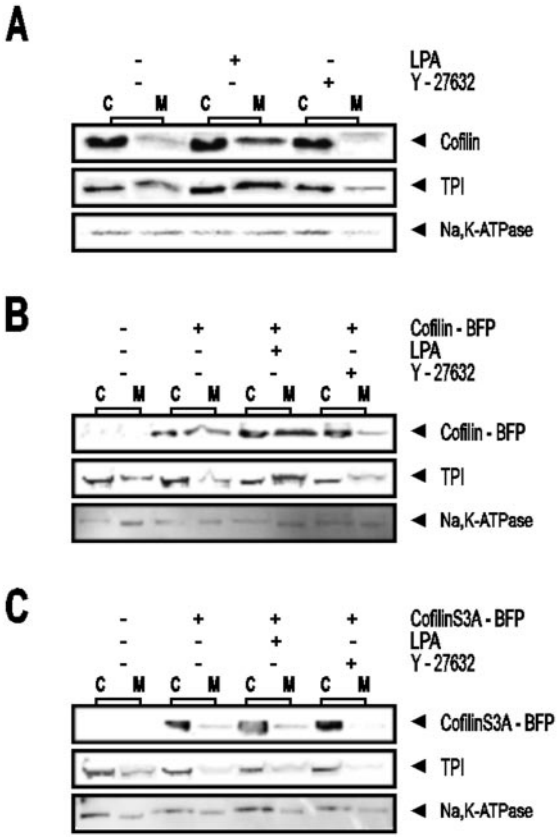

FIG. 2. **Cofilin is translocated from the cytosol to the plasma membrane together with TPI by LPA.** *A*, HeLa cells were incubated with 10 μM LPA or 10 μM Y-27632 for 30 min after serum starvation. Then the cells were separated into cytosol (*C*) and membrane (*M*) fractions. Each cell fraction was blotted sequentially with anti-cofilin polyclonal antiserum, anti-TPI polyclonal antiserum, and anti-Na,K-ATPase polyclonal antibody. *B*, HeLa cells were transiently transfected with cofilin-BFP. After transfection, the cells were incubated with 10 μM LPA or 10 μM Y-27632 for 30 min following serum starvation. Cell fractionation and blotting were performed as described above, replacing anti-cofilin polyclonal antiserum with anti-GFP polyclonal antibody. *C*, HeLa cells were transiently transfected with cofilin S3A-BFP, fractionated, and blotted with various antisera as described in *B*.

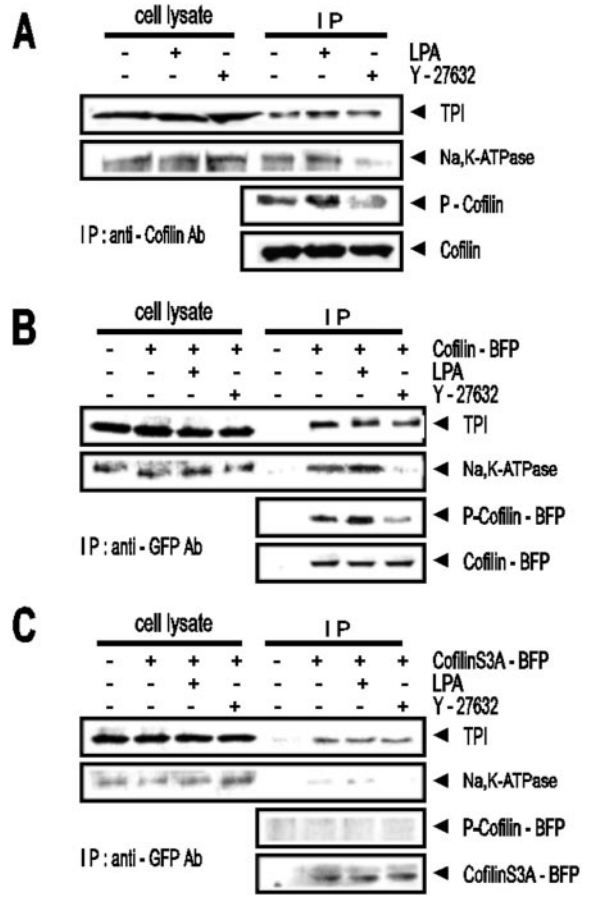

FIG. 3. **Phosphorylated cofilin interacts with TPI and Na,K-ATPase simultaneously through the activation of Rho by LPA.** *A*, HeLa cells were incubated with 10 μM LPA or 10 μM Y-27632 for 30 min after serum starvation. Immunoprecipitates were prepared from 1 mg/ml HeLa cell extracts with anti-cofilin polyclonal antiserum. Total cell lysates (50 μg) and immune complexes (*IP*) were blotted sequentially with anti-TPI polyclonal antiserum, anti-Na,K-ATPase polyclonal antibody, anti-phosphoserine polyclonal antibody, and anti-cofilin polyclonal antiserum. *B*, HeLa cells were transiently transfected with cofilin-BFP. Immunoprecipitation and blotting were performed as described above, replacing anti-cofilin polyclonal antiserum with anti-GFP polyclonal antibody. *C*, HeLa cells were transiently transfected with cofilin S3A-BFP. Immunoprecipitation and blotting were then performed as described in *B*.

ing protein, we used cofilin clone 267, which was short of 44 residues from the N terminus, as a bait in the yeast two-hybrid screening. However, we used rat full-length cofilin cDNA for further experiments.

To confirm that cofilin interacts with TPI *in vivo*, we performed coimmunoprecipitation experiments using appropriately tagged antisera (Fig. 1*A*). Expression plasmids of both HA-tagged rat cofilin (HA-cofilin) and blue fluorescence-tagged rat TPI (TPI-BFP) were cotransfected transiently into HeLa cells. Immunoprecipitates were prepared from HeLa extracts with anti-HA monoclonal antibody and immunoblotted with anti-GFP polyclonal antibody. HA-cofilin coprecipitated 54.7 kDa of the TPI-BFP only from transfected cells (Fig. 1*A*). To determine whether cofilin interacts simultaneously with both TPI and Na,K-ATPase, we blotted the coprecipitated complex sequentially with rabbit Na,K-ATPase polyclonal antibody. 100 kDa of endogenous Na,K-ATPase was also detected only from transfected cells, suggesting that cofilin is complexed with TPI as well as Na,K-ATPase at the same time (Fig. 1*A*). This finding is in accord with our previous study (1).

To confirm the above coimmunoprecipitation results, we examined, using confocal immunofluorescence microscopy, whether endogenous cofilin is complexed simultaneously with TPI and Na,K-ATPase in HeLa cells (Fig. 1*B*). When HeLa cells in serum-containing media were labeled with either rat anti-cofilin polyclonal antiserum (*blue*) or rabbit anti-TPI polyclonal antiserum (*green*), we observed that endogenous cofilin was found distributed with polarized pattern in about half of the cell, whereas endogenous TPI was found distributed equally throughout the cell (Fig. 1*B*). On staining with monoclonal anti-Na,K-ATPase antibody (*red*), endogenous Na,K-ATPase was also found throughout the cell but distributed mainly at the plasma membrane (Fig. 1*B*). As can be seen in Fig. 1*B*, *middle panel*, cofilin was colocalized with TPI as shown by merged *azure* color in the cytoplasm. Cofilin was also colocalized with Na,K-ATPase as well as TPI at the plasma membrane as shown by merged *white* color in the membrane region (Fig. 1*B*, *right bottom panel*, *magnified*). Colocalization of cofilin, TPI, and Na,K-ATPase at the plasma membrane region was observed only in serum-containing media but not in serum-free media. This may be because the serum phosphorylates cofilin via a Rho-ROCK-LIM kinase-mediated signaling pathway (23, 32–34).

The Cofilin-TPI Complex Is Translocated from the Cytosol to the Plasma Membrane upon Activation of the Rho Signaling Pathway—Phosphorylation of cofilin inhibits its actin depolymerizing activity. Cofilin is phosphorylated by LPA, which acti-

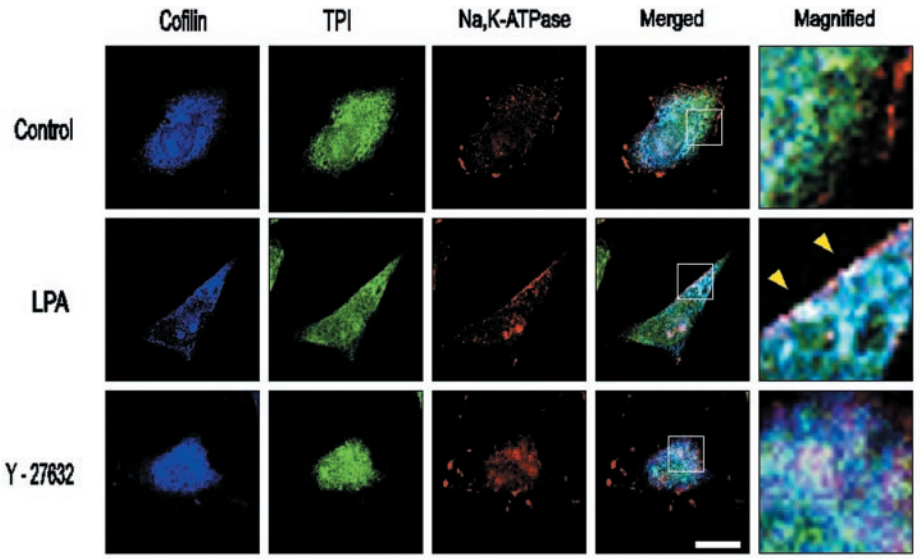

FIG. 4. **Cofilin associates with TPI and Na,K-ATPase simultaneously at the plasma membrane region via Rho activation by LPA.** HeLa cells were incubated with 10 μM LPA or 10 μM Y-27632 for 30 min after serum starvation and fixed with 3.7% formaldehyde. After fixing, endogenous cofilin (*blue*), TPI (*green*), and Na,K-ATPase (*red*) were stained with rat anti-cofilin polyclonal antiserum, rabbit anti-TPI polyclonal antiserum, and mouse anti-Na,K-ATPase monoclonal antibody, respectively. The merged images of three molecules were magnified and showed colocalization at the plasma membrane (*white*). *Bar*, 20 μm.

vates Rho GTPase. This phosphorylation is inhibited by Y-27632, a specific inhibitor of ROCK which is a downstream effector of Rho and phosphorylates LIM kinase (23).

We therefore investigated whether the translocation of cofilin and TPI to the plasma membrane is under the control of a Rho-mediated signaling pathway (Fig. 2). After treatment of HeLa cells with LPA following serum starvation, translocation of both endogenous cofilin and TPI to the plasma membrane was increased remarkably (Fig. 2*A*). On the other hand, inhibition of ROCK by Y-27632 (23) decreased the translocation (Fig. 2*A*). Endogenous Na,K-ATPase in membrane fraction was not affected by treatment with either LPA or Y-27632 (Fig. 2*A*). Based on these data, it appears that cofilin bound to TPI may translocate to the plasma membrane to form local complexes with Na,K-ATPase and that the translocation process is under the control of a Rho-mediated signaling pathway.

Cofilin exists in dephosphorylated and phosphorylated (on the serine 3 residue) forms (22–25). To investigate the role of phosphorylation of cofilin in the translocation phenomenon, we constructed BFP-tagged wild type cofilin (cofilin-BFP) and a mutant cofilin, in which serine 3 was replaced with alanine (cofilin S3A-BFP) and used them for transient transfection of HeLa cells. Translocation of the overexpressed cofilin-BFP and endogenous TPI to the plasma membrane was increased upon treatment with LPA and decreased upon treatment with Y-27632, whereas endogenous Na,K-ATPase was not affected (Fig. 2*B*). However, neither overexpressed cofilin S3A-BFP nor endogenous TPI was changed in the plasma membrane fraction, even after treatment with either LPA or Y27632 (Fig. 2*C*), indicating that phosphorylated cofilin complexed with TPI translocates to the plasma membrane.

To obtain more evidence that phosphorylated cofilin complexed with TPI translocates to the membrane and associates with Na,K-ATPase via a Rho-mediated signaling pathway, we introduced expression plasmids of cofilin-BFP and cofilin S3A-BFP into HeLa cells and performed coimmunoprecipitation experiments (Fig. 3). Immunoprecipitates were prepared from LPA- and Y-27632-treated HeLa cell lysates after serum starvation and coprecipitated with either cofilin polyclonal antiserum or GFP polyclonal antibody. We blotted the coprecipitated complexes sequentially with the following antibodies: anti-Na,K-ATPase polyclonal antibody, anti-TPI polyclonal antiserum, anti-cofilin polyclonal antibody, and anti-GFP polyclonal antibody. We also examined the phosphorylated forms of both endogenous cofilin and overexpressed cofilin by blotting the coprecipitated complexes with anti-phosphoserine polyclonal antibody. As shown in Fig. 3, the phosphorylated forms of endogenous cofilin (Fig. 3*A*) and cofilin-BFP (Fig. 3*B*) were increased by LPA but decreased by Y-27632. Endogenous Na,K-ATPase immunoprecipitated with anti-cofilin antibody or anti-GFP antibody was also increased by LPA but decreased by Y-27632 (Fig. 3, *A* and *B*). We could not detect the phosphorylated cofilin and Na,K-ATPase in immunoprecipitates from cofilin S3A-BFP cell extracts by the treatment of either LPA or Y-27632 (Fig. 3*C*), which indicates that only phosphorylated cofilin is able to associate with Na,K-ATPase. However, there was a consistent amount of TPI in all of the immunoprecipitates, and this was not affected by treatment with either LPA or Y-27632. Taken together, these results suggest that the interaction of cofilin with TPI is not dependent on phosphorylation of cofilin, which in turn suggests that both phosphorylated and dephosphorylated forms of cofilin can bind to TPI, but only the phosphorylated cofilin complexed with TPI is able to associate with Na,K-ATPase.

Next, we performed confocal immunofluorescence microscopy to chase the movement of cofilin and TPI upon the treatment of LPA and Y27632 in HeLa cells (Fig. 4). After treatment with LPA and Y-27632 following serum starvation, HeLa cells were labeled with cofilin polyclonal antiserum, TPI polyclonal antiserum, or Na,K-ATPase monoclonal antibody. Endogenous Na,K-ATPase was detected at the plasma membrane as well as in the cytoplasm, whereas endogenous cofilin and TPI were distributed throughout the cell excluding the plasma membrane in serum-starved HeLa cells (Fig. 4). They did not colocalize with Na,K-ATPase at the plasma membrane in serum-starved HeLa cells (Fig. 4, merged and magnified panels in *Control*). However, in LPA-treated HeLa cells, endogenous cofilin and TPI became associated with Na,K-ATPase at the plasma membrane as shown by merged *white* colors (Fig. 4, merged and magnified panels in *LPA*). In contrast to treatment with LPA, treatment with Y-27632 caused the accumulation of these proteins mainly in the nucleus, as shown by merged *white* colors (Fig. 4, merged and magnified panels in *Y-27632*). These results confirm that the association of cofilin with Na,K-ATPase at the plasma membrane requires phosphorylation of cofilin via a Rho-mediated signaling pathway.

Phosphorylated Cofilin Is Involved in Feeding Glycolytic Fuel to Na,K-ATPase—Based on earlier reports that ATP from glycolysis is required to regulate Na,K-ATPase (35–37) and that glycolytic enzymes are associated with structural proteins such

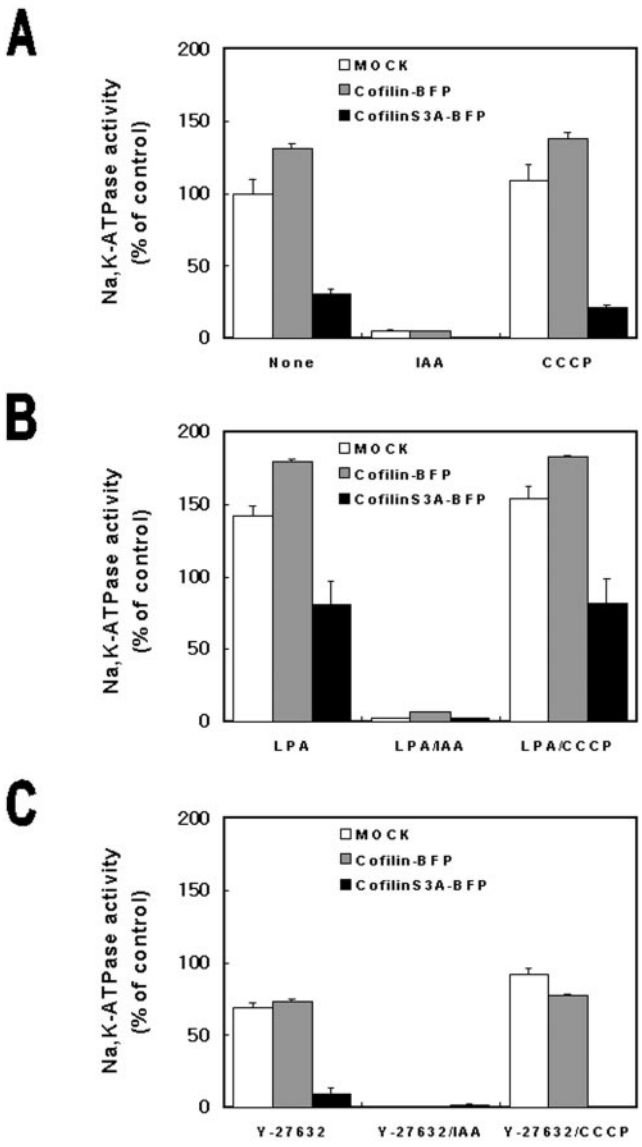

FIG. 5. **Activation of Rho by LPA increases Na,K-ATPase activity, and inhibition of glycolysis by IAA decreases Na,K-ATPase activity.** *A*, HeLa cells were transiently transfected with cofilin-BFP or cofilin S3A. After serum starvation, the cells were incubated with 1 mM ouabain for 15 min and treated with 50 μM IAA or 0.1 μM CCCP for 30 min. $^{86}Rb^+$ uptake was accomplished by adding of 2 μCi/ml $^{86}Rb^+$ and counted in triplicate. *B*, HeLa cells were treated as described above except for treatment with 10 μM LPA before 50 μM IAA or 0.1 μM CCCP treatment. *C*, HeLa cells were treated as described above except for treatment with 10 μM Y-27632 before 50 μM IAA or 0.1 μM CCCP treatment. The *bar graph* shows the results of three independent experiments ± S.E.

as actin, microtubules, and plasma membrane (28–31), we speculated that ATP produced by a glycolytic enzyme cluster including TPI may be channeled to the Na,K-ATPase and that the process is mediated via phosphorylated cofilin. To verify this, cofilin-BFP and cofilin S3A-BFP were transiently expressed in HeLa cells, and Na,K-ATPase activity was measured using ouabain-sensitive $^{86}Rb^+$ uptake assay (Fig. 5). Consistent with our previous report (1), Na,K-ATPase activity of cofilin-BFP-transfected cells was about 31% higher than that of mock-transfected cells (Fig. 5*A*). In contrast, the Na,K-ATPase activity of cofilin S3A-BFP-transfected cells was decreased (Fig. 5*A*). To determine whether Na,K-ATPase activity is dependent on glycolysis or oxidative phosphorylation, we employed IAA, a relatively specific irreversible inhibitor of glyceraldehyde 3-phosphate dehydrogenase, and CCCP, a protonophore oxidative phosphorylation inhibitor (Fig. 5*A*). In mock, cofilin-BFP, and cofilin S3A-BFP-transfected cells, Na,K-ATPase activity was decreased significantly (to 0–5%) in the presence of IAA (Fig. 5*A*). However, in the presence of CCCP, mock and cofilin-BFP-transfected cells exhibited slightly increased Na,K-ATPase activity, and cofilin S3A-BFP-transfected cells exhibited slightly lower activity (Fig. 5*A*). As suggested by Okamoto *et al.* (37), the slight increase in Na,K-ATPase activity in mock and cofilin-BFP-transfected cells produced by CCCP may be caused by a compensatory increase in glycolysis. These data suggest that glycolysis, but not oxidative phosphorylation, is effective in providing ATP to Na,K-ATPase, through a process that requires the phosphorylated cofilin.

The studies described in Fig. 5*A* were also performed after treatment with LPA and Y-27632. As expected, after LPA treatment, Na,K-ATPase activity of mock-, cofilin-BFP-, and cofilin S3A-BFP-transfected cells increased to 142, 180, and 80%, respectively, (Fig. 5*B*). However, after LPA treatment, Na,K-ATPase activity in all three transfected cells remarkably decreased in the presence of IAA but was almost unaffected in the presence of CCCP (Fig. 5*B*). Y-27632 treatment caused, in all three transfected cells, decreases in Na,K-ATPase activity, which was decreased further by IAA and unaffected by CCCP (Fig. 5*C*). These results indicate again that glycolytic ATP is required for Na,K-ATPase activity and that the glycolytic fuel is supplied by the phosphorylated cofilin via a Rho-mediated signaling pathway.

Cofilin Changes the Intracellular Sodium Concentration via a Rho-mediated Signaling Pathway—Because Na,K-ATPase is reported to be involved in the extrusion of intracellular sodium, we wondered whether phosphorylation of cofilin, which regulates this enzyme, also affects the intracellular sodium concentration $[Na^+]_i$. To check this out, we performed flow cytometry experiments (FACS) using the cell-permeable sodium-binding fluorescent indicator, sodium green, and measured the $[Na^+]_i$ (Fig. 6). FACS of HeLa cells in the presence of LPA showed a remarkable decrease in the intracellular sodium of a population of cells, probably through the increased Na,K-ATPase activity (Fig. 6*A*). On the other hand, FACS showed little change in the intracellular sodium concentration in the presence of Y-27632 (Fig. 6*A*). These results suggest that sodium transport to outside of the cells may be facilitated by the activation of Rho signaling, which presumably phosphorylates cofilin and activates sodium activity.

We also tested whether $[Na^+]_i$ is affected by a glycolytic pathway or oxidative pathway, using specific inhibitors of IAA and CCCP (Fig. 6*B*). As expected, in the presence of IAA, the population of cells that have an increased $[Na^+]_i$ was increased, whereas in the presence of CCCP, there was no difference from the control (Fig. 6*B*). These results suggest that a glycolytic pathway, rather than an oxidative pathway, provides the energy demands for Na,K-ATPase activity.

To clarify the relationship between glycolytic energy fuel and cofilin, in the context of its regulation by a Rho-mediated signaling pathway, we treated HeLa cells with LPA and Y-27632 as well as IAA and CCCP, as described in ouabain-sensitive $^{86}Rb^+$ uptake experiments (Fig. 6*C*). As expected, in the presence of IAA, the population of cells that had an increase in $[Na^+]_i$ was increased by pretreatment with either LPA or Y-27632 (Fig. 6*C*). However, both in the absence and presence of CCCP, $[Na^+]_i$ decreased after pretreatment with LPA but increased after pretreatment of Y-27632 (Fig. 6*C*). Taken together, these results suggest that Rho and ROCK play an important role in the regulation of $[Na^+]_i$ probably through the phosphorylation of cofilin.

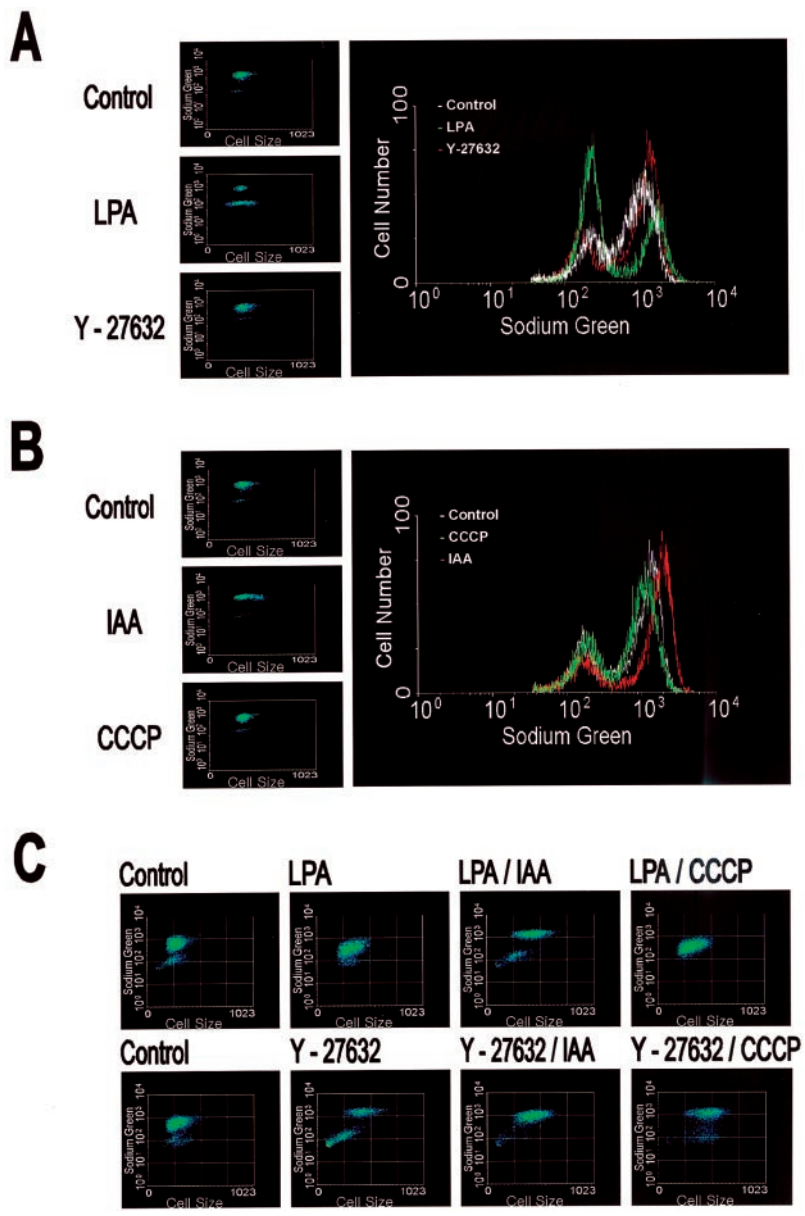

FIG. 6. **Activation of Rho by LPA decreases the intracellular sodium concentration, and inhibition of glycolysis by IAA increases the intracellular sodium concentration.** *A*, serum-starved HeLa cells were treated with 10 μM LPA or 10 μM Y-27632 for 30 min and trypsinized. Then the cells were loaded with sodium green tetraacetate (final concentration, 8 μM). After incubation for 30 min, cells were washed with PBS three times and analyzed on a flow cytometer. The data were transformed into density plots and histograms using CellQuest software. *B*, serum-starved HeLa cells were treated with 50 μM IAA or 0.1 μM CCCP for 30 min and analyzed as described above. *C*, serum-starved HeLa cells were treated with LPA or Y-27632 before treatment with IAA or CCCP and analyzed as described above. These data represent the averages of at least three independent experiments.

DISCUSSION

This study demonstrated the following: (*a*) cofilin is phosphorylated by LPA through the Rho signaling cascade, Rho/ROCK/LIM kinase; (*b*) both phosphorylated and dephosphorylated cofilin S interacts with TPI; (*c*) phosphorylated cofilin complexed with TPI is translocated to the plasma membrane and becomes associated with Na,K-ATPase; and (*d*) the TPI-glycolytic enzyme complex feeds glycolytic fuel to Na,K-ATPase. In Fig. 7, we propose a pathway for Na,K-ATPase regulation by the cofilin-TPI complex in HeLa cells.

The two major sources of ATP in the cells are aerobic glycolysis and mitochondrial oxidative phosphorylation. It is believed that oxidative ATP supports the mechanical activity of the cell, whereas glycolytic ATP fuels energy for membrane functions (38). Indeed, many membrane proteins such as the ATP-sensitive K^+ channel, Ca^{2+} pump, Na,K-ATPase, and L-type Ca^{2+} channel, are known to use glycolytic ATP preferentially rather than oxidative ATP (39). Recently, direct binding of the glycolytic enzyme aldolase to a proton pump was demonstrated (40). In the present work, we demonstrated that a glycolytic enzyme, TPI, associates indirectly with the large cytoplasmic domain of the sodium pump through phosphorylated cofilin. Our previous

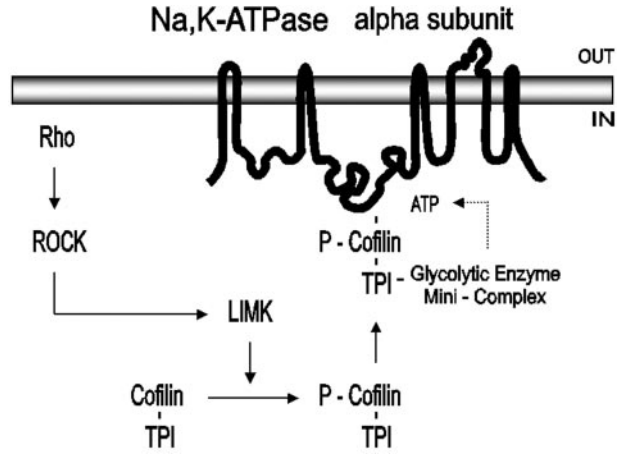

FIG. 7. **Proposed mechanism for Na,K-ATPase regulation.** Cofilin is phosphorylated by LPA through the Rho signaling cascade, Rho/ROCK/LIM kinase, and both dephosphorylated cofilin and phosphorylated cofilin S interact with TPI. The phosphorylated cofilin-TPI complex is translocated to the plasma membrane and becomes associated with Na,K-ATPase. At the same time, TPI forms a glycolytic enzyme minicomplex feeding glycolytic fuels to Na,K-ATPase.

demonstration, employing a yeast two-hybrid assay, that the large cytoplasmic domains of glucose transporter 1, glucose transporter 4, cystic fibrosis transmembrane conductance regulator, and plasma membrane Ca-ATPase did not interact with cofilin (41) suggests that the interaction with cofilin may not be a general phenomenon for feeding glycolytic fuels to the transmembrane proteins. Thus, it appears that glycolytic energy for the membrane function is provided via two different mechanisms, namely, one involving direct interaction of a plasma membrane protein such as a proton pump with glycolytic enzymes, and the other involving indirect interaction of a plasma membrane protein such as a sodium pump with glycolytic enzyme via cofilin mediator.

It has been shown that glycolytic enzymes such as aldolase and glyceraldehyde-3-phosphate dehydrogenase interact directly with structural proteins such as actin and microtubules, whereas TPI binds indirectly (30, 42, 43). Previously, we identified, in yeast two-hybrid screening studies, both TPI and cofilin as binding partners of the α subunit of Na,K-ATPase. However, when TPI was reintroduced into transformants containing Na,K-ATPase, the interaction was not reproducible, suggesting that TPI does not bind directly to Na,K-ATPase (data not shown). The present study, demonstrating that cofilin interacts with TPI and that this interaction is not dependent on the phosphorylation of cofilin, suggests that both phosphorylated and dephosphorylated cofilin can bind to TPI. Thus, it seems that TPI interacts indirectly, through phosphorylated cofilin with Na,K-ATPase, and through dephosphorylated cofilin, with structural proteins.

Dephosphorylated cofilin is essential to actin dynamics. It becomes inactive through phosphorylation at serine 3 residue, in a process regulated by LIM kinase (22–25). Phosphorylation of LIM kinase depends on several small GTPase signaling cascades, known as Rho/ROCK and Rac or Cdc42/Pak sequences (22–25, 44). Although the signaling pathway for cofilin phosphorylation is well defined, the function of the inactive form of the phosphorylated cofilin has not been clear until now. The present study is the first demonstration that the phosphorylated cofilin functions as a glycolytic ATP feeder for the ATP-hydrolyzing sodium pump.

Rho is an important signaling element that regulates the cytoskeleton and interacts with other small GTPases, as well as other signaling elements, to regulate various cell functions (45, 46). In the present study, we observed that Rho is involved in the regulation of intracellular sodium, probably through Na,K-ATPase activation, which requires the phosphorylated cofilin and TPI complex. Rho also stimulates Na^+/H^+ exchanger activity through ROCK, and activation of the Na^+/H^+ exchanger requires the glycolytic ATP (47–49). Because both Na,K-ATPase and the Na^+/H^+ exchanger are involved in the regulation of sodium homeostasis, Rho also may play an important role in the maintenance of intracellular sodium.

In summary, we have described studies indicating how glycolytic enzymes are associated with the plasma membrane and what role they play in supporting ion pump activity in relative isolation from other metabolic enzyme cascades. The advantage of this system would be that the noted close juxtaposition and the functional coupling of energy producing system and energy-consuming enzymes may allow a fast and efficient adaptation of metabolic flow to fluctuating cellular conditions.

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REFERENCES

1. Lee, K., Jung, J., Kim, M., and Guidotti, G. (2001) *Biochem. J.* **353,** 377–385
2. Bamburg, J. R. (1999) *Annu. Rev. Cell Dev. Biol.* **15,** 185–230
3. Bamburg, J. R., and Bray, D. (1987) *J. Cell Biol.* **105,** 2817–2825
4. Yonezawa, N., Nishida, E., Koyasu, S., Maekawa, S., and Ohta, Y. (1987) *Cell Struct. Funct.* **2,** 443–452
5. Abe, H., Ohshima, S., and Obinata, T. (1989) *J. Biochem.* (*Tokyo*) **106,** 696–702
6. Abe, H., Obinata, T., Minamide, L. S., and Bamburg, J. R. (1996) *J. Cell Biol.* **132,** 871–885
7. Moons, A. L., Janmey, P. A., Louie, K. A., and Drubin, D. G. (1993) *J. Cell Biol.* **120,** 421–435
8. Nagaoka, R., Abe, H., Kusano, K., and Obinata, T. (1995) *Cell Motil. Cytoskel.* **30,** 1–7
9. Moriyama, K., and Yahara, I. (2002) *J. Cell Sci.* **115,** 1591–1601
10. Pfannstiel, J., Cyrklaff, M., Habemann, A., Stoeva, S., Griffiths, G., Shoeman, R., and Faulstich, H. (2001) *J. Biol. Chem.* **276,** 49476–49484
11. Baorto, D. M., Mellado, W., and Shelanski, M. L. (1992) *J. Cell Biol.* **117,** 357–367
12. Heyworth, P. G., Robinson, J. M., Ding, J., Ellis, B. A., and Badwey, J. A. (1997) *Histochem. Cell Biol.* **108,** 221–233
13. Kanamori, T., Hayakawa, T., Suzuki, M., and Titani, K. (1995) *J. Biol. Chem.* **270,** 8061–8067
14. Meberg, P. J., Ono, S., Minamide, L. S., Takahashi, M., and Bamburg, J. R. (1998) *Cell Motil. Cytoskel.* **39,** 172–190
15. Ohta, Y., Nishida, E., Sakai, H., and Miyamoto, E. (1989) *J. Biol. Chem.* **264,** 16143–16148
16. Okada, K., Takano-Ohmuro, H., Obinata, T., and Abe, H. (1996) *Exp. Cell Res.* **22,** 116–122
17. Samstag, Y., Eckerskorn, C., Wesselborg, S., Henning, S., Wallich, R., and Meuer, S. C. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 4494–4498
18. Takuma, T., Ihida, T., Yokoyma, N., Tamura, S., and Obinata, T. (1996) *J. Biochem.* (*Tokyo*) **120,** 35–41
19. Abe, H., Nagaoka, R., and Obinata, T. (1993) *Exp. Cell Res.* **206,** 1–10
20. Saito, T., Lamy, F., Roger, P. P., Lecocq, R., and Dumont, J. E. (1994) *Exp. Cell Res.* **212,** 49–61
21. Niwa, R., Ohashi, K. N., Takeichi, M., Mizuno, K., and Uemura, T. (2002) *Cell* **108,** 233–246
22. Arber, S., Barbayannis, F. A., Hanser, H., Schneider, C., Stanyon, C. A., Bernard, O., and Caroni, P. (1998) *Nature* **393,** 808–809
23. Maekawa, M., Ishizaki, T., Boku, S., Watanabe, N., Fujita, A., Iwamatsu, A., Obinata, T., Ohashi, K., Mizuno, K., and Narumiya, S. (1999) *Science* **285,** 895–898
24. Sumi, T., Matsumoto, K., Takai, Y., and Nakamura, T. (1999) *J. Cell Biol.* **147,** 1519–1532
25. Yangs, N., Higuchi, O., Ohashi, K., Nagata, K., Wada, A., Kangawa, K., Nishida, E., and Mizuno, K. (1998) *Nature* **393,** 809–812
26. Yonezawa, N., Homma, Y., Yahara, I., Sakai, H., and Nishida, E. (1991) *J. Biol. Chem.* **266,** 17218–17221
27. Kusano, K., Abe, H., and Obinata, T. (1999) *Mol. Cell Biochem.* **190,** 133–141
28. Balaban, R. S., and Bader, J. P. (1984) *Biochim. Biophys. Acta* **804,** 419–426
29. Cantiello, H. F. (1995) *Am. J. Physiol.* **269,** F637–F643
30. Clarke, F. M., and Masters, C. J. (1975) *Biochim. Biophys. Acta* **381,** 37–46
31. Nelson, J. W., and Hammerton, R. W. (1989) *J. Cell Biol.* **108,** 893–902
32. Geneste, O. J., Copeland, W., and Treisman, R. (2002) *J. Cell Biol.* **157,** 831–838
33. Nobes, C. D., and Hall, A. (1995) *Cell* **81,** 53–62
34. Ridley, A. J., and Hall, A. (1992) *Cell* **70,** 389–399
35. Paul, R. J. (1983) *Am. J. Physiol.* **244,** C399–C409
36. Dizon, J., Burkhoff, D., Tauskela, J., Whang, J., Cannon, P., and Katz, J. (1998) *Am. J. Physiol.* **274,** H1082–H1089
37. Okamoto, K., Wang, W., Rounds, J., Chambers, E. A., and Jacobs, D. O. (2001) *Am. J. Physiol.* **281,** E479–E488
38. Weiss, J., and Hiltbrand, B. (1985) *J. Clin. Invest.* **75,** 436–447
39. Hardin, C. D., Zhang, C., Kranias, E. G., Steenaart, N. A., Raeymaekers, L., and Paul, R. J. (1993) *Am. J. Physiol.* **265,** H1326–H1333
40. Lu, M., Holliday, L. S., Zhang, L., Dunn, W. A., Jr., and Gluck, S. L. (2001) *J. Biol. Chem.* **276,** 30407–30413
41. Kim, M., Jung, J., Park, C.-S., and Lee, K. (October 1, 2002) *Biochimie* (*Paris*), DOI: 10.1016/50300-9084(02)00004-4
42. Stephan, P., Clarke, F., and Morton, D. (1986) *Biochim. Biophys. Acta* **873,** 127–135
43. Walsh, J. L., Keith, T. J., and Knull, H. R. (1989) *Biochim. Biophys. Acta* **999,** 64–70
44. Edwards, D. C., Sanders, L. C., Bokoch, G. M., and Gill, G. N. (1999) *Nat. Cell Biol.* **1,** 253–259
45. Takai, Y., Sasaki, T., Tanaka, K., and Nakanishi, H. (1995) *Trends Biochem. Sci.* **20,** 227–231
46. Dickson, B. J. (2001) *Curr. Opin. Neurobiol.* **11,** 103–110
47. Tominaga, T., Ishizaki, T., Narumiya, S., and Barber, D. L. (1998) *EMBO J.* **17,** 4712–4722
48. Szaszi, K., Kurashima, K., Kapus, A., Paulsen, A., Kaibuchi, K., Grinstein, S., and Orlowski, J. (2000) *J. Biol. Chem.* **27,** 28599–28606
49. Sugiyama, S., Satoh, H., Nomura, N., Terada, H., Watanabe, H., and Hayashi, H. (2001) *Mol. Cell. Biochem.* **217,** 153–161

Interaction of Cofilin with Triose-phosphate Isomerase Contributes Glycolytic Fuel for Na,K-ATPase via Rho-mediated Signaling Pathway

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