

# Protein Phosphatase Type-1, Not Type-2A, Modulates Actin Microfilament Integrity and Myosin Light Chain Phosphorylation in Living Nonmuscle Cells

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**Abstract.** Dynamic reorganization of the actin microfilament networks is dependent on the reversible phosphorylation of myosin light chain. To assess the potential role of protein phosphatases in this process in living nonmuscle cells, we have microinjected the purified type-1 and type-2A phosphatases into the cytoplasm of mammalian fibroblasts. Our studies reveal that elevating type-1 phosphatase levels led to the rapid (within 30 min) and fully reversible disassembly of the actin microfilament network as determined by immunofluorescence analysis. In contrast, microinjection of equivalent amounts of the purified type-2A phosphatase had no effect on actin microfilament organization. Metabolic labeling of cells after injection of purified phosphatases was used to analyze changes in protein phosphorylation. Concomitant with the disassembly of the actin microfilaments induced by type-1

phosphatase, there was an extensive dephosphorylation of myosin light chain. No such change was observed when cells were injected with type-2A phosphatase. In addition, after extraction of fibroblasts with Triton X-100, the type-1 phosphatase could be specifically localized by immunofluorescence to a fibrillar network of microfilaments. Furthermore, neutralizing type-1 phosphatase activity *in vivo* by microinjection of an affinity-purified antibody, prevented the reorganization of actin microfilaments that we had previously described following injection of cAMP-dependent protein kinase. These data support the notion that type 1 and type-2 phosphatases have distinct substrate specificity in living cells, and that type-1 phosphatase plays a predominant role in the dephosphorylation of myosin light chain and thus in the modulation of actin microfilament organization *in vivo* in intact nonmuscle cells.

REVERSIBLE protein phosphorylation is well known to play an implicit role in the mechanisms that regulate actomyosin contractility (1, 18–20, 27; reviewed in references 32, 43). There has been a consensus that the increased phosphorylation of the 20–22-kD myosin light chain component of the myosin complex coincides with the increased contractility of smooth and skeletal muscle tissue (2, 5, 19, 20, 41; reviewed in references 2, 27, 32, 43), and is related to changes in actin organization and cell shape in nonmuscle cells (6, 33). A calcium/calmodulin-dependent enzyme, myosin light chain kinase (MLCK),<sup>1</sup> catalyzes this reaction (3, 17; reviewed in references 32, 43). More controversial has been understanding the mechanisms which bring about the regulation of this enzyme in living muscle cells, since in addition to its activation by calmodulin (17; reviewed in reference 32), MLCK activity can be modulated through direct phosphorylation by the cAMP-dependent pro-

tein kinase (A-kinase) (3, 17, 28; reviewed in references 32, 43). We have recently shown that inhibition of MLCK by microinjection of anti-MLCK antibodies into the mammalian cell line REF-52 led to the dephosphorylation of myosin light chain, and the disassembly of the actin microfilament network (33). Moreover, a similar effect was obtained when cells were injected with the catalytic subunit for A-kinase and coincided with the increased phosphorylation of MLCK (33). Since these events were accompanied by the dephosphorylation of the myosin light chains, we therefore postulated that myosin light chain dephosphorylation was an integral process in actomyosin relaxation in nonmuscle cells. Similar effects of A-kinase had been observed previously in studies of smooth muscle tissue (17, 20, 28; reviewed in reference 43), where treatment of skinned muscle fibers with agents that activate A-kinase led to muscle relaxation. These studies (and others; reviewed in reference 32) were restricted, however, by the difficulty in observing or identifying the concomitant dephosphorylation of myosin light chains and relaxation of actomyosin in the tetanized smooth muscle fibers (20). Whereas there is some recent evidence that cAMP-

1. *Abbreviations used in this paper:* A-kinase, cAMP-dependent protein kinase; MLC, myosin light chain; MLCK, myosin light chain kinase; PP-1, type-1 protein phosphatase; PP-2A, type-2A protein phosphatase; REF-52, rat embryo fibroblast.

dependent relaxation of smooth muscle fibers coincides with myosin dephosphorylation (19), there still remain clear cases (also cited in reference 19) where either mechanisms independent of cAMP bring about the relaxation of smooth muscle or the maintenance of contractile force occurs after the dephosphorylation of myosin (reviewed in reference 32). However, addition of purified phosphatase to skinned fibers did cause decrease in tension suggesting that myosin light chain (MLC) dephosphorylation is involved in muscle relaxation (9, 10, 26, 29).

Whereas the mechanisms that control myosin phosphorylation by MLCK are now better understood, less is known of the enzymes that bring about MLC dephosphorylation in vivo. Indeed, in comparison to our understanding of protein kinases, knowledge of the regulation, structure, and substrate specificity of protein phosphatases is underdeveloped. The two predominant and best studied serine and threonine protein phosphatases, type-1 and type-2A (PP-1 and PP-2A, respectively) (reviewed in references 4, 16), show distinct functional properties, in particular their sensitivity to specific inhibitor proteins (reviewed in references 4, 16). However, as reviewed by Cohen (16), PP-1 and PP-2A show broad and overlapping specificities in vitro (although there is evidence that these phosphatases catalyze the dephosphorylation of certain substrates at different rates [4, 13, 16] and their precise functions in vivo are as yet unknown. Since both enzymes have been previously proposed to be involved in the dephosphorylation of the myosin light chain (9, 10, 14, 15, 22, 26, 35, 39, 45; reviewed in references 16, 43), we chose to examine the consequences of elevating intracellular phosphatase levels through microinjection of the purified type-1 and type-2A phosphatases in rat embryo fibroblasts. These studies reveal that only type-1 phosphatase induced rapid and reversible changes in microfilament organization, which mimic those observed after inhibition of MLCK activity (33). Furthermore, microinjection of type-1 phosphatase induced the dephosphorylation of the myosin light chain whereas type-2A did not. These studies demonstrate a high degree of substrate specificity between the two enzymes in living fibroblasts, not necessarily anticipated from previous in vitro analysis, and an important role for type-1 phosphatase in the regulation of actomyosin.

## Materials and Methods

### Microinjection and Cell Culture

Rat embryo fibroblast (REF-52), were cultured in DME supplemented with 8.0% FCS in a humidified atmosphere containing 5.0% CO<sub>2</sub>. Cells were grown and subcultured onto glass coverslips for immunofluorescence and microinjection as described elsewhere (33). For microinjection studies, cells were microinjected with purified phosphatase enzymes (or antibodies) diluted immediately before microinjection into an injection buffer (100 mM K<sup>+</sup> glutamate, 39 mM K<sup>+</sup> citrate, pH 7.3) containing 0.5 mg/ml inert immunoglobulins (to act subsequently as an inert marker in identifying microinjected cells) (34).

### Phosphatases and Kinase Purification and Use

The homogeneous 33-kD catalytic fragment of type-1 protein phosphatase (PP-1) was purified from rabbit skeletal muscle as described elsewhere (11). In most experiments, PP-1, one of four preparations, was diluted to a final concentration of 0.5 mg/ml (with a sp act ~11,000–17,000 U/mg [25]); we also assayed a range of concentrations from 0.1–0.75 mg/ml). The catalytic subunit type-2A protein phosphatase (one of two preparations) was purified

to homogeneity from bovine cardiac muscle by ethanol precipitation as described before (35). Both the native (complexed to regulatory subunit) and catalytic preparations of PP-2A were microinjected in the concentration range 0.1–1.6 mg/ml. The phosphatase was diluted from a 1.3–1.8 mg/ml solution (sp act ~8,000–10,000 U/mg) to the same specific catalytic activity as type-1 preparations. We have used maximum catalytic activity of each phosphatase directed against their best in vitro substrate, i.e., phosphorylase-a for PP-1 (11) and isolated MLC for PP-2A (35) in equilibrating the amounts of each phosphatase injected. Indeed, since PP-1 is two- to three-fold less active than PP-2A towards isolated MLC, normalizing the activities of the two enzymes toward this substrate would have resulted in our underestimating the appropriate activity for PP-2A. To compensate for the apparent differences in in vitro reactivity of PP-1 and PP-2A for myosin light chain (isolated or complexed) we have injected a four- to five-fold range of activities for either enzyme. The catalytic subunit of cAMP-dependent protein kinase (A-kinase) was purified from beef heart as described (7). Cells were injected with a 0.5 mg/ml solution of A-kinase diluted in injection buffer. Proteins were stored at –20°C before use. Activities of all proteins were confirmed before microinjection.

### Immunofluorescence

Microinjected cells growing on glass coverslips were fixed at various times after injection in 3.7% formalin in PBS for 5 min. After brief extraction in –20°C acetone, actin microfilaments were visualized with rhodamine-conjugated phalloidin (rh-phalloidin), a specific stain for F-actin (a generous gift from Prof. T. Weiland, Max Planck Institute, Heidelberg, Federal Republic of Germany). Cells were simultaneously stained for the co-injected inert marker antibody with affinity-purified fluorescein conjugated anti-rabbit antibodies and mounted in Airvol-205 before examination and photography on an Axiophot photomicroscope as described elsewhere (34). To examine the distribution of phosphatase type-1 in REF-52 cells, cells were extracted in 100 mM K<sup>+</sup>-Hepes (pH 7.2), 1 mM MgSO<sub>4</sub>, 0.3% (vol/vol) Triton X-100 for 30 s. Immediately afterward cells were fixed in formalin and extracted with acetone as described above and subsequently stained for the distribution of phosphatase type-1 and affinity-purified sheep antibodies raised against the catalytic subunit of type-1 phosphatase purified from rabbit skeletal muscle (11). Primary antibody distribution was revealed using fluorescein-conjugated rabbit anti-sheep Igs (Cappel, Organon Technika, Fresnes Cedex, France). Cells were concomitantly stained for the distribution of actin using rh-phalloidin as described above.

For studies in which cells were injected with antibodies directed against type-1 phosphatase (injected over a range of concentrations from 0.1 to 1.0 mg/ml), the same affinity-purified sheep antibody as used above was diluted into injection buffer containing type-2A phosphatase or the cAMP-dependent protein kinase. Injection solutions also contained an inert marker antibody. Cells were fixed and stained for the distribution of actin as described above.

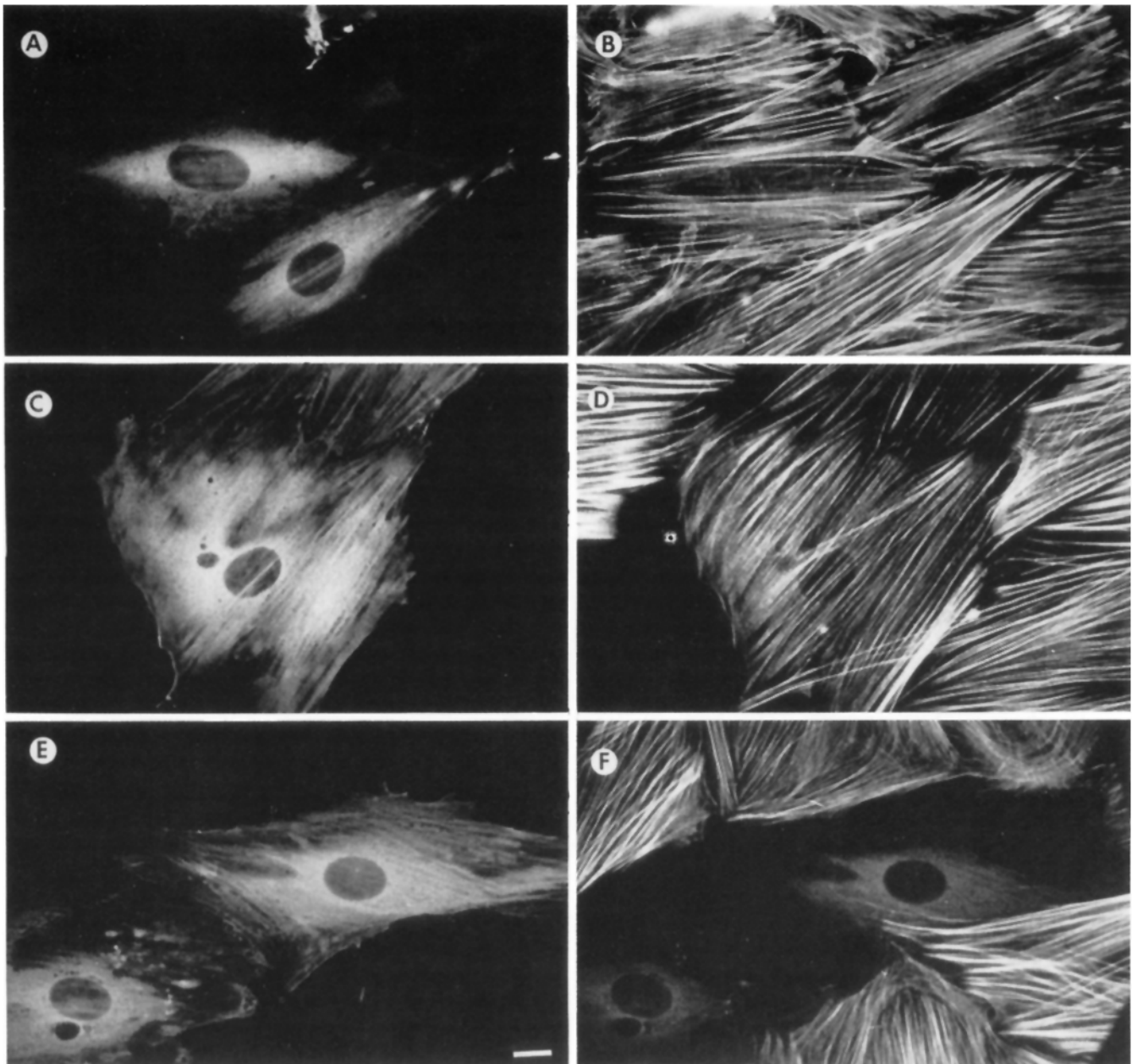
### Metabolic Labeling

Cells growing on small glass coverslips were microinjected and metabolically pulse labeled as described previously (33). Phosphatase proteins were microinjected in the same buffer as described above with the exception that the marker antibody was omitted. After labeling phosphoprotein patterns were analyzed by two-dimensional PAGE before autoradiography as described before (33).

## Results

### Microfilament Reorganization Occurs after Microinjection of Type-1 Phosphatase, Not Type-2A Phosphatase

We have previously shown that the distribution of actin microfilaments in mammalian fibroblasts can be markedly altered through activation of the cAMP-dependent A-kinase or through inactivation of the MLCK, and this reorganization was also accompanied by the pronounced dephosphorylation of MLC (33). To identify the phosphatase responsible for this MLC dephosphorylation, we used the same cells as in our previous study, rat embryo fibroblasts REF-52 cells.

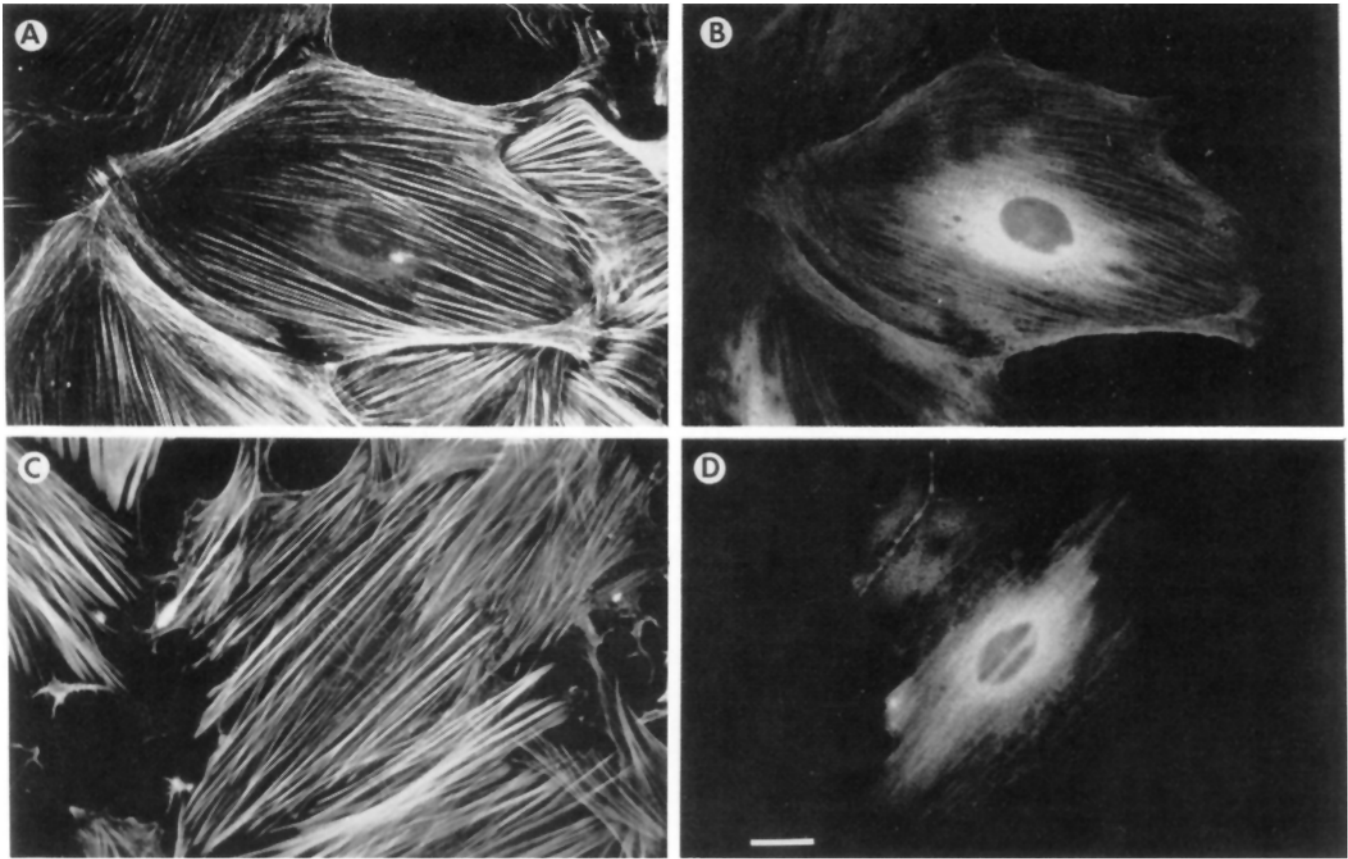


**Figure 1.** Microinjection of protein phosphatase type-1 induces actin microfilament disassembly. Rat embryo fibroblasts (REF-52) growing on glass coverslips were microinjected with either microinjection buffer or the purified catalytic fragment of PP-1 (0.5 mg/ml, ~15,000 U/mg, 7,500 U/ml). At different times thereafter the cells were fixed and stained for the distribution of actin microfilaments as described in Materials and Methods. All solutions for microinjection contained an inert antibody to act as marker in identifying injected cells. Shown are fluorescence micrographs of the distribution of the coinjected marker antibody (*A*, *C*, and *E*), or the distribution of actin microfilaments in the same cells (*B*, *D*, and *F*). (*A* and *B*) Cells microinjected with microinjection buffer alone; (*C* and *D*) cells microinjected with PP-1 and fixed 15 min thereafter; and (*E* and *F*) cells microinjected with PP-1 and fixed 30 min afterwards. Bar, 10  $\mu$ m.

Cells were synchronized into the G<sub>1</sub> phase of the cell cycle by serum starvation and refeeding (34) since we have observed that the amount, activity, and distribution of a specific inhibitor for type-1 phosphatase, inhibitor-2, fluctuates in a cell cycle-dependent manner. Purified PP-1 was obtained by the method of Brautigam et al. (11) from rabbit skeletal muscle. For microinjection studies, purified phosphatase was dialyzed into an injection buffer containing 100 mM K<sup>+</sup>-glutamate, 40 mM K<sup>+</sup>-citrate, 1.0 mM MgSO<sub>4</sub>, pH 7.25 sup-

plemented with 0.3 mg/ml rabbit anti-mouse Igs to act as an inert marker in identifying injected cells.

Microinjection of the buffer alone (Fig. 1, *A* and *B*) has little or no effect on the distribution of actin microfilaments as shown in *B*, with microinjected cells containing a similar complex distribution of thick and thin filament bundles. In contrast, within 15 min of injecting PP-1 (0.5 mg/ml, ~8,000 U/ml), the actin microfilaments in injected cells (marked by antibody in Fig. 1 *C*) have begun to reorganize into thicker

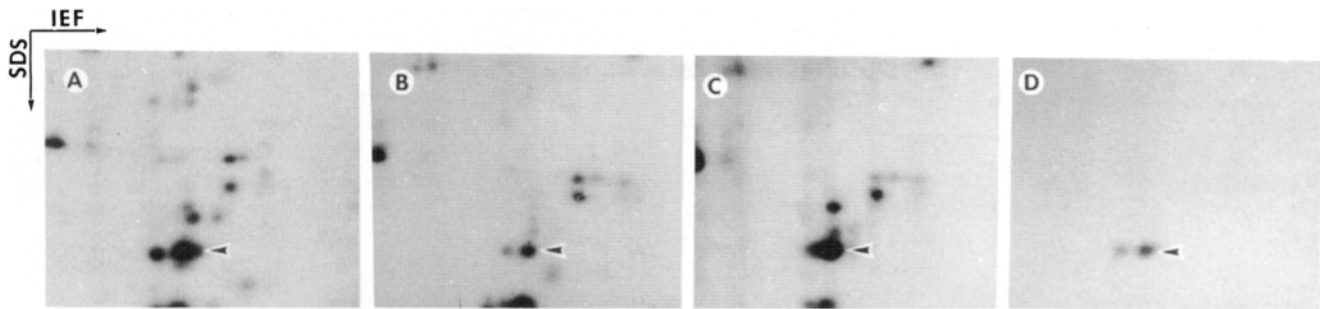


**Figure 2.** Microinjection of type-2A phosphatase does not modulate microfilament organization. Rat embryo fibroblasts were microinjected with solutions of PP-2A before fixation and staining for the organization of microfilaments as described in Materials and Methods. Cells were microinjected with either PP-2A alone (at 1.7 mg/ml,  $\sim 10,000$  U/mg, 17,000 U/ml) or together with an anti-PP-1 antibody (0.5 mg/ml) which inhibits type-1 phosphatase activity. All microinjection solutions also contained an inert marker antibody. Shown are fluorescence micrographs of cells stained for the distribution of actin (A and C) and for the microinjected marker antibody (B and D). A and B show cells microinjected with purified PP-2A alone. C and D show cells microinjected with PP-2A and anti-PP-1 antibodies. Bar, 10  $\mu$ m.

fibers resulting in an apparent decrease in the overall density of the actin staining in comparison to surrounding cells (D). By 30 min after injection, injected cells (Fig. 1 E) have continued to reorganize their actin and now contain little or no detectable actin filament staining (Fig. 1 F). A similar reorganization of actin can also be observed when injected cells are stained with four different anti-actin antibodies or analyzed by whole-mount electron microscopy (data not shown), indicating that the loss of actin staining does not reflect an immunological artifact. Incubating cells for longer periods reveals that the filaments have partly reformed by 60 min after microinjection, and little or no difference in actin distribution can be discerned when comparing PP-1 injected or uninjected cells 90 min after injection (data not shown). We observed similar effects on actin microfilament organization over quite a wide range of concentrations from 0.3 (5,000 U/ml) to 0.75 (12,000 U/ml) mg/ml PP-1 in the needle (reflecting 0.03–0.07 mg/ml injected intracellular concentration). Below 0.3 mg/ml the effects on filament organization were less pronounced, and below 0.1 mg/ml no change in organization could be observed (data not shown).

The effects of PP-1 on actin organization could be abolished in a number of different ways. Preincubation of purified phosphatase with affinity-purified anti-PP-1 antibodies (which neutralizes PP-1 activity *in vitro*) and subsequent

removal of the antibody-phosphatase complex with protein A-Sepharose, completely abolished the reorganization of actin (data not shown), strongly suggesting that the presence of active PP-1 in the injection solution accounted for the changes seen. This was also confirmed by injecting the anti-PP-1-phosphatase complex into the cells which also resulted in no change in actin organization (data not shown). Furthermore, the effect on filament organization could be abolished by boiling the preparation for 2 min before injection (data not shown). These data (and those showing that microinjection of the buffer alone have no effects on actin) strongly link the changes in actin staining to the presence of active PP-1. We have also observed similar changes in actin organization after PP-1 injection in a number of other cell lines including Swiss 3T3, rat-2, bovine aortic fibroblasts, and human diploid fibroblasts (data not shown). Since type-1 and type-2A phosphatases exhibit overlapping substrate reactivity *in vitro*, we next examined if elevating intracellular type-2A phosphatase would also induce actin filament reorganization. In experiments similar to those described above, REF-52 cells were injected with a solution of purified catalytic subunit of type-2A phosphatase (PP-2A), purified from bovine cardiac muscle (35). To ensure that studies were comparable, the same units of maximum catalytic activity of either phosphatases were used for microinjection studies. (PP-1



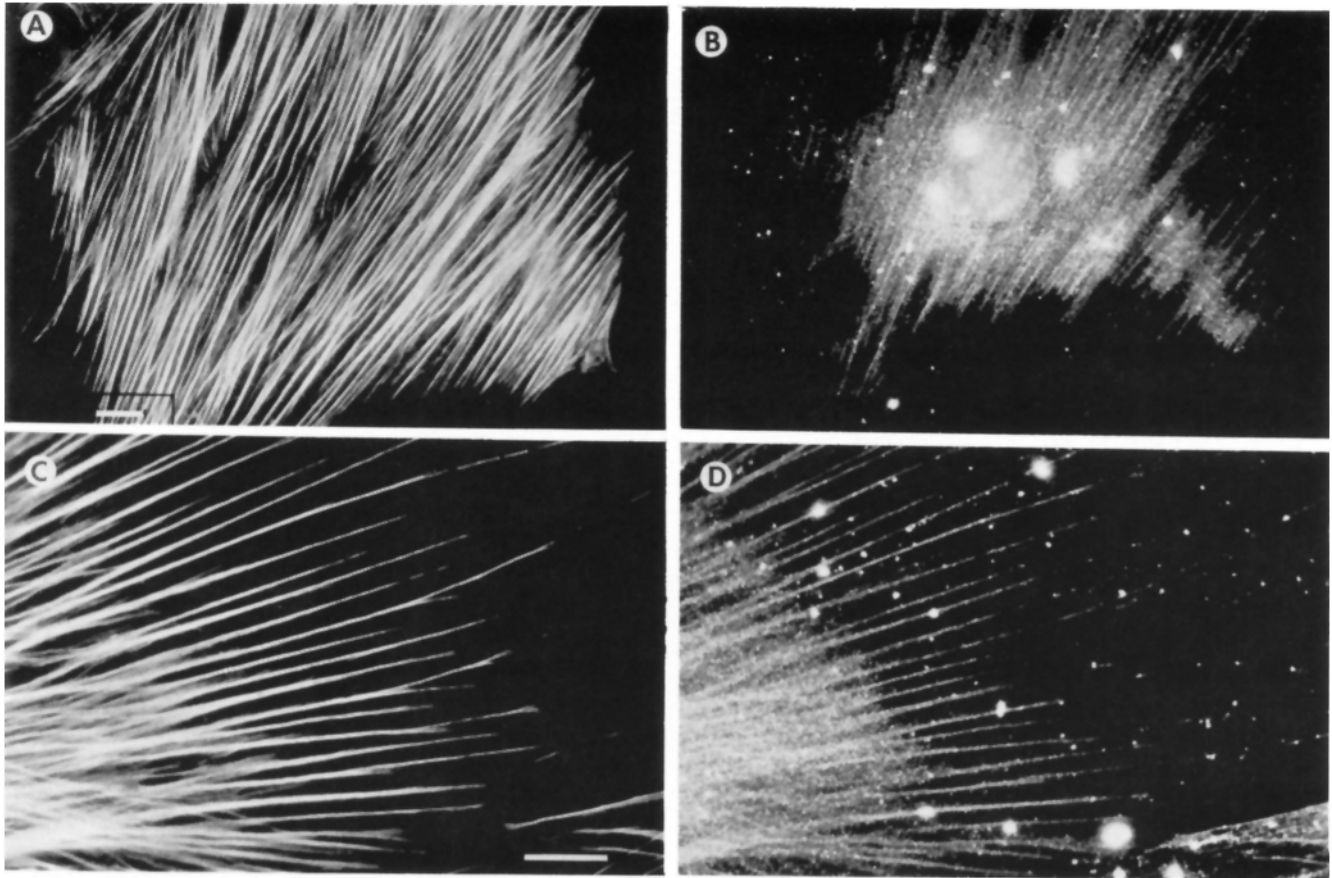
**Figure 3.** Dephosphorylation of myosin light chain accompanies activation of type-1 phosphatase but not type-2A. Rat embryo fibroblasts growing on 2 mm<sup>2</sup> glass coverslips were microinjected with either microinjection buffer, purified phosphatase type-1 (0.5 mg/ml), or purified phosphatase type-2A (1.7 mg/ml). Immediately afterwards cells were incubated in 5  $\mu$ l of [<sup>32</sup>P]orthophosphate in DME for 30 min. At the end of the labeling period, cells were lysed in boiling sample buffer and analyzed by two-dimensional PAGE as described in Materials and Methods. Shown are autoradiograms of the region of the gels that include myosin light chain. *A* shows myosin light chain phosphorylation in cells injected with microinjection buffer, *B* shows the same region of the gel from cells injected with phosphatase type-1, *C* shows a similar region from cells injected with type-2A phosphatase. *D* shows a similar region of a gel after injection of PP-2A and after transfer to nitrocellulose. The nitrocellulose was subsequently reacted with rabbit anti-myosin light chain and developed for anti-rabbit. Shown are the positions where the myosin light chain reacted with this antibody. Marked on the autoradiographs and the blot is the position of the myosin light chain (arrows), and the direction of the first- and second-dimension electrophoresis. The acidic side of the gel is to the right.

was assayed with phosphorylase-a as substrate [11], PP-2A with isolated MLC as substrate [35]). Since the isolated myosin light chain differs in its capacity to act as substrates for PP-1 and PP-2A (being an approximately two- to threefold better substrate for PP-2A than PP-1), injecting equal activities towards this substrate would have led us to underestimate the amount of PP-2A required for injection (although it would not have changed the result). Fig. 2 shows the effects of microinjection of PP-2A (1.7 mg/ml, 15,000–17,000 U/ml) on actin filament organization. The cells injected with PP-2A (marked by control antibody staining in Fig. 2 *B*), show only very subtle changes in organization of actin when compared to the surrounding control cells (shown in Fig. 2 *A*). We did observe a small proportion of the cells showing a more pronounced effect on actin organization, but this never reached the extent observed after injection of PP-1. (Indeed, the subtle changes were similar to those observed when lower concentrations [0.1 mg/ml] of PP-1 were microinjected.) Since there is evidence that type-1 activity may contaminate preparations of PP-2A (~5% [12]), we proceeded to reanalyze the effects of PP-2A in the presence of antibodies inhibitory to phosphatase type-1 activity and that have no effect towards type-2A activity *in vitro* (12). When a solution of PP-2A (17,000 U/ml) containing 0.5 mg/ml anti-PP-1 antibodies was microinjected into REF-52 cells, injected cells (Fig. 2 *D*) showed no change in actin distribution (Fig. 2 *C*) when compared to surrounding cells. This absence of an effect of PP-2A injected in the presence of anti-PP-1 was observed over a wide range of concentrations of PP-2A, indicating that the subtle effects on actin distribution seen when injecting PP-2A alone were most likely due to low levels of contaminating PP-1 in the PP-2A preparation. This conclusion was also confirmed by passing the preparation of PP-2A through heparin-Sepharose, which removes possible contaminating PP-1, a treatment that resulted in effectively abolishing the minor effects previously observed on actin filaments even at high concentrations of injected PP-2A (1.7 mg/ml). The amount of PP-2A injected here (1.7 mg/ml), represents approximately fivefold more PP-2A activity than the minimal activity of PP-1

found to have an effect on actin filament organization. We have also observed that injection of the native holoenzyme form of PP-2A has no effect on actin organization (data not shown). Moreover, over expression of PP-2A after microinjection of a plasmid (pCMV, bearing a cytomegalovirus promoter) encoding the PP-2A catalytic subunit, did not induce any changes in cell actin organization (although showing some other effects on the cells, like the highest concentration of PP-2A enzyme used here, these effects are currently under investigation) (data not shown).

#### ***Dephosphorylation of Myosin Light Chain in Response to Microinjection of Type-1 Phosphatase, but Not Type 2A Phosphatase, Coincides with Actin Reorganization***

Since dephosphorylation of myosin light chain correlated with the reorganization of actin microfilaments (33), we therefore tested to see if the reorganization of actin induced by injection of PP-1 also was accompanied by dephosphorylation of myosin light chain. Cells were plated on small glass coverslips and after injecting each cell on the coverslip (equal numbers for each experiment) with injection buffer, or PP-1 or PP-2A, the coverslips were transferred to humidified chambers and incubated with [<sup>32</sup>P]orthophosphate for 30 min. After labeling, cells were solubilized and analyzed for changes in phosphoproteins by two-dimensional PAGE. We had previously identified and localized MLC on our gel system (33). As shown in Fig. 3 *A*, confirming our previous findings, in control cells or cells injected with buffer alone, the myosin light chain appears as two phosphoprotein spots, one more heavily phosphorylated and running as a more acidic isoform. Cells microinjected with PP-1 show a marked diminution in the labeling of MLC within 30 min, particularly on the more heavily phosphorylated acidic form (Fig. 3 *B*). In some experiments we observed the complete dephosphorylation of this acidic form of MLC leaving only the more basic phosphoisoform. The dephosphorylation of MLC after injection of PP-1 was most pronounced when



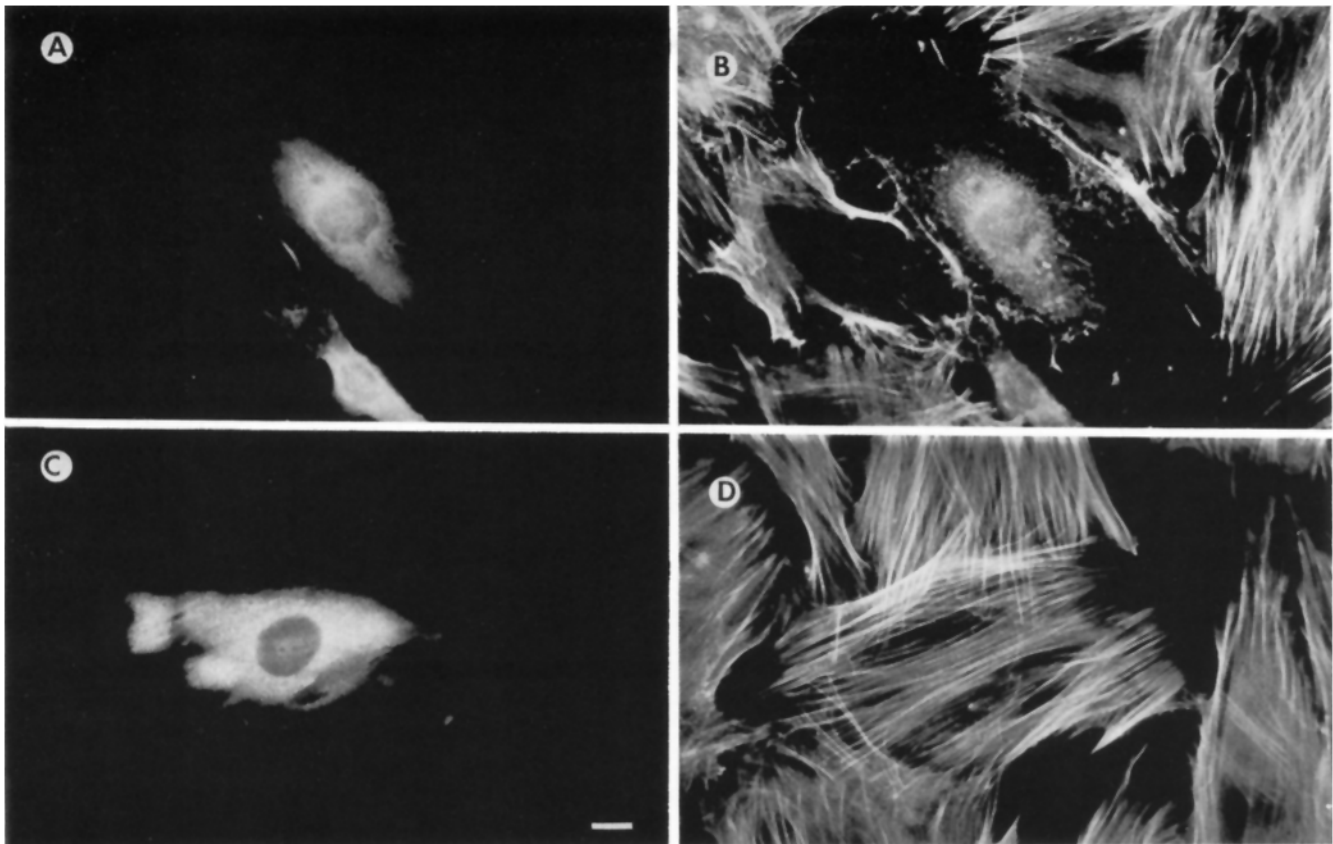
**Figure 4.** Immunocytochemical localization of PP-1 in REF-52 cells fixed after brief detergent extraction. REF-52 cells were grown on coverslips and extracted for 30 s in 0.3% Triton X-100 in 10 mM Hepes, pH 7.5, followed by fixation in 3.7% formalin and acetone extraction, before staining for the distribution of actin and PP-1 as described in Materials and Methods. Shown are fluorescence micrographs of the distribution of actin (A and C) and PP-1 (B and D). Bars: (B) 10  $\mu$ m; (D) 5  $\mu$ m.

cells were labeled from 15 to 45 min after injection, which coincides temporally with the most extensive actin reorganization. When PP-2A phosphatase was microinjected (Fig. 3 C), there was little or no difference in MLC phosphorylation between control cells (Fig. 3 A) and cells injected with PP-2A (Fig. 3 C). To confirm that these proteins were indeed MLC we performed Western immunoblotting of cells after injection of phosphatases. Fig. 3 D shows a typical Western blot using anti-MLC (a generous gift from Dr. James Stull, University of Texas, Dallas, Texas). Again, as we have described previously, in our gel system MLC resolves as two spots, the more heavily staining acidic spot resolving exactly over the position of the more heavily  $^{32}$ P-labeled isoform of MLC. Since we do not observe any other proteins reacting with these antibodies, we must conclude that the constitutively low level phosphorylated isoform resolves closely or to the same position as the unphosphorylated isoform. From this data it is clear that the phosphoisoform of MLC which shows the most prominent dephosphorylation after injection of PP-1, and not PP-2A, corresponds to the form that we have previously shown to decrease its phosphorylation after injection of A-kinase or antibodies that inactivate MLCK *in vivo* (33). Although no changes in MLC phosphorylation were observed after injection of PP-2A, we did observe a few other proteins on the two-dimensional PAGE, which showed changes in phosphorylation. However, none of these proteins appeared

to correspond to known actin binding proteins (for example, heavy myosin) and since most of these are as yet unidentified further research will be required to ascertain the potential implication of this effect that does not clearly relate to the present study on actomyosin. However, these data indicate that the PP-2A preparation we are using is active inside the REF-52 cells. Taken together, these results provide additional evidence that the disassembly of actin microfilaments in fibroblasts involves the dephosphorylation of MLC and indicate that this dephosphorylation of MLC appears to be catalyzed predominantly by type-1 phosphatase. The selectivity of this effect provides some of the first evidence of differential substrate specificity for phosphatases in living cells.

#### ***Type-1 Phosphatase Antisera Stain Predominantly Actin Microfilaments in Extracted Cells***

Providing further support for the potential role of type-1 phosphatase in actin filament organization, we have observed by immunofluorescence staining using an affinity-purified anti-PP-1 antibody, the decoration of the actin filament network. In cells fixed after a brief extraction with 0.3% Triton X-100, staining for PP-1 revealed a fibrillar pattern (Fig. 4, B-D), exactly overlaying the staining for actin microfilaments (Fig. 4, A-C) produced by rhodamine phalloidin. We also observed some actin filament staining in cells that had



**Figure 5.** Coinjection of anti-PP-1 antibodies with the cAMP-dependent protein kinase (A-kinase) prevents the disassembly of actin microfilaments induced by injection of A-kinase alone. The purified catalytic subunit of cAMP-dependent protein kinase (0.5 mg/ml) with and without affinity-purified antibodies directed against phosphatase type-1 (0.5 mg/ml) were microinjected into rat embryo fibroblasts growing on glass coverslips. 45 min thereafter, coverslips were fixed and stained for the distribution of an inert antibody coinjected to identify microinjected cells and for the distribution of actin. Shown are fluorescence micrographs of the distribution of marker antibody (*A* and *C*) or actin (*B* and *D*). *A* and *B* show cells injected with the kinase alone, *C* and *D* show cells injected with the kinase and anti-PP-1 simultaneously. Bar, 10  $\mu$ m.

not been extracted (data not shown), implying that the actin staining pattern is not an artifact of the extraction procedure. Indeed the staining pattern could be abolished by preincubating the antibody with purified PP-1, but not with PP-2A (data not shown). It is also possible to observe some nuclear localization of PP-1 (in Fig. 4 *B*) in agreement with other previous studies using proteins fractionated from isolated nuclei (24, 31). Interestingly, although the pattern is faint, there does not appear to be any clear evidence of striated staining characteristic similar to the patterns seen for some of the other actin binding regulatory proteins (for example,  $\alpha$ -actinin, myosin, and MLCK [21, 23]).

***Microfilament Disassembly Induced by A-Kinase Is Prevented by Coinjection of Antibodies Specific for Protein Phosphatase Type-1 or Phosphatase Inhibitor-2***

Our previous studies demonstrated that microinjection of A-kinase induced disassembly of actin microfilaments through a mechanism involving MLCK inactivation, and the resulting dephosphorylation of MLC (33). The finding that microinjection of PP-1 induces the disassembly of the actin microfilaments in a manner similar to that observed after MLCK inactivation, suggests that type-1 phosphatase may play a role in the dephosphorylation of MLC that accompanied the reor-

ganization of the microfilaments after A-kinase activation. To test this possibility, we examined the effect of coinjecting the catalytic subunit of A-kinase (0.5 mg/ml) plus affinity-purified anti-PP-1 antibodies (0.5 mg/ml), which specifically neutralize type-1 phosphatase activity *in vitro* (12).

As shown in Fig. 5, elevation of intracellular A-kinase levels through microinjection of the catalytic subunit of A-kinase together with inert antibodies rapidly induced the disassembly of microfilaments such that injected cells (Fig. 5 *A*) showed little or no actin microfilament staining (Fig. 5 *B*) 60 min after injection. In marked contrast, we observed no such effects in cells coinjected with A-kinase and antibodies that neutralize type-1 phosphatase, and by 60 min after injection, injected cells (Fig. 5 *C*) still contain a distribution of microfilaments indistinguishable from that in surrounding uninjected cells (Fig. 5 *D*). This effect was observed using a wide range of concentrations of A-kinase from 0.1 to 3.0 mg/ml. Increasing the concentration of anti-PP-1 antibody coinjected with A-kinase still prevented actin reorganization by A-kinase, whereas lowering the concentration of the antibody below 0.1 mg/ml led to actin reorganization, just as observed in cells injected with A-kinase alone. Confirming this effect of anti-PP-1, coinjection of purified phosphatase inhibitor-2 (0.3 mg/ml) with A-kinase also prevented microfila-

ment disassembly in a fashion similar to anti-PP-1 (data not shown). These results confirm that the effect of A-kinase on the actin microfilaments indeed depends upon the presence of active phosphatase type-1, since neutralizing this enzyme in living cells prevented the disassembly of actin filaments normally observed after A-kinase microinjection. In further support of this conclusion, we have performed the same experiment with cells treated with the phosphatase inhibitor okadaic acid (8, 30, 45). When cells were treated with low concentrations of okadaic acid (10 nM), injection of A-kinase induced effects on actin filaments similar to those we have described before or shown in Fig. 5 B. At this concentration okadaic acid is an effective inhibitor of PP-2A only (8, 30). However, when the same experiment is performed in the presence of 1  $\mu$ M okadaic acid (which inhibits both PP-1 and PP-2A), we observed no effects on actin organization after injection of A-kinase (data not shown). Taken together with the results of injecting purified phosphatases, these results imply an integral role for protein phosphatase type-1 in microfilament organization.

## Discussion

Through microinjection of the purified catalytic subunits of type-1 and type-2A phosphatases, we examined the involvement of these proteins in modulating the organization of actin microfilaments in living nonmuscle cells.

The increased tension of different muscle types is associated with an overall increase in phosphorylation of the light chains of myosin ( $M_r$  20–22 kD) (1, 2, 5, 17, 19, 20, 27, 41), which appears to involve activation of a calcium-calmodulin-dependent protein kinase (MLCK) (1–3, reviewed in references 32, 43) although other kinases can phosphorylate MLC (44). Phosphorylation of MLCK by A-kinase results in a reduction in MLCK activity since it occurs at the calmodulin binding site (17, 19, 28, reviewed in reference 43). We have shown recently that inactivation of MLCK in living fibroblasts by microinjection of cAMP-dependent protein kinase (A-kinase) or anti-MLCK antibodies resulted in microfilament disassembly and MLC dephosphorylation (33). The present study, using microinjection of phosphatases, provides further confirmation that in nonmuscle cells, dephosphorylation of MLC, most likely at the MLCK site, results in actin filament disassembly. As reviewed in references 27 and 43, the potential mechanisms to account for such an effect may involve changes in myosin-MgATPase activity and the conversion of myosin monomers from 10S to 6S conformation, both events being regulated by MLC phosphorylation.

Protein phosphatases reactive with MLC have been isolated from smooth muscle (22, 26, 29, 37–39, 45, reviewed 16, 40), as well as from cardiac (36, 37, 42) and skeletal muscle (14, 15, reviewed in reference 16) and appear to be related to either type-1 (14, 15, 42, reviewed in reference 16) or type 2A enzymes (8, 10, 26, 30). Both a polycation-modulated phosphatase holoenzyme (9) and the catalytic subunit of a skeletal protein phosphatase (41) have been found to decrease isometric tension of skinned smooth muscle and skeletal muscle fibers respectively. Both these protein phosphatase preparations are likely to be classed as type-2A phosphatases (16). It remains unclear whether the activity observed with skinned muscle fibers resulted from cross-contamination of these preparations with type-1 phosphatase activity,

as pointed out in the present study, or whether reactivity with type-2A phosphatase was due to changes in structure of actomyosin during preparation of skinned fibers, rendering it susceptible to dephosphorylation since, indeed, isolated MLC are substrates *in vitro* for PP-2A (35). One possible source of error in identifying the phosphatases responsible for dephosphorylating myosin light chain arises from categorizing the phosphatase as type-1 or type-2A. For example many of the *in vitro* properties of the phosphatases from smooth muscle are not entirely consistent with the functional criteria of type-1 since, although they preferentially dephosphorylate the  $\beta$ -subunit of phosphorylase kinase and bind heparin-agarose (like type-1 phosphatase), they are insensitive to inhibitor 2 (40). As reviewed by Cohen (16), there is strong evidence that a type-1 phosphatase enzyme, PP-1M, and not a type-2A enzyme, is responsible for the dephosphorylation of intact actomyosin, despite the capacity of purified type-2A enzymes to dephosphorylate isolated myosin light chains and in some cases induce relaxation of intact skinned smooth muscle fibers. Alternatively, both classes of phosphatase may dephosphorylate sites on MLC, but these sites may be different. For example, as reported by Erdodi et al. (22), PP-1-related phosphatase more effectively dephosphorylates the MLCK sites on actomyosin whereas a type-2A-related phosphatase activity preferentially brought about the dephosphorylation of a threonine at the putative protein kinase-C phosphorylation site (for example, see reference 43). Therefore, the identity of the phosphatase(s) responsible for myosin light chain dephosphorylation at the MLCK site (which is the site involved in the dephosphorylation-dependent relaxation of actomyosin) in living cells has not been rigorously established. Here, we have shown that in living nonmuscle cells, the endogenous MLC phosphatase can be blocked by anti-PP-1 antibodies, thereby preventing microfilament reorganization induced by A-kinase. Interestingly, our observations that PP-1 is implicit in actomyosin regulation after A-kinase injection suggests that phosphatase inhibitor-1 (the A-kinase activated inhibitor specific for PP-1 [for example, see reference 16]) does not play an active role in modulating PP-1M (the form of PP-1 bound to actomyosin; 16) activity in REF-52 cells. Microinjection of purified skeletal muscle type-1 phosphatase also produces morphological reorganization of the actin microfilaments in rat embryo fibroblasts and decreased myosin light chain phosphorylation, whereas type-2A catalytic subunit induced only minimal effects on microfilament organization or MLC phosphorylation and these could be blocked by coinjection of anti-PP-1 antibodies.

Whereas our data strongly indicate that PP-2A does not induce dephosphorylation of MLC *in vivo*, or changes in actin organization, we do not consider that these reflect a general lack of activity of the PP-2A preparations for several reasons. Firstly, the same results were obtained with a number of different preparations of PP-2A, the activity of which was verified *in vitro* during the microinjection experiments. Furthermore, whereas there appeared to be no changes in myosin phosphorylation, a few other proteins showed alterations in phosphorylation after microinjection of PP-2A. Moreover, using plasmids that overexpress active PP-2A catalytic subunit we have also observed no changes in actin organization (while such an injection does produce other effects in cells, as also observed with the highest concentrations of PP-2A



enzyme). Finally, we show that while inhibiting PP-2A has no effect on the reorganization of actin filaments by A-kinase (using low concentrations of okadaic acid), the inhibition of PP-1 (and PP-2A) by high concentrations of this compound does prevent A-kinase-induced actin reorganization. Altogether, these results demonstrate a high degree of in vivo substrate specificity for type-1 and type-2A protein phosphatases towards intact intracellular actomyosin, and show that only type-1 protein phosphatase operates in vivo to dephosphorylate the light chains of nonmuscle (smooth muscle type) myosin.

This conclusion is consistent with the recent biochemical evidence showing that type-1 phosphatase associates with myofibrils (14, 15, 42). Substantial amounts of PP-1 remain bound to either skeletal (14) or cardiac muscle myofibrils (42) after extensive detergent extraction. These activity measurements are in agreement with our localization of PP-1 on microfilaments by immunofluorescence staining after Triton X-100 extraction. Interestingly, the staining pattern revealed by anti-PP-1 along the microfilament under these conditions (Fig. 4) seemed to be continuous. There was no evidence of the more common striated staining seen with many of the other actin binding or regulatory proteins such as myosin light chain kinase, myosin light chain, or alpha-actinin (21, 23). The absence of striated actin filament staining suggests that the phosphatase does not bind to (or localizes to) one single actin binding protein. It may bind to two proteins which are adjacent to one another on the actin since this has been shown previously to produce complete staining pattern even if the staining pattern of either protein alone is striated (23).

Our results implicating type-1 phosphatase as the predominant myosin phosphatase in nonmuscle (smooth muscle type) actomyosin are consistent with the effects of okadaic acid (8, 45) and calyculin (30), two toxins that induce contraction of smooth muscle fibers. Whereas the okadaic acid result was obtained at concentrations where the toxin was inhibitory to both type-1 and type-2A protein phosphatases, calyculin A induced muscle contraction at concentrations where it was 50-fold more inhibitory to type-1 phosphatase (30).

The present data indicate that in mammalian cells type-1 phosphatase plays an important role in regulating actomyosin activity. This role is consistent with our recent observations that throughout the majority of the mammalian cell cycle, the type-1 phosphatase is localized in the cytoplasm where the bulk of the actin is also found (Lamb, N. J. C., D. L. Brautigan, and A. Fernandez, manuscript in preparation). While further work is still required to confirm that PP-1 acts upon the same sites on myosin light chain that are phosphorylated by MLCK in living cells and dephosphorylated during A-kinase induced actin disassembly, the present studies illustrate for the first time that type-1 phosphatase plays a major role in modulating the actin microfilament cytoskeleton in living nonmuscle cells.

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