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Evidence for a Calpeptin-sensitive Protein-tyrosine Phosphatase Upstream of the Small GTPase Rho

A NOVEL ROLE FOR THE CALPAIN INHIBITOR CALPEPTIN IN THE INHIBITION OF PROTEIN-TYROSINE PHOSPHATASES*

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Activation of the thiol protease calpain results in proteolysis of focal adhesion-associated proteins and severing of cytoskeletal-integrin links. We employed a commonly used inhibitor of calpain, calpeptin, to examine a role for this protease in the reorganization of the cytoskeleton under a variety of conditions. Calpeptin induced stress fiber formation in both forskolin-treated REF-52 fibroblasts and serum-starved Swiss 3T3 fibroblasts. Surprisingly, calpeptin was the only calpain inhibitor of several tested with the ability to induce these effects, suggesting that calpeptin may act on targets besides calpain. Here we show that calpeptin inhibits tyrosine phosphatases, enhancing tyrosine phosphorylation particularly of paxillin. Calpeptin preferentially inhibits membrane-associated phosphatase activity. Consistent with this observation, *in vitro* phosphatase assays using purified glutathione *S*-transferase fusion proteins demonstrated a preference for the transmembrane protein-tyrosine phosphatase-*α* over the cytosolic protein-tyrosine phosphatase-1B. Furthermore, unlike wide spectrum inhibitors of tyrosine phosphatases such as pervanadate, calpeptin appeared to inhibit a subset of phosphatases. Calpeptin-induced assembly of stress fibers was inhibited by botulinum toxin C3, indicating that calpeptin is acting on a phosphatase upstream of the small GTPase Rho, a protein that controls stress fiber and focal adhesion assembly. Not only does this work reveal that calpeptin is an inhibitor of protein-tyrosine phosphatases, but it suggests that calpeptin will be a valuable tool to identify the phosphatase activity upstream of Rho.

The small GTP-binding protein Rho belongs to the larger Ras family of small GTPases and has been demonstrated to regulate cytoskeletal reorganization, stimulating the formation of stress fibers and focal adhesions in cultured cells (1). Characteristic of all Ras family members, Rho functions as a "molecular switch" in the cell (2–4), cycling between inactive GDP-bound and active GTP-bound states. The rate of activation and inactivation of this GTPase is regulated by a number of proteins, including guanine nucleotide exchange factors, GTPase-activating proteins, and guanine nucleotide dissociation inhibitors (2). Although considerable insight has been gained into the mechanisms regulating small GTPases such as Rho, many components along the Rho signaling pathway remain to be identified.

Protein tyrosine phosphorylation and/or dephosphorylation events play a major role in the Rho-mediated regulation of cytoskeletal reorganization. Evidence for this comes from the use of inhibitors of protein-tyrosine kinases such as genistein (5, 6), erbstatin (6), and tyrphostin 25 (7), which have been demonstrated to inhibit the activation of Rho and prevent cytoskeletal assembly. Consistent with this, studies using protein-tyrosine phosphatase (PTPase)[1] inhibitors, such as pervanadate (6, 8, 9) and phenylarsine oxide (PAO) (10, 11), have demonstrated induction of stress fibers in serum-starved cells. Since these inhibitors block the enzymatic activity of a wide range of kinases and phosphatases, it is unclear which enzyme(s) are responsible for Rho regulation in these instances. In addition, studies artificially overexpressing soluble PTPases (through microinjection or scrape loading) resulted in disassembly of stress fibers and focal adhesions (12). Although there is increasing evidence for PTPases as important players in the regulation Rho and cytoskeletal reorganization (13–16), the identity of the enzyme(s) lying upstream of Rho activation remains elusive.

The second messenger cAMP also exerts dramatic effects on cytoskeletal architecture. Elevation of cAMP in a variety of cell types induces loss of actin stress fibers and focal adhesions, rounding of cells, and in some cases detachment from the underlying substratum (17–19). Increases in intracellular cAMP also decrease the phosphorylation of multiple proteins, including the tyrosine phosphorylation of focal adhesion proteins paxillin (20) and $pp125^{FAK}$ (21) and the phosphorylation of myosin light chain (22). Although the mechanism(s) eliciting the effects of cAMP appear to be complex, recent studies (23–27) point toward a role for this cyclic nucleotide in the down-regulation of Rho. There may be multiple ways in which cAMP can regulate cytoskeletal reorganization, and they may all participate to some extent in the cytoskeletal disassembly associated with cAMP elevation.

One potential candidate that may play a role in the disassembly of cytoskeletal structures is the ubiquitous thiol protease calpain. This enzyme localizes to focal adhesions (28, 29), where it has been shown to participate in the limited proteolysis of numerous structural and signaling proteins associated with these adhesions (30–34). The proteolytic actions of calpain have been postulated to destabilize focal adhesions and sever

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[1] The abbreviations used are: PTP or PTPase, protein-tyrosine phosphatase; CI-1, calpain inhibitor 1; GST, glutathione *S*-transferase; PAO, phenylarsine oxide; DMEM, Dulbecco's modified Eagle's medium.

the linkage between the extracellular matrix and the contractile cytoskeleton of the cell (35, 36). Consistent with this, several recent reports have confirmed a role for calpain in the down-regulation of cell processes such as spreading (37), migration (36), and platelet-mediated clot retraction (35, 38). The possible participation of calpain in events requiring remodeling of the cytoskeleton prompted us to investigate the role of this protease in disassembly of the cytoskeleton. Surprisingly, we found that treatment of cells with calpeptin, an inhibitor of calpain, was sufficient to induce stress fiber formation, focal adhesions, and cell contractility. This effect was specific for calpeptin and could not be mimicked by other calpain inhibitors. Furthermore, calpeptin-induced formation of stress fibers and focal adhesions was inhibited by botulinum toxin C3, suggesting that the calpeptin target was upstream of Rho. We report that calpeptin cross-reacts with another family of enzymes, PTPases, which also possess a critical cysteine in their active site. Using this knowledge, we provide evidence for the existence of a PTPase activity, associated with the membrane fraction of cells, which acts upstream of Rho.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Calpeptin, PAO, calpain inhibitor 1 (CI-1), recombinant calpastatin, and E64d were obtained from Calbiochem-Novabiochem. Forskolin was purchased from Biomol Research Laboratories (Plymouth Meeting, PA). Dibutyryl cAMP and Protein A-Sepharose were from Sigma. Glutathione-Sepharose 4B was obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). Transfer polyvinylidene difluoride membranes were obtained from Millipore Corp. (Bedford, MA). Radiochemicals were from NEN Life Science Products. A cyclic AMP ^{3}H assay system was purchased from Amersham International (Buckinghamshire, United Kingdom). Antiphosphotyrosine polyclonal, antiphosphotyrosine PY20, and anti-paxillin monoclonal antibodies were purchased from Transduction Laboratories (Lexington, KY). Anti-mouse peroxidase-conjugated IgG was from Jackson Laboratories (West Grove, PA). Fluorescein-conjugated rabbit IgG and rabbit anti-mouse IgG were from Chemicon (Temecula, CA). Rhodamine-conjugated phalloidin was from Molecular Probes, Inc. (Eugene, OR). Fluorescein-conjugated anti-human IgG for microinjection was from ICN/Cappel (Aurora, OH). GST constructs for the recombinant PTPases and botulinum toxin C3 were generous gifts from various research laboratories. The catalytic domain of PTP-1B (PTP-37K-1B) (Dr. J. Dixon, University of Michigan), cytoplasmic domain of PTPα (Dr. M. Thomas, Washington University, St. Louis, MO), PTEN (Dr. N. Tonks, Cold Spring Harbor, NY), and C3 exotransferase (Dr. L. Feig, Tufts University, Boston, MA).

Cell Culture—Swiss 3T3 fibroblasts and REF-52 fibroblasts were maintained in DMEM as described previously (6). Swiss 3T3 fibroblasts were serum-starved by incubation in DMEM lacking serum for at least 24 h.

Drug Treatment—In some experiments, cells were preincubated with vehicle alone (Me_2SO) (0.1%, v/v) or one of the following compounds: calpeptin (0.1–1.0 mg/ml), E-64d (100 μM), CI-1 (10 μM), pervanadate (50 μM), EGTA/$MgCl_2$ (1 and 2 mM, respectively), or PAO (1 μM). Cells were incubated with these compounds for 30 min at 37 °C, unless otherwise indicated.

Immunofluorescence Microscopy—Immunofluorescence microscopy was performed as described previously (6).

Preparation of Whole Cell Lysates and Subcellular Fractions—Whole cell lysates were prepared as described previously (6). For isolation of cytosol and membrane fractions, adherent cells were scraped into resuspension buffer (5 mM Tris, pH 7.6, 0.25 M sucrose, 2 mM EDTA, 10 μg/ml leupeptin, 25 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride), sonicated six times (8 s each), and then centrifuged at 1,000 $\times$ *g* to remove intact cells and nuclei. Cytosol and membrane fractions were isolated by centrifugation at 100,000 $\times$ *g* for 30 min. The membrane pellet was resuspended in Triton X-100-containing buffer. Measurement of protein concentration was determined using the Coomassie Protein Assay Reagent, with bovine serum albumin as a standard, following the manufacturer's instructions (Pierce).

Immunoprecipitation and Immunoblotting—Immunoprecipitation of paxillin or phosphotyrosine-containing proteins was performed as described previously (6). Cell lysates or immunoprecipitated samples were separated by 10% SDS-polyacrylamide gel electrophoresis (39) under reducing conditions and then transferred to polyvinylidene difluoride membranes. Western blots were performed as described by Towbin *et al.* (40). Blots were developed using SuperSignal Substrate for Western blotting (Pierce).

Preparation of Pervanadate—Vanadate stock solution was prepared essentially as described (41). Pervanadate (50 mM) was prepared by combining 100 μl of vanadate, 88 μl of Tris-buffered saline, and 12 μl of 30% hydrogen peroxide. The pervanadate solution was used within 5 min of preparation.

Measurement of Intracellular cAMP Levels—Intracellular cAMP levels were measured as described by Yuan *et al.* (42), using a ^{3}H-labeled cyclic AMP assay system according to the manufacturer's instructions (Amersham Pharmacia Biotech).

Growth and Purification of GST Fusion Proteins—The recombinant catalytic subunit of PTP-1B (PTP-37K-1B), the cytoplasmic tail of PTPα, PTEN, and C3 exotransferase were expressed as GST fusion proteins in *Escherichia coli* (BL21) as described previously (43).

Microinjection of C3 Exotransferase or Calpastatin—Cells were grown on coverslips and injected with either 100 μg/ml C3 exotransferase or 2 mg/ml recombinant calpastatin (diluted in microinjection buffer (5 mM Tris, pH 7.6, 150 mM NaCl, 5 mM $MgCl_2$ and 0.1 mM dithiothreitol)). Cells were coinjected with fluorescein-conjugated anti-human IgG in order to detect injected cells. Cells were returned to the tissue culture incubator to allow for recovery (30–60 min).

In Vitro PTPase Assays—Whole cell lysates or subcellular fractions from forskolin-treated (10 μM, 30 min) REF-52 or serum-starved (24 h) Swiss 3T3 fibroblasts were prepared as described previously (44). [^{32}P]poly(Glu:Tyr) substrate was prepared according to the method of Burridge and Nelson (45). All assays were performed within the linear range with respect to protein-tyrosine phosphatase activity. Samples were diluted in phosphatase assay buffer (50 mM Tris, pH 7.0, 1 mg/ml BSA, 0.3% β-mercaptoethanol, 50 μM EDTA) to a final volume of 100 μl. Reactions were started by the addition of [^{32}P]poly(Glu:Tyr) (approximately 40,000 cpm) and incubated at 30 °C for 5–20 min. Reactions were terminated by the addition of an equal volume of 20% trichloroacetic acid. Tubes were incubated on ice for 10 min and vortexed, and the precipitated protein was pelleted by centrifugation in a microcentrifuge at 12,000 rpm for 2 min. The supernatant was removed and added to 2 ml of scintillation fluid. Phosphatase activity was determined by the amount of free label released into the supernatant measured by scintillation counting, minus the amount of label released in the absence of lysate. Results are expressed as the mean of several independent experiments.

RESULTS

Activation of the thiol protease calpain has been demonstrated to result in the down-regulation of a number of cellular processes including cell spreading, migration, and contraction (35–38). These processes are all dependent on reorganization of the cytoskeleton. In addition, calpain is localized to focal adhesions, where its activation has been shown to correlate with the proteolysis and loss of various structural and signaling proteins (30–34) from these sites and the disruption of integrin-cytoskeletal linkages (35, 36). These studies imply a potentially important role for calpain in the disassembly of cytoskeletal architecture. To investigate this possibility, we searched for cell models that could easily be manipulated to induce cytoskeletal disassembly. We chose a rat fibroblast cell line (REF-52) as one of our cell models, since these cells have been demonstrated to rapidly and reversibly lose stress fibers and focal adhesions in response to agents that elevate intracellular levels of cAMP (*e.g.* forskolin, dibutyryl cAMP) (17, 46). With this cell model, we set out to determine whether calpain was involved in the disassembly of stress fibers and focal adhesions induced by elevated cAMP levels.

Calpeptin Blocks the Disassembly of Stress Fibers—In order to study the role of calpain inside the cell, it was necessary to perturb the function of this protease. Several synthetic inhibitors have been produced that block calpain activity (47, 48). One such inhibitor is calpeptin (49), a dipeptide aldehyde that was designed to bind specifically to the critical cysteine residue in the active site of calpain, preventing the binding and subsequent proteolysis of calpain substrates (48). Therefore, we be-

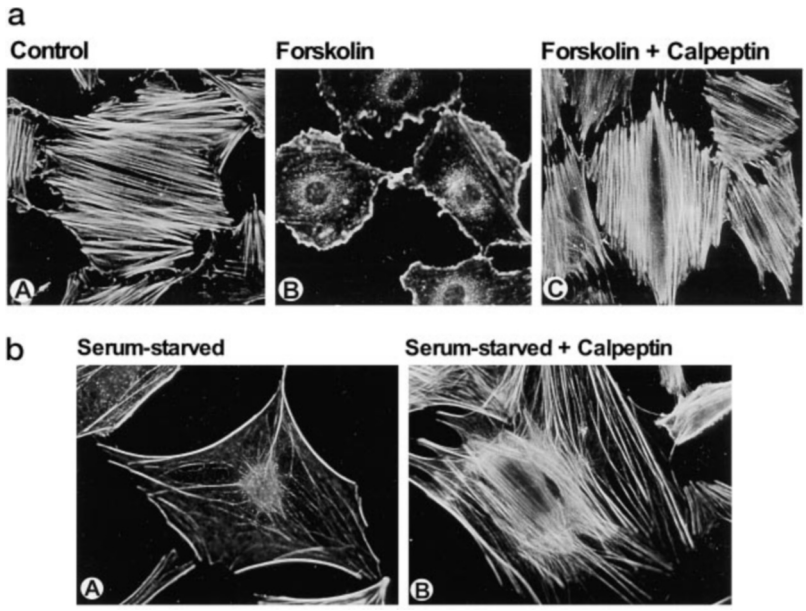

FIG. 1. **Calpeptin induces the formation of stress fibers.** REF-52 and Swiss 3T3 fibroblasts were cultured in complete DMEM and plated onto coverslips. *a*, REF-52 fibroblasts were preincubated with vehicle (Me_2SO) alone (*A* and *B*) or 100 μg/ml calpeptin (*C*) for 30 min. Cells were then treated with vehicle (Me_2SO) alone (*A*) or 10 μM forskolin (*B* and *C*) for 30 min. *b*, Swiss 3T3 fibroblasts were placed in serum-free medium and starved for 24 h. Cells were then treated with vehicle (Me_2SO) alone (*A*) or 100 μg/ml calpeptin for 30 min (*B*). Both REF-52 and Swiss 3T3 cells were fixed and processed for fluorescence microscopy as described under "Experimental Procedures." Cells were visualized by staining with rhodamine-conjugated phalloidin.

gan our examination of the role of calpain in regulating the disassembly of stress fibers in our chosen cell model, using calpeptin. Under normal culture conditions, REF-52 fibroblasts displayed a striking array of thick actin stress fibers (Fig. 1*a*, *A*). This organization was dramatically lost in response to treatment with 10 μM forskolin (elevation of cAMP) (Fig. 1*a*, *B*). However, when these cells were treated with the calpain inhibitor calpeptin (100 μg/ml), prior to forskolin treatment, the cells retained their normal actin organization and did not lose their stress fibers (Fig. 1*a*, *C*). We found this effect to be dose-dependent, with noticeable effects at concentrations of calpeptin as low as 20 μg/ml (data not shown). To ensure that the effects of calpeptin were not unique to forskolin, we also performed similar studies using the cAMP derivative, dibutyryl cAMP. Consistent with forskolin treatment, we found that calpeptin could prevent the dibutyryl cAMP-induced loss of stress fibers in REF-52 fibroblasts (data not shown). As a further control, we measured intracellular cAMP levels using a ^{3}H-labeled cAMP assay system and confirmed that calpeptin was not merely acting by lowering cAMP levels inside the cell (data not shown). These results suggest that calpeptin was able to block the disassembly of the cytoskeleton induced by intracellular cAMP elevation.

The calpeptin-mediated effects on cAMP-induced disassembly could be due to blocking disassembly or alternatively promoting assembly of stress fibers. To explore the possibility that calpeptin might promote stress fiber assembly, we examined the effect of calpeptin on serum-deprived Swiss 3T3 cells. When Swiss cells are starved of serum for up to 24 h, their actin stress fibers will disassemble (Fig. 1*b*, *A*). Previous studies have demonstrated that the addition of serum or serum components such as lysophosphatidic acid will rapidly induce the formation of stress fibers in starved cells via the activation of Rho (1). Strikingly, when calpeptin was added to the serum-free culture medium, Swiss 3T3 cells rapidly (within 30 min) reformed their stress fibers (Fig. 1*b*, *B*), a response reminiscent of serum or lysophosphatidic acid addition. These results are consistent with those obtained using REF-52 fibroblasts and suggest that calpeptin can promote the formation of stress fibers.

Calpain Is Not the Target for Calpeptin-mediated Inhibition of Stress Fiber Disassembly—Although calpeptin has proven to be an effective inhibitor of the calpain-mediated proteolysis of several calpain substrates (30–34), like many other inhibitors its specificity at higher concentrations is questionable. To confirm that calpain was the protein targeted for inhibition by calpeptin, we tested a number of other calpain inhibitors widely used in the literature. These inhibitors were used at concentrations previously demonstrated to block calpain activity (35, 38). Surprisingly, preincubation of REF-52 fibroblasts (Fig. 2) or Swiss 3T3 fibroblasts (data not shown) with E64d (100 μM) (Fig. 2*a*, *D*) or CI-1 (10 μM) (Fig. 2*a*, *E*) did not prevent the forskolin-induced disassembly of stress fibers. Since calpain is a calcium-dependent protease, we tried chelation of calcium, which is an effective method of inhibiting calpain activation. However, consistent with CI-1 and E64d, incubation of REF-52 fibroblasts with a combination of EGTA (1 mM) and Mg^{2+} (2 mM) (Fig. 2*a*, *F*) had no effect on the forskolin-induced disassembly of stress fibers. Finally and most convincingly, the introduction of recombinant calpastatin (the endogenous and highly specific inhibitor of calpain) into these cells did not block forskolin-induced events (Fig. 2*b*). These results suggested that calpain was not the target of calpeptin in these studies and thus not responsible for the disassembly of stress fibers under conditions of elevated intracellular cAMP or deprivation of serum.

Further evidence ruling out calpain as the target of calpeptin in these experiments came from studies in which we demonstrated that calpeptin could reverse stress fiber disassembly, even after forskolin treatment had been administered. REF-52 fibroblasts were incubated for 30 min with forskolin, conditions that would allow for the complete disruption of stress fibers (Fig. 2*c*, *A*). Calpeptin was then added to the culture medium, in the continued presence of forskolin, and the cells incubated for various time periods (Fig. 2*c*, *B–D*). Reappearance of stress fibers was evident as early as 10 min after calpeptin addition, and full recovery of stress fibers was achieved within 30 min. This result is inconsistent with the notion that calpeptin is inhibiting the activation of calpain, since proteolytic cleavage of calpain substrates cannot be "reversed." Furthermore, new protein synthesis could not be responsible for the restoration of stress fibers by calpeptin, since this process would not be significant in the time scale of this experiment. Taken together, these observations indicate that calpain is not the responsible protease regulating stress fiber disassembly and suggest that calpeptin is inhibiting some other protein(s) responsible for cytoskeletal remodeling.

Calpeptin Mediates Its Effects via the Inhibition of PTPases—Calpeptin is a dipeptide aldehyde whose mode of inhi-

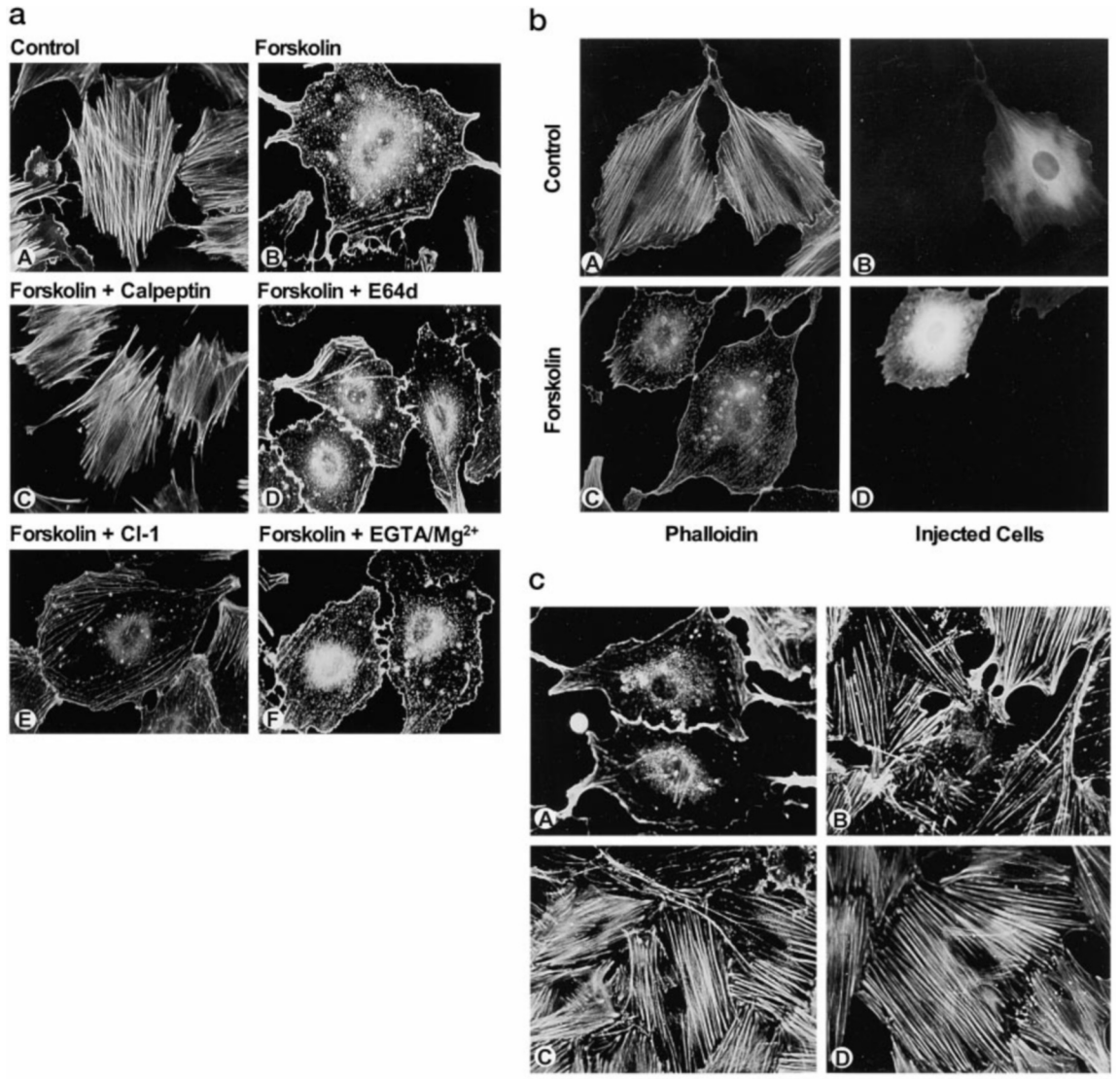

FIG. 2. **Calpeptin-mediated induction of stress fibers is not mediated through inhibition of calpain.** REF-52 fibroblasts cultured in complete DMEM were plated onto coverslips. *a*, cells were preincubated with vehicle (Me_2SO) alone (*A* and *B*), 100 μg/ml calpeptin (*C*), 100 μM E64d (*D*), 20 μM CI-1 (*E*), or 1 mM EGTA and 2 mM $MgCl_2$ (*F*) for 30 min. Cells were then treated with vehicle (Me_2SO) alone (*A*) or 10 μM forskolin (*B–F*) for 30 min. *b*, cells were injected with recombinant calpastatin (2.0 mg/ml) along with fluorescein-conjugated anti-human IgG to identify injected cells. Cells were then treated with vehicle (Me_2SO) (*A* and *B*) or forskolin (10 μM) for 30 min (*C* and *D*). *c*, all cells were treated with 10 μM forskolin for 30 min (*A–D*). Following this, 100 μg/ml calpeptin was added, and cells were incubated for 0 (*A*), 10 (*B*), 20 (*C*), or 30 (*D*) min. Cells were visualized by staining with rhodamine-conjugated phalloidin, as described under "Experimental Procedures."

bition is via binding the active site cysteine of calpain (48, 49). Since our results suggested that calpeptin was not promoting stress fiber formation through inhibition of calpain, it was necessary to determine other potential targets for the inhibitor. One other group of enzymes with a critical cysteine residue in their active site is the PTPases (50). To investigate the possibility that calpeptin may inhibit PTPases, we initially examined the tyrosine phosphorylation pattern in both REF-52 and Swiss 3T3 fibroblasts, under conditions where calpeptin had previously been demonstrated to induce stress fiber formation (see Fig. 1, *a* and *b*). We found that the tyrosine phosphorylation of several proteins was enhanced by calpeptin treatment (Fig. 3*a*). In particular, we noted a dramatic increase in tyrosine phosphorylation of a protein migrating as a broad band above the 64-kDa molecular mass marker. By performing immunoprecipitations of REF-52 cell lysates, we were able to identify this protein as paxillin (Fig. 3*b*), a 68-kDa adapter protein localized to focal adhesions. Paxillin has previously been demonstrated to undergo rapid dephosphorylation in response to intracellular cAMP elevation (20). Consistent with this, we also found that paxillin was rapidly dephosphorylated upon treatment with forskolin in REF-52 fibroblasts (Fig. 3*c*). Furthermore, in correlation with immunofluorescence studies demonstrating a calpeptin-mediated reversal of stress fiber disassembly in forskolin-treated cells (Fig. 2*c*), the dephosphorylation of paxillin was completely reversed upon the addition of calpeptin (Fig. 3*c*). These results are consistent with inhibition of a tyrosine phosphatase. Our attempts to determine the identity of other proteins migrating around 36–40 and 110–130 kDa that displayed changes in tyrosine phosphorylation (albeit minor) were not successful. We examined the tyrosine phosphorylation status of $pp125^{FAK}$ and $p130^{cas}$, which have previously been demonstrated to change in correlation with the regulation of stress fibers and focal adhesions (8, 51–57). We could not detect any significant changes in the tyrosine phosphorylation of either of these proteins in response to calpeptin. Therefore, it appears at least in this system that paxillin, but not $p130^{cas}$ or $pp125^{FAK}$, displays changes in tyrosine phosphorylation during the calpeptin-mediated induction of stress fibers.

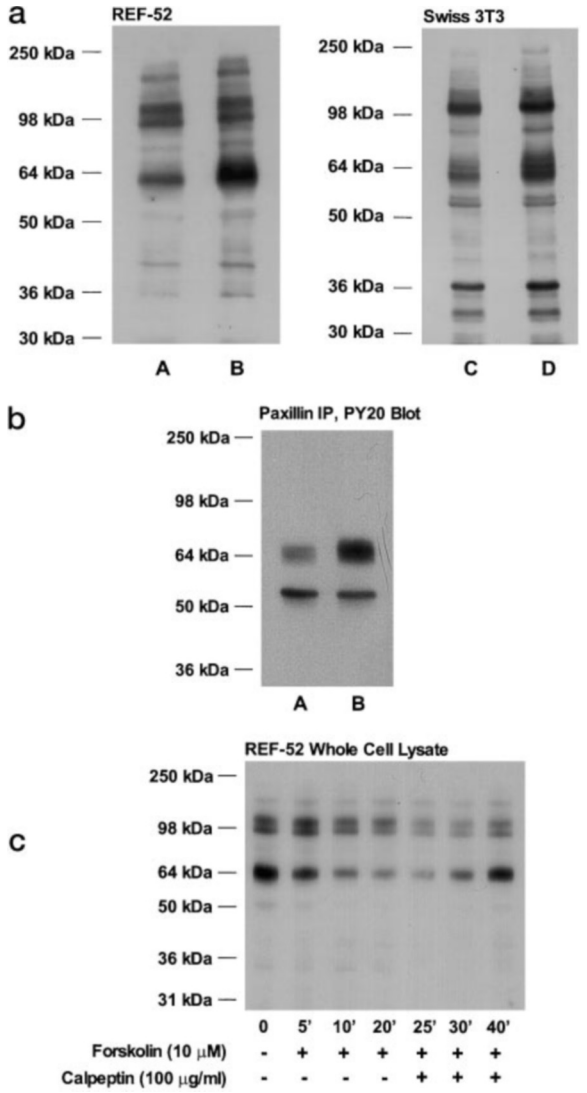

FIG. 3. **Calpeptin enhances tyrosine phosphorylation of cellular proteins, including paxillin.** REF-52 and Swiss 3T3 cells were cultured in complete DMEM. REF-52 cells were pretreated with vehicle (Me_2SO) alone (*A*) or 100 μg/ml calpeptin (*B*) for 30 min, followed by incubation with 10 μM forskolin for 30 min. Swiss cells were serum-starved for 24 h and then incubated with vehicle (Me_2SO) alone (*C*) or 100 μg/ml calpeptin (*D*) for 30 min. Whole cell lysates were prepared as described under "Experimental Procedures." *a*, equal amounts of protein were analyzed by 10% SDS-polyacrylamide gel electrophoresis, followed by immunoblotting with an antiphosphotyrosine antibody (PY20). *b*, equal amounts of proteins from REF-52 cell lysates were used to immunoprecipitate paxillin, as described under "Experimental Procedures." Immunoblot analysis of immunoprecipitates was performed using an anti-phosphotyrosine antibody (PY20). A lower band of approximately 55 kDa present in both lanes represents the mouse IgG heavy chain. *c*, REF-52 fibroblasts were incubated with 10 μM forskolin for the indicated times. Following 20 min of forskolin incubation, 100 μg/ml calpeptin was included in the cell medium. After the completion of the time course, whole cell lysates were prepared as described previously, and equal amounts of lysate were analyzed by immunoblotting using an anti-phosphotyrosine monoclonal antibody, PY20.

To confirm the ability of calpeptin to act as a PTPase inhibitor, we next examined its ability to inhibit PTPase activity in *in vitro* assays using an exogenous substrate. Whole cell lysates from Swiss 3T3 and REF-52 fibroblasts were prepared as described under "Experimental Procedures" and assayed for their ability to remove the radiolabeled phosphate from [^{32}P]poly-(Glu:Tyr). The addition of cell lysates to the assay resulted in the release of radiolabel from the substrate (Fig. 4, *a* and *b*, *DMSO*). However, when calpeptin was included in these assays, PTPase activity in both Swiss 3T3 and REF-52 fibroblast cell lysates was reduced by a small yet significant level (10–20%) (Fig. 4, *a* and *b*, 100 μg/ml). It is important to note that calpeptin was unable to fully inhibit the phosphatase activity in these cell lysates, even at higher concentrations (Fig. 4, *a* and *b*, 1.0 mg/ml). This suggests that calpeptin may not be a very efficient inhibitor of PTPases or, alternatively, that it may only target a subset of phosphatases in the whole cell lysate.

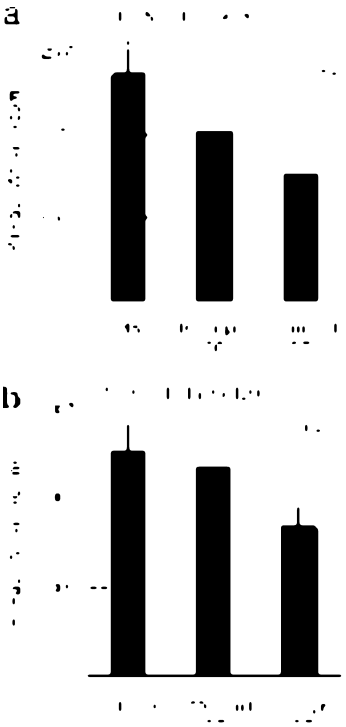

FIG. 4. **Calpeptin directly inhibits whole cell lysate PTPase activity *in vitro*.** Swiss 3T3 and REF-52 fibroblasts were cultured in complete DMEM. Whole cell lysates were prepared from forskolin-treated REF-52 fibroblasts (*a*) or serum-starved Swiss 3T3 cells (*b*) as described under "Experimental Procedures." *In vitro* PTPase assays were performed on equal amounts of protein from cell lysates as described, in the presence of vehicle (Me_2SO (*DMSO*)) alone or 100 μg/ml or 1.0 mg/ml calpeptin. All assays were performed within the linear range with respect to PTPase activity. Phosphatase activity is expressed as the amount of ^{32}P label released from [^{32}P]poly(Glu:Tyr) and reflects the mean ± S.D. from several independent experiments.

Calpeptin Preferentially Targets Membrane-associated PTPases—PTPases can be divided into two broad categories, the cytosolic enzymes and the transmembrane receptor type PTPases (58, 59). We examined the possibility that calpeptin may preferentially inhibit the activity of one of these categories by performing *in vitro* PTPase assays on cytosolic and membrane fractions prepared from REF-52 fibroblasts. Interestingly, calpeptin appeared to exert a greater inhibitory effect on the phosphatase activity present in the membrane fraction of REF-52 fibroblasts (79% inhibition) when compared with the cytosolic fraction (35% inhibition) (Fig. 5*a*). This suggests that calpeptin preferentially inhibits either transmembrane PTPases or, alternatively, PTPases that are localized to the membrane through their association with membrane proteins. Consistent with an action on transmembrane PTPases, we found that the effect of calpeptin on the phosphatase activity of GST-PTPα (Fig. 5*b*, *II*), a transmembrane PTPase, was greater than its effect on the cytosolic PTPase GST-PTP-1B (Fig. 5*b*, *I*). We also tested the ability of this inhibitor to block the activity of a phosphatase with dual specificity, PTEN, and found that calpeptin exerted a moderate inhibitory effect on PTEN (Fig. 5*b*, *III*). In addition, preliminary kinetic studies performed on the

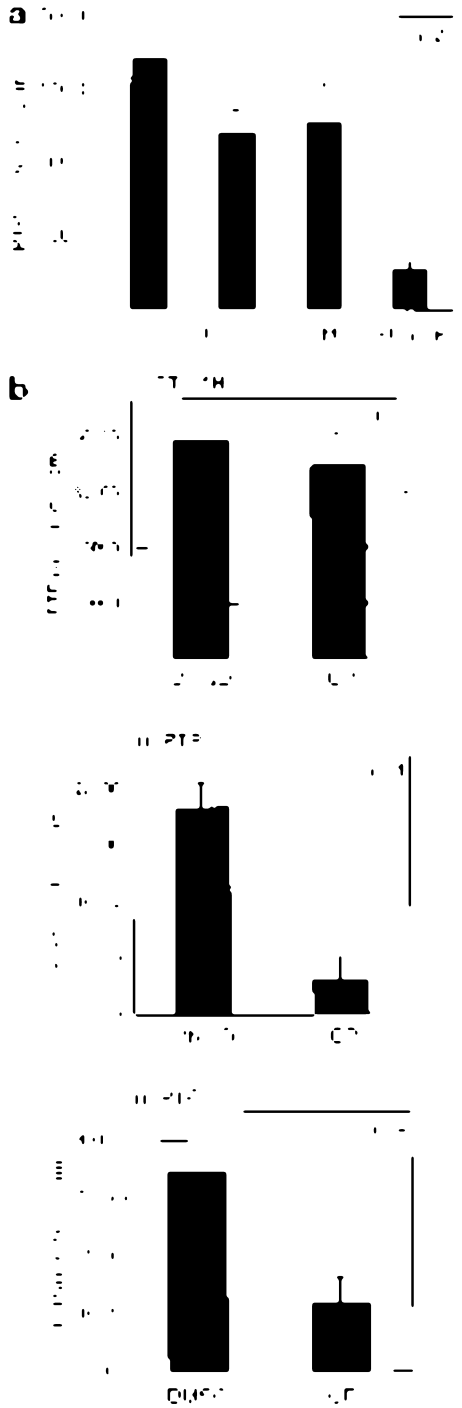

FIG. 5. **Calpeptin exerts greater inhibitory effects on membrane-associated PTPases *in vitro*.** Cytosolic (*Cyt*) and membrane (*PM*) fractions from forskolin-treated REF-52 fibroblasts (*a*) and GST-PTP-1B (*I*), GST-PTPα (*II*), and GST-PTEN (*III*) (*b*) were prepared as described under "Experimental Procedures." *In vitro* PTPase assays were performed in the absence or presence of calpeptin (100 μg/ml (*a*) or 1.0 mg/ml (*b*)) as described for Fig. 4.

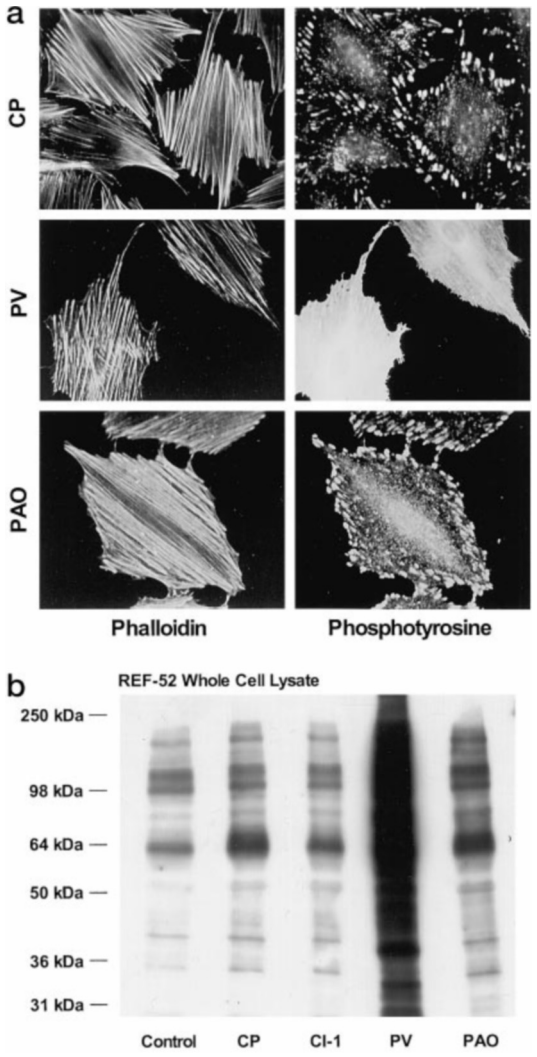

FIG. 6. **The effect of various PTPase inhibitors on forskolin-induced disassembly of stress fibers and protein-tyrosine phosphorylation.** REF-52 fibroblasts were cultured in complete DMEM on coverslips or 100-mm tissue culture dishes. Cells were pretreated with vehicle (Me_2SO) alone (control), 100 μg/ml calpeptin (*CP*), 10 μM CI-1, 50 μM pervanadate (*PV*), or 1 μM PAO for 30 min. Cells were then treated with 10 μM forskolin for 30 min. *a*, cells were visualized by double staining for actin and phosphotyrosine using rhodamine-conjugated phalloidin and a polyclonal antiphosphotyrosine antibody followed by a fluorescein-conjugated anti-rabbit secondary antibody. *b*, whole cell lysates were prepared as described under "Experimental Procedures," and equal amounts of proteins were analyzed by immunoblot analysis using an anti-phosphotyrosine antibody, PY20.

mode of calpeptin-mediated inhibition of PTPα suggested that inhibition is most likely competitive in nature (data not shown). This is similar to the mechanism of its inhibition of calpain and consistent with an active site-directed inhibitor.

Comparisons made between the calpeptin-mediated inhibition of PTPases and that of two widely used PTPase inhibitors (pervanadate and PAO) provided further evidence that calpeptin was selectively inhibiting a subset of PTPases. Both pervanadate (8, 9) and PAO (10, 11) have previously been demonstrated to induce stress fiber formation. In our cell system, pervanadate and PAO both prevented the forskolin-mediated disassembly of stress fibers in REF-52 fibroblasts (Fig. 6*a*, *PV* and *PAO*) and increased tyrosine phosphorylation of cellular proteins, including paxillin (Fig. 6*b*, *PV* and *PAO*). Pervanadate is an irreversible and universal inhibitor of PTPases (58), as demonstrated by its ability to induce an extremely high level of tyrosine phosphorylation (Fig. 6, *a* and *b*, *PV*). In contrast, PAO, which has been suggested to selectively inhibit membrane PTPases (61), targets only those PTPases containing vicinal thiol groups, and induces only a modest increase in tyrosine phosphorylation (Fig. 6, *a* and *b*, *PAO*). Calpeptin appeared to mimic the response displayed by PAO (Fig. 6, *a* and *b*, *CP*) and induced only a small increase in the tyrosine phosphorylation in REF-52 fibroblasts.

We have used a number of experimental strategies in an attempt to identify the calpeptin-sensitive membrane-associ-

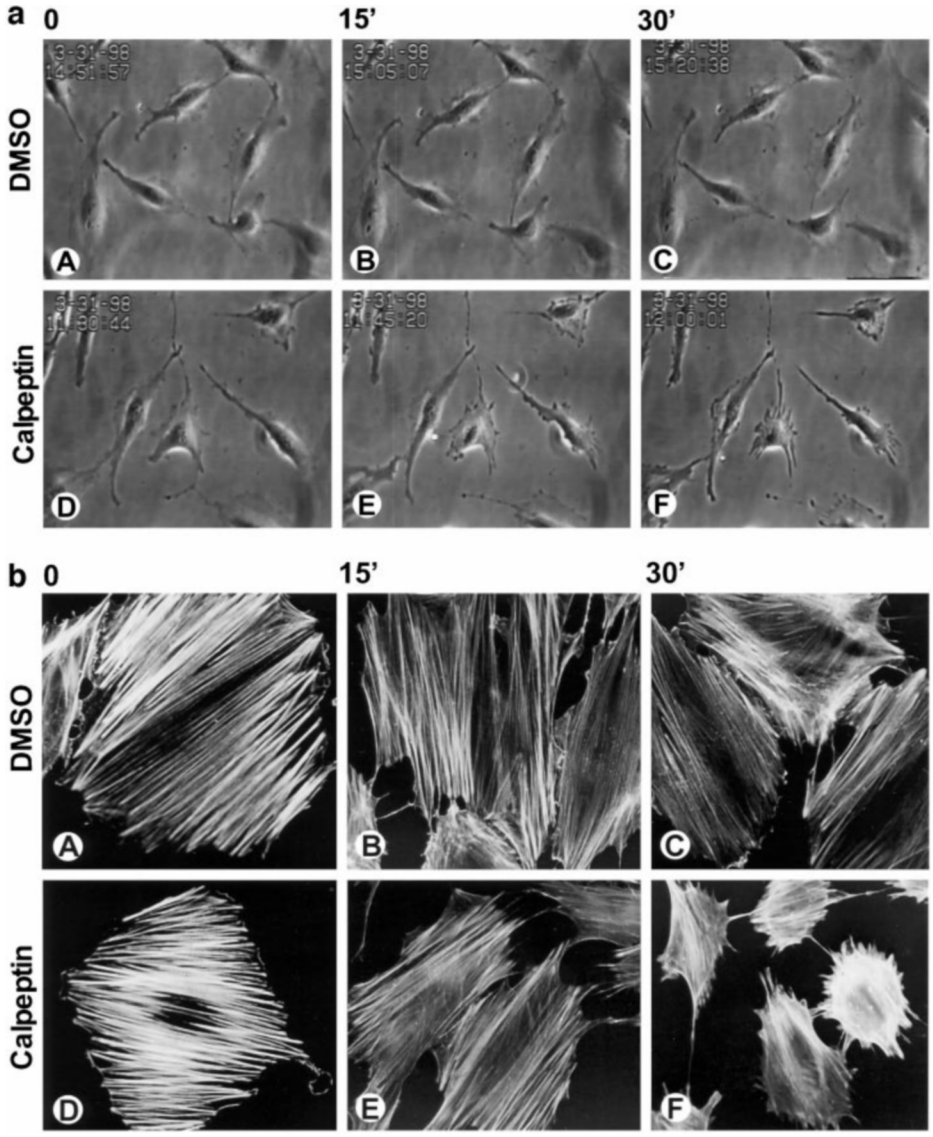

FIG. 7. **Calpeptin induces cell contractility.** REF-52 fibroblasts were cultured in complete DMEM on 60-mm tissue culture dishes or coverslips. Cells were treated with vehicle (Me_2SO (*DMSO*)) alone or 100 μg/ml calpeptin for 30 min. *a*, the morphology of live cells was monitored in the presence of 25 mM Hepes, pH 7.3, and recorded using video microscopy. *b*, cells cultured on coverslips were fixed and processed for fluorescence microscopy. Cells were visualized by staining for actin using rhodamine-conjugated phalloidin. *Panels* depict cells after 0, 15, and 30 min of incubation with Me_2SO (*A–C*) or calpeptin (*D–F*). Note the smaller "contracted phenotype" of the cells at 15 and 30 min.

ated PTPase. One of these was the PTPase in-gel assay (45), which provides a method of detecting phosphatase activity while identifying the molecular weight of the enzyme. We have tried to modify this assay to identify the calpeptin-sensitive PTPase by including calpeptin in the renaturation procedure. This technique relies on effective renaturation of the enzyme(s) following SDS-polyacrylamide gel electrophoresis, and although it works well for cytoplasmic PTPases, it is unfortunately ineffective for studying membrane PTPases, since they do not renature sufficiently to detect any significant activity. Therefore, if calpeptin is primarily inhibiting membrane PTPases, it is not surprising that we did not detect changes in any of the bands appearing in this assay.

Calpeptin Induces Cell Contractility—The formation of actin-rich stress fibers and the clustering of integrins and signaling proteins to focal complexes are hallmark responses of activation of the small GTPase Rho. Rho activation has also been shown to stimulate cell contractility (62, 63). Since calpeptin was able to induce the formation of stress fibers and focal adhesions, we were interested in determining whether calpeptin could also induce cell contractility. REF-52 fibroblasts were incubated with calpeptin for a time period of 30 min, and cells were monitored in real time using video microscopy (Fig. 7*a*). Alternatively, cells were fixed and stained at certain points throughout the same time course to examine their actin stress fibers (Fig. 7*b*). Fig. 7 depicts sequential frames, each 15 min apart and covering a length of 30 min. As demonstrated in the control, where the vehicle alone (Me_2SO (*DMSO*)) (Fig. 7*a*, *A–C*) was added to the medium, the morphology of the cells did not change dramatically over the 30-min time period. However, when calpeptin was added to the medium, a significant contraction of the cells occurred (Fig. 7*a*, *D–F*), which was evident even as early as 15 min. This contractile morphology is strikingly similar to that observed in Swiss 3T3 fibroblasts microinjected with constitutively activated recombinant Rho (64). Contractility of fibroblasts was also observed by Rhodamine-phalloidin staining of cells incubated with calpeptin at similar time points (Fig. 7*b*). These results suggest that calpeptin not only induces the formation of stress fibers but also regulates the contractile behavior of cells, both of which are associated with activation of Rho.

Calpeptin Inhibits a PTPase Upstream of Rho—Since the effect of calpeptin on cells appeared to mimic Rho activation, we next examined if the effects of calpeptin were being exerted upstream or downstream of Rho. The Rho inhibitor, C3 exotransferase, was microinjected into REF-52 fibroblasts, and cells were allowed to recover for 60 min. Within this time, cells injected with C3 lost their stress fibers as a consequence of Rho inactivation. Cells were then treated with calpeptin. If acting at a site upstream of Rho, we would expect that calpeptin

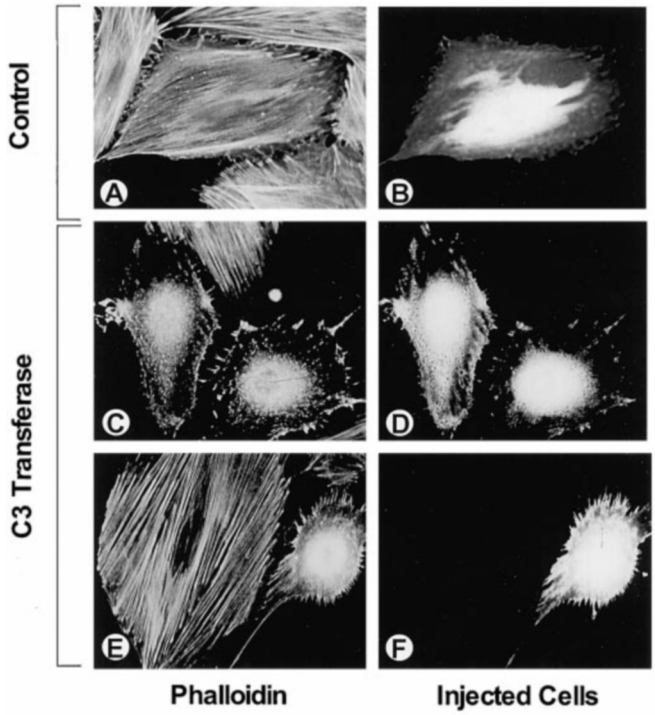

FIG. 8. **Calpeptin exerts its actions upstream of Rho.** REF-52 fibroblasts cultured in complete DMEM on coverslips were microinjected with fluorescein-conjugated anti-human IgG (*A* and *B*) or coinjected with fluorescein-conjugated anti-human IgG and 100 μg/ml C3 exotransferase (*C–F*), as described under "Experimental Procedures." Cells were allowed to recover for 60 min and then treated with 100 μg/ml calpeptin for 30 min. Cells were visualized by staining for actin using rhodamine-conjugated phalloidin. Injected cells were identified by positive fluorescein staining. Note that cells injected with C3 lack stress fibers even in the presence of calpeptin.

would not be able to bypass C3-inactivated Rho and induce stress fiber formation in injected cells. However, if calpeptin was acting downstream of Rho activation, stress fiber formation should be induced upon calpeptin treatment. As demonstrated in Fig. 8 (*C–F*), calpeptin was not able to reverse the disassembly of stress fibers induced by C3. This suggests that calpeptin is acting on a PTPase upstream of Rho. Studies are under way to identify the PTPase targeted by calpeptin.

DISCUSSION

The Rho family of small GTPases play an essential role in actin reorganization (3, 4). Therefore, their regulation has important implications for processes such as embryonic development, cell migration, and wound healing. The elucidation of signaling components regulating Rho and its family members is consequently integral to a better understanding of these basic cellular events. The studies presented here define a novel role for calpeptin, a widely used inhibitor of calpain, as an inhibitor of PTPases. In doing so, we have identified a calpeptin-sensitive PTPase activity that acts as a regulator of Rho and cytoskeletal reorganization. The calpeptin-sensitive PTPase described in this report induces a number of the characteristic responses associated with Rho activation, such as formation of stress fibers and focal adhesions, as well as the induction of cell contractility. Furthermore, its actions are blocked by the Rho inhibitor C3 exotransferase, suggesting that it is acting upstream of Rho. Although numerous guanine nucleotide exchange factors have been identified that act upstream of Rho to regulate its activation state, signaling events linking cell receptor stimulation and guanine nucleotide exchange factor activity are relatively unexplored.

Involvement of PTPases is not a new concept in the area of Rho regulation. Studies using various inhibitors of tyrosine kinases (genistein, erbstatin, tyrphostin 25) (5–7) or phosphatases (pervanadate, PAO) (8–11) have implicated tyrosine phosphorylation events both upstream and downstream of Rho activation. But information regarding the responsible enzymes mediating this regulation remains to be identified. Recent reports have provided some insight into the potential identity of some PTPases with the ability to regulate cell migration and stress fiber and focal adhesion formation. The tumor suppressor PTEN, a phosphatase with dual specificity, was identified as a regulator of cell migration and spreading (15). 2-Fold overexpression of PTEN down-regulated focal adhesions and stress fibers. However, whereas we did not detect any significant changes in $pp125^{FAK}$ tyrosine phosphorylation, a 60% decrease in the tyrosine phosphorylation of this protein was observed in response to PTEN expression, with no detectable changes in the tyrosine phosphorylation of paxillin. In addition, more recent studies suggest that the cellular effects of PTEN may not be the result of modulating the enzyme's tyrosine phosphatase activity but may be attributed to the phosphatidylinositol lipid phosphatase activity of PTEN (65–68). Yu *et al.* (13) have reported that cells deficient in functional SHP-2, a ubiquitous cytosolic PTPase, exhibit delayed spreading, reduced cell migration, an increased number of focal adhesions, and reduced $pp125^{FAK}$ dephosphorylation. However, deletion of SHP-2 from cells appears to have no dramatic effects on the tyrosine phosphorylation of paxillin, in contrast with the calpeptin-sensitive PTPase described in this report. Finally, recent studies have demonstrated that overexpression of PTP-1B, also a cytosolic PTPase, in 3Y1 rat fibroblasts not only resulted in impaired cell adhesion but also interfered with cell spreading, migration, cytoskeletal architecture, and focal adhesion formation (14). However, a separate study using different cell lines showed no effect on cell morphology when overexpressing PTP-1B (69). Instead, this latter study showed decreased focal adhesions when an inactive form of the PTPase was overexpressed.

A role for calpain in the regulation of PTPase activity has been reported. Calpain has been shown to proteolyze the cytosolic PTPases PTP-1B (32) and PTP-MEG (44), increasing their enzymatic activity. Although we demonstrate reduced PTPase activity with the addition of the calpain inhibitor calpeptin, several lines of evidence rule out calpain activation as responsible for changes in phosphatase activity in this report. First, we could not demonstrate a role for calpain in forskolin-induced or serum deprivation-induced disassembly of stress fibers, using any other calpain inhibitor available, including the highly specific calpastatin. Second, chelation of calcium, which inhibits calcium-dependent calpain activation, does not mimic calpeptin-induced cellular effects. Third, forskolin treatment or serum starvation of cells does not appear to induce the characteristic cleavage of PTP-1B (32, 38) associated with calpain activation.[2] Therefore, we can conclude that changes in PTPase activity seen in our studies are not the consequence of calpain activation.

The second important issue brought to light in this report concerns the specificity of calpeptin. The original design of calpeptin as a calpain inhibitor focused on manipulating the interaction formed between the active site of the protease and its substrates (48, 49). As is the case with other cysteine proteases, the protease domain of calpain contains a cysteine residue (Cys^{108}) that is essential for its proteolytic activity. This cysteine residue participates in the formation of a thioester intermediate between the protease and its substrate.

[2] S. M. Schoenwaelder and K. Burridge, unpublished observations.

Other residues surrounding the active site cysteine may also play some role in this process. This information was used to design an inhibitor that would (in theory) recognize calpain but not other cysteine proteases. Unfortunately, although calpeptin does exhibit higher specificity toward calpain than other calpain inhibitors that were produced in the past, when used at higher concentrations it has also been demonstrated to inhibit other cysteine proteases such as papain (48). We now report that calpeptin inhibits another species of enzyme containing an active site cysteine, the PTPases. We find that concentrations of calpeptin thought previously to be specific for calpain (20–100 μg/ml) also appear to inhibit a subset of PTPases both *in vitro* and *in vivo*. Preliminary kinetic analyses suggest that calpeptin's mode of PTPase inhibition is most likely competitive, similar to that of its inhibition of calpain. This new information highlights the dangers of relying solely on the results provided by a single pharmacological inhibitor.

In conclusion, we have identified a PTPase activity, associated with the membrane fraction of cells, which appears to play an important role upstream in the regulation of the small GTPase Rho. From our preliminary characterizations of this enzyme activity, it appears to effectively induce the formation of stress fibers, focal adhesions, and cell contractility, similar to the actions of Rho activators such as serum or lysophosphatidic acid. Although little is known about the identity of this membrane-associated phosphatase, we hope to utilize the novel application of calpeptin as a PTPase inhibitor to establish its identity and define some of the signaling components that lie upstream of Rho in the regulation of cytoskeletal reorganization.

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