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# Focal Adhesion and Stress Fiber Formation Is Regulated by Tyrosine Phosphatase Activity

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**Tyrosine phosphorylation of cytoskeletal proteins plays an important role in the regulation of focal adhesions and stress fiber organization. In the present study we examined the role of tyrosine phosphatases in this process using p125FAK and paxillin as substrates. We show that tyrosine phosphatase activity in Swiss 3T3 cells was markedly increased when actin stress fibers were disassembled by cell detachment from the substratum, by serum starvation, or by cytochalasin D treatment. This activity was blocked by phenylarsine oxide, an inhibitor of a specific class of tyrosine phosphatases characterized by two vicinal thiol groups in the active site. Phenylarsine oxide treatment of serum-starved cells induced increased tyrosine phosphorylation of p125FAK and paxillin in a dose-dependent manner and induced assembly of focal adhesions and actin stress fibers, showing that inhibition of one or more phenylarsine oxide-sensitive tyrosine phosphatases is a sufficient stimulus for triggering focal adhesion and actin stress fiber formation in adherent cells.** © 1996 Academic Press, Inc.

## INTRODUCTION

Cell adhesion to the extracellular matrix (ECM)<sup>2</sup> influences diverse cellular processes including cell migration and differentiation [1]. Cells in culture adhere to ECM components at discrete contact sites termed focal adhesions, dynamic structures containing clusters of integrins bound to the ECM and a large number of cytoplasmic proteins, some of which (e.g., talin, vinculin, and  $\alpha$ -actinin) appear to be mainly structural, while others (e.g., p125FAK, p60c-src, protein kinase

C, and tensin) are likely to play a role in the cellular response to extracellular signals [2–4].

The nature of the interaction between the components of focal adhesions is complex and little is known about how assembly and disassembly are regulated. The formation of focal adhesions involves members of the Rho family of GTP-binding proteins [5] and increased tyrosine phosphorylation of several focal adhesion-associated proteins such as p125FAK, paxillin, and tensin [6–11]. The tyrosine kinase p125FAK is a good candidate for regulating turnover of focal adhesions [12–15]. Tyrosine phosphorylation and recruitment to focal adhesion of p125FAK is an early event in the adhesion-induced signaling pathway [8, 11], and it has been shown that the level of tyrosine phosphorylation of this protein correlates with the assembly of focal adhesion and actin stress fibers [14].

The steady-state levels of tyrosine phosphorylation of substrate proteins are determined by the balance between the activities of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPases). While a great deal is known about the importance of the PTKs as regulators of key cellular events, much less information exists about the physiological role and regulation of PTPases, with one contributing factor being the difficulty in obtaining suitably phosphorylated purified substrates for assay. In addition to modulating PTK function [16], PTPases have been shown to play both positive and negative roles in diverse cellular processes including lymphocyte maturation and activation [17, 18], cell cycle control [19, 20], signal transduction [21–28], and development [29, 30]. Like the PTKs, the PTPases exist in both receptor-like and cytoplasmic forms [31, 32]. Although there is significant basal PTPase activity in cells, it has become apparent that the activities of PTPases are regulated in a sophisticated manner, and the idea that PTPases act constitutively to reverse the action of regulated PTKs has proved to be far from true [21, 33–36].

Two chemical inhibitors, phenylarsine oxide (PAO) and vanadate, have been used to study the role of PTPases in a variety of cell types. PAO, a trivalent

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<sup>2</sup> Abbreviations used: ECM, extracellular matrix; PTK, protein tyrosine kinase; PTPase, protein tyrosine phosphatase; PAO, phenylarsine oxide; CD, cytochalasin D; DTT, dithiothreitol; LPA, lysophosphatidic acid.

arsenical compound, is known to react with two thiol groups of closely spaced protein cystenyl residues to form stable dithioarsine rings. The complex cannot be reversed by monothiols, but in the presence of low-molecular-weight dithiols, such as 2,3-dimercaptopropanol or 1,4-dithiothreitol, the binding is competitively reversed [37–39]. Vanadate strongly inhibits numerous PTPases, presumably because many of the reactions catalyzed by these enzymes proceed via a transition state containing a penta-coordinate intermediate [40], and such a structure is much more stable for vanadate than phosphate [41]. The different mechanisms of action may explain why PAO and vanadate have different effects on some cell systems [42, 43].

Our previous studies [14] showed that inhibition of PTPases with vanadate or PAO rendered actin filaments partially resistant to cytochalasin D-induced disassembly, suggesting that PTPases might play a major role in the regulation of cell adhesion. We have now developed an assay for PTPase activity using p125FAK and paxillin as substrates. We show that conditions which lead to focal adhesion and actin stress fiber disassembly result in stimulation of phosphotyrosine phosphatase activity. Moreover, inhibition of this activity with the relatively selective covalent PTPase inhibitor PAO induces the formation of focal adhesions and actin fibers. These data suggest that inhibition of specific PTPases may be a major mechanism involved in focal adhesion organization during cell adhesion.

## MATERIALS AND METHODS

**Cell culture.** Mouse Swiss 3T3 cells were obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, UK) and cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Paisley, Scotland) supplemented with 10% fetal calf serum (Advanced Protein Products, Brierly Hill, West Midlands, UK), 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin.

**Cell treatments.** Stock solutions of PAO (10 mM), cytochalasin D (CD) (1 mg/ml), and lysophosphatidic acid (LPA) (1 mg/ml) (all from Sigma, Poole, Dorset, UK) were prepared in DMSO. A freshly prepared mixture of 1 mM sodium orthovanadate and 1 mM hydrogen peroxide in water, which yields vanadyl hydroperoxide ( $\text{Van}^+$ ), was also used. Immediately before each experiment, aliquots of the stock solutions were diluted in prewarmed serum-free DMEM and added to cell cultures for the indicated times.

**Antibodies.** FAK4 polyclonal antibody and FAK9.2 monoclonal antibody used in immunoprecipitation and in Western blotting detection of the p125FAK, respectively, were prepared in our laboratory as described previously [14]. Mouse monoclonal anti-phosphotyrosine antibody PY20 was purchased from Transduction Laboratories (Technology Drive, Nottingham, UK). A mouse monoclonal anti-vinculin antibody and a mouse monoclonal anti-paxillin antibody were generous gifts from Dr. V. E. Koteliansky (CNRS-Institut Curie, Paris, France) and Dr. C. E. Turner (State University of New York, Syracuse, NY), respectively. TRITC-conjugated goat anti-mouse IgG was purchased from Amersham (Aylesbury, UK).

**Indirect immunofluorescence microscopy.** For immunofluorescence studies, cells were grown to 90% confluence on circular (1-cm diameter) glass coverslips. After treatments, cells were washed

quickly with twice PBS, fixed for 10 min in 3.7% (v/v) paraformaldehyde in PBS, and permeabilized with 0.5% Triton X-100, 3.7% formaldehyde in PBS for 5 min. The coverslips were then incubated with 1% BSA in PBS for 30 min. Permeabilized cells were incubated at room temperature for 60 min with the appropriate primary antibody, washed five times with PBS, and stained with a mixture of TRITC-conjugated goat anti-mouse IgG (1:100) and FITC-conjugated phalloidin (from Sigma, for F-actin detection) (1:100) in PBS for 60 min. After five washes with PBS, the coverslips were mounted on glass slides in PBS-glycerol (1:1) and examined using a Zeiss Axiophot epifluorescence microscope. Photographs were taken on Ilford HP5 Plus film (ASA 400).

**Immunoprecipitation of phosphotyrosine-containing proteins.** Cell monolayers on 15-cm dishes were treated with 5  $\mu$ M PAO in DMEM for 10 min to inhibit tyrosine phosphatases [14]. After two washes with an ice-cold stop solution (5 mM EDTA, 10 mM NaF, 10 mM  $\text{Na}_4\text{P}_2\text{O}_7$ , 0.5 mM  $\text{Na}_3\text{VO}_4$  in PBS), the cells were detergent extracted in 700  $\mu$ l of lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.25% sodium deoxycholate, 5 mM EDTA, 10 mM NaF, 10 mM  $\text{Na}_4\text{P}_2\text{O}_7$ , 0.5 mM  $\text{Na}_3\text{VO}_4$ , 1  $\mu$ M PAO, 10  $\mu$ g/ml leupeptin, 4  $\mu$ g/ml pepstatin, and 0.1 U/ml aprotinin) (all from Sigma) and cell debris were removed by centrifugation (MSE microcentaur microfuge) at 13,000 rpm for 30 min at 4°C. Protein concentration of cell extracts was determined by the Bradford protein assay method using a commercially available kit (Bio-Rad Labs., Hercules, CA). To perform specific immunoprecipitations, the lysate was preabsorbed with a mixture of protein A and goat anti-mouse IgG coupled to agarose beads (both from Sigma) for 1 h at 4°C; the beads were then removed by centrifugation. For p125FAK immunoprecipitation, the FAK4 polyclonal antibody (1:200) was added to the supernatant, and immunocomplexes were bound to protein A coupled to agarose beads by incubation overnight at 4°C. When p125FAK and paxillin were coprecipitated, both anti-p125FAK polyclonal antibody (1:200) and anti-paxillin monoclonal antibody (1:25) were added to supernatant, and immunocomplexes were bound to a mixture of protein A and goat anti-mouse IgG coupled to agarose beads.

**Protein tyrosine phosphatase assay.** Cells (one 9-cm dish per point), treated for 10 min at 37°C as indicated, were washed twice with ice-cold PBS and scraped into 1 ml of ice-cold PTPase assay buffer [50 mM Hepes (pH 7.4), 0.5% Triton, 2 mM EDTA, 5 mM dithiothreitol (DTT), 10  $\mu$ g/ml leupeptin, 4  $\mu$ g/ml pepstatin, and 0.1 U/ml aprotinin]. In some experiments the sulfhydryl-containing compound DTT was omitted. After clarification at 13,000 rpm (20 min at 4°C), the supernatant was transferred to a new tube and protein concentration was measured by the Bradford protein assay method. Lysates were adjusted to 200  $\mu$ g/ml and tested for PTPase activities by monitoring their ability to dephosphorylate tyrosine-phosphorylated immunoprecipitated substrates (p125FAK or the mixture p125FAK-paxillin; see above) adsorbed to protein A-agarose. The immunoprecipitated substrates were washed in five changes of PTPase assay buffer and split in equal aliquots. The measurement of PTPase activity in cell lysates was performed in PTPase assay buffer by incubating, at 37°C, an aliquot of tyrosine-phosphorylated substrates with an appropriate dilution of cell lysates in a final volume of 250  $\mu$ l. After the indicated incubation time, the reaction mixture was put on ice and diluted by adding 1 ml of ice-cold PTPase stop solution (0.5% Triton, 0.1% SDS, 100  $\mu$ M  $\text{Na}_3\text{VO}_4$  in TBS). The substrates were then recovered by centrifugation, washed two times, and boiled in 70  $\mu$ l of Laemmli's buffer for 5 min. Western blotting was performed to determine residual tyrosine-phosphorylated substrates as described below. To test the direct effect of PAO on PTPase activity, cell lysates were pretreated for 5 min on ice with 5  $\mu$ M PAO prior to assay for phosphotyrosine phosphatase activity.

**Western blotting.** Total lysates or immunoprecipitates boiled in Laemmli's buffer were resolved in a 7% SDS-polyacrylamide gel and blotted onto nitrocellulose filters using a semidry apparatus (Novablot, Pharmacia) according to the manufacturer's instructions. The

filters were washed extensively with TBS-T (150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 0.3% Tween 20), blocked at 42°C for 1 h with 5% BSA in TBS-T, and incubated overnight with an anti-phosphotyrosine antibody (mAb PY20) diluted in TBS/1% BSA (1:3000). The filters were then washed three times with TBS-T, incubated for 2 h with anti-mouse IgG peroxidase conjugate (1:3500) (Sigma), and developed using an ECL detection method (Amersham, UK) as per the manufacturer's directions. Where necessary, the primary and secondary antibodies were stripped from the filter and the filters reprobed with an anti-p125FAK antibody (mAb FAK 9.2).

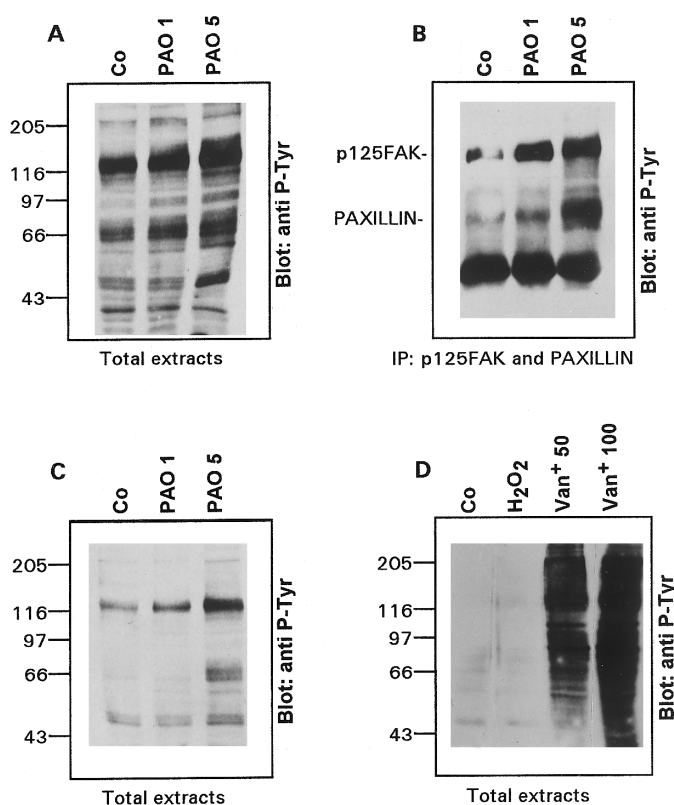
## RESULTS

### *Cytoskeleton Disassembly Leads to Activation of PTPases*

Changes in the tyrosine phosphorylation state of several cytoskeletal proteins correlate with changes in the structure of the actin cytoskeleton [14, 44, 45]. To test whether PTPases play a major role in regulating tyrosine phosphorylation during the assembly of focal adhesions, we developed a method to directly measure phosphotyrosine phosphatase activity on potential endogenous substrates such as the focal adhesion proteins p125FAK and paxillin. To prepare these substrates, confluent Swiss 3T3 cells were treated with the PTPase inhibitor PAO (5  $\mu$ M) for 10 min. This treatment results in a marked accumulation of tyrosine-phosphorylated p125FAK and paxillin, as detected by Western blot experiments (Figs. 1A and 1B). Tyrosine-phosphorylated p125FAK and paxillin were then immunoprecipitated from the cellular lysate and used as substrates in a cell-free PTPase assay (see Materials and Methods). In some assays p125FAK alone was used as PTPase substrate.

To define the experimental conditions for the assay, we initially measured the PTPase activity in lysates from adherent cells versus suspended cells. In suspended cells actin stress fibers are lost and p125FAK is almost completely dephosphorylated [11]. As shown in Figs. 2A and 2B, suspended Swiss 3T3 cells exhibited higher PTPase activity on p125FAK than adherent cells. This activity was largely inhibited either by PAO treatment of intact cells (Figs. 2A and 2B) or by direct addition of PAO to the cell lysates (not shown). The inhibition of PTPase activity by PAO was largely reversed by addition of dithiothreitol (Figs. 2A and 2B), consistent with the ability of PAO to react with two vicinal thiol groups, essential for the activity of some PTPases [38]. Dose-response (Figs. 2C and 2D) and time-course (not shown) experiments showed that the PTPase activity was linear within the experimental ranges. Thus, lysates of suspended cells have an enhanced ability to dephosphorylate p125FAK compared to lysates of adherent cells.

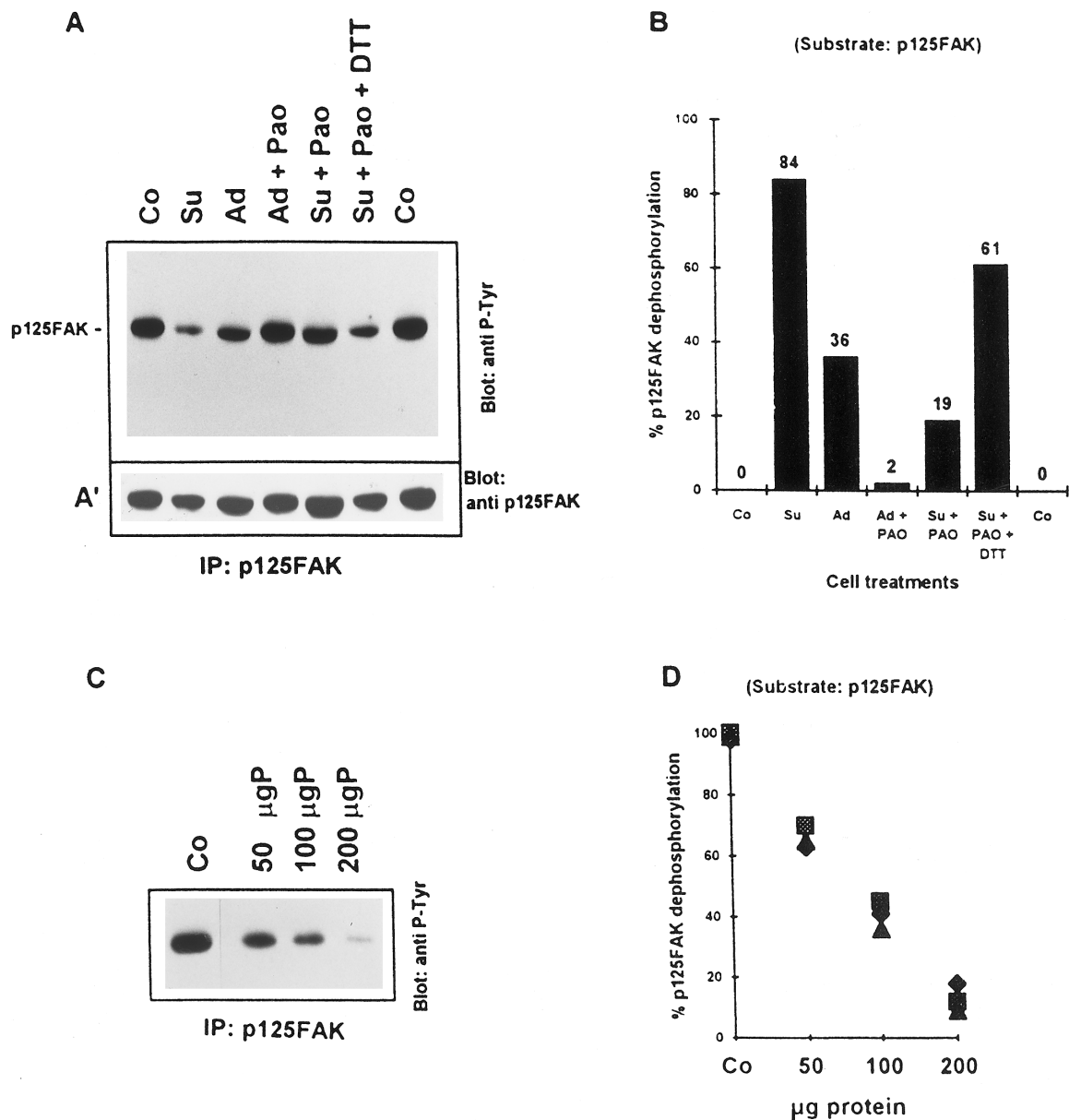
Culturing Swiss 3T3 cells overnight in serum-free medium results in the loss of actin stress fibers and vinculin/paxillin-containing focal adhesions without



**FIG. 1.** Serum- and PTPase inhibitor-dependent tyrosine phosphorylation of cellular proteins in Swiss 3T3 cells. (A) Cells maintained in 10% serum were treated for 10 min with the indicated micromolar concentrations of phenylarsine oxide (PAO) or with the carrier DMSO (Co), and cell lysates were Western blotted with the anti-phosphotyrosine antibody PY20. Molecular weight markers (kilodaltons) are indicated on the left. (B) The same cell lysates used in A were immunoprecipitated with a mixture of anti-p125FAK and anti-paxillin antibodies and Western blotted with the anti-phosphotyrosine antibody PY20. The positions of p125FAK and paxillin are indicated on the left. (C, D) Serum-starved cells were treated for 10 min with the indicated micromolar concentration of phenylarsine oxide (PAO), vanadyl hydroperoxide (Van<sup>+</sup>), or hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) or were left untreated (Co) and processed as indicated in A. Equal amounts of protein were loaded in each lane. The exposure time in A, B, and C was 2 min whereas in D it was 30 s. At the exposure level shown in D, bands on lanes Co and H<sub>2</sub>O<sub>2</sub> are not readily discernible.

cell detachment from the substratum [44, 46]. Serum deprivation also results in p125FAK and paxillin dephosphorylation [44; see also Fig. 1, compare A and C]. We, therefore, measured PTPase activity in control and serum-starved Swiss 3T3 cells. As shown in Figs. 3A and 3B, both p125FAK and paxillin were more rapidly dephosphorylated in the presence of lysates from serum-starved cells than in those from control cells maintained in 10% serum. The increase in PTPase activity in starved cells was inhibited by pretreatment of cells with PAO (not shown).

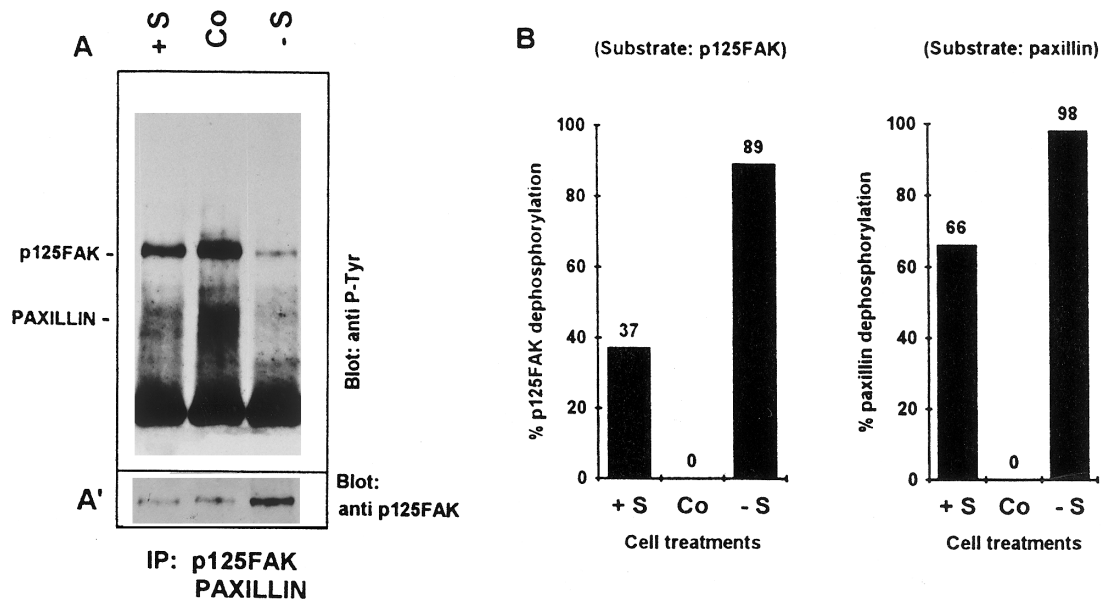
We also examined cells treated with CD, a drug that



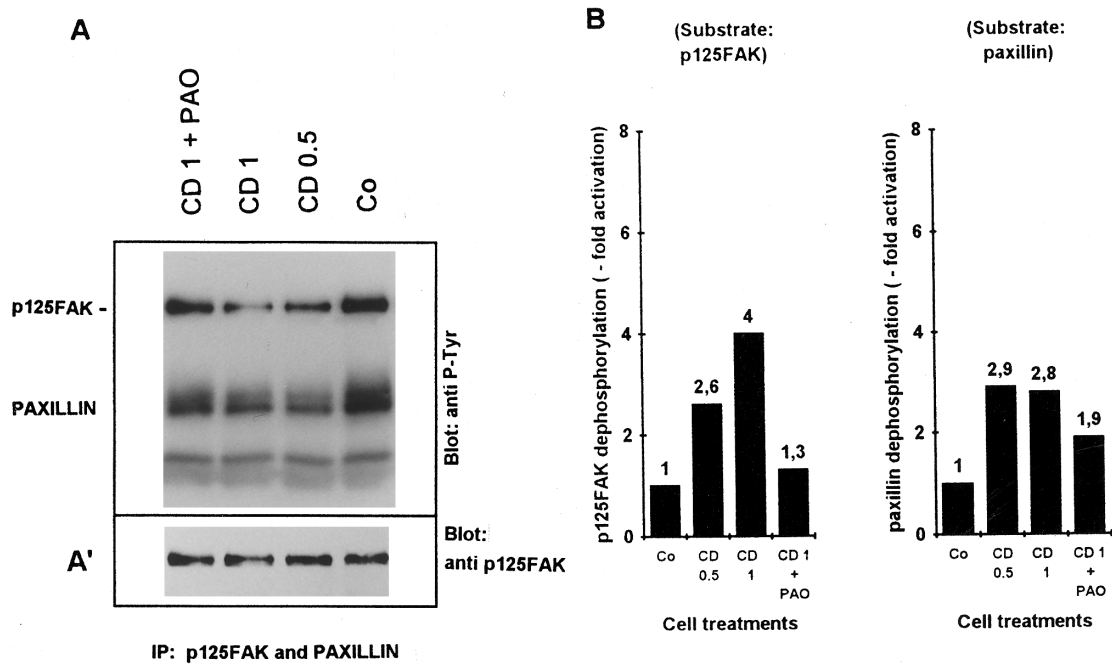
**FIG. 2.** *In vitro* PTPase assay on p125FAK substrate. (A) Swiss 3T3 cells in suspension (Su) or adherent on culture dish (Ad) were left untreated or treated for 10 min with 5  $\mu$ M PAO, alone or together with 1 mM DTT. To test PTPase activity cell lysates were incubated with immunoprecipitated p125FAK as described. The blot was immunostained with the anti-phosphotyrosine PY20. In the lanes indicated with "Co" (Control) the tyrosine phosphorylation level of p125FAK substrate in the absence of cell lysate is shown. (B) The diagram represents a densitometric analysis of the level of p125FAK dephosphorylation after *in vitro* PTPase assay. The results are plotted as percentage of controls (Co). (A') The blot shown in A was stripped and reprobed with the anti-p125FAK antibody, to test p125FAK loading in all lanes. (C) Dose response of PTPase activity. The immunoprecipitated p125FAK substrate was incubated for 10 min in a 250- $\mu$ l final volume with cell lysates containing the indicated protein concentrations and processed as in A. (D) Densitometric analysis of three different dose-response experiments are plotted as percentage of p125FAK dephosphorylation referred to control level (Co).

induces disassembly of the actin stress fibers and dephosphorylation of p125FAK [14, 47] without detaching cells from the culture dish. As shown in Figs. 4A and 4B, treatment of standard cell cultures with 0.5–1  $\mu$ M cytochalasin D for 10 min resulted in an enhanced PTPase activity in cellular lysates as measured by p125FAK and

paxillin dephosphorylation. This increase in PTPase activity was completely blocked by PAO (Figs. 4A and 4B). We conclude that disassembly of actin stress fibers is a necessary and sufficient condition for induction of a PAO-sensitive PTPase activity able to dephosphorylate focal adhesion proteins.



**FIG. 3.** Serum starvation in Swiss 3T3 cells leads to PTPase activation. (A) Cells were maintained in 10% serum (+S) or serum starved for 16 h (-S) and cell lysates were incubated with p125FAK and paxillin to test PTPase activity as described. The tyrosine phosphorylation levels of p125FAK and paxillin in the absence of cell lysates is shown (Co). (A') The blot has been immunostained with the anti-phosphotyrosine PY20, then stripped and reprobred with the anti-p125FAK antibody, to show loading in all lanes. (B) Densitometric analysis of the blot reported in A. The values are represented as percentage of p125FAK and paxillin dephosphorylation referred to control level (Co).



**FIG. 4.** Cytochalasin D treatment leads to PTPase activation. (A) Swiss 3T3 cells maintained in 10% serum were left untreated (Co) or treated with 0.5-1  $\mu$ M CD or with 1  $\mu$ M CD plus 5  $\mu$ M PAO. Cell lysates were then used for PTPase assay on the substrates p125FAK and paxillin as described. (A') The blot was immunostained with the anti-phosphotyrosine PY20, then stripped and reprobred with the anti-p125FAK antibody, to show loading in all lanes. (B) Densitometric analysis of the blot reported in A. The PTPase activity is represented as fold activation of p125FAK and paxillin dephosphorylation referred to control level (Co).

### PAO Induces Focal Adhesion Assembly and Stress Fiber Formation

Since the PTPase activity induced by disassembly of actin stress fibers is sensitive to PAO, we used this inhibitor to test whether modulation of PTPase activity can affect the organization of the actin cytoskeleton. Thus, we analyzed the effect of PAO in Swiss 3T3 cells in which stress fibers and focal adhesions were disassembled by serum starvation. As shown in Figs. 5A and 5B, serum-starved cells lack actin stress fibers and phosphotyrosine-containing focal adhesions. PAO treatment of these cells induced a dose-dependent formation of actin filaments and phosphotyrosine-containing focal adhesions (Figs. 5C–5F). Interestingly, at low doses (0.5–1  $\mu$ M) PAO stimulated a very rapid increase in phosphotyrosine clusters, observable within 5 min of addition (Fig. 5D). The response was maximal with 5  $\mu$ M PAO at 5–10 min after addition, when the phosphotyrosine clusters clearly colocalized with the ends of newly formed stress fibers (Figs. 5E and 5F). Vinculin (Figs. 5G–5J) and paxillin (not shown) staining for focal adhesions gave similar results.

We also tested the effect of PAO treatment on the level of tyrosine phosphorylation of p125FAK and paxillin. When serum-starved 3T3 cells were exposed to PAO, the tyrosine phosphorylation of p125FAK and paxillin was restored to levels close to those in cells grown in 10% serum (compare Figs. 1A and 1C). Vanadyl hydroperoxide, another known PTPase inhibitor, also allowed recovery of tyrosine-phosphorylated proteins in serum-starved cells (Fig. 1D) and induced focal adhesion and stress fiber assembly [44]. However, vanadyl hydroperoxide treatment induced a much broader profile of tyrosine-phosphorylated cellular proteins, compared to the more selective PAO (compare Figs. 1C and 1D). Thus, PAO treatment induces the organization of focal adhesions and stress fibers in serum-starved Swiss 3T3 cells as well as a parallel increase in p125FAK and paxillin tyrosine phosphorylation.

To examine the effects of PAO on CD-induced disruption of actin stress fibers and focal adhesions, Swiss 3T3 cells grown under standard conditions were treated with 1  $\mu$ M CD or with 1  $\mu$ M CD plus 1 and 5  $\mu$ M PAO for 10 min. CD-treated cells showed a loss of actin stress fibers and paxillin-containing focal adhesions (Figs. 6C and 6D) compared with untreated cells (Figs. 6A and 6B). In contrast, cells treated with CD plus PAO retained numerous paxillin-containing focal adhesions (Figs. 6F and 6H). Actin stress fibers were

also present, but were largely restricted to the cell margins and were notably absent from the central region of the cell (Figs. 6E and 6G).

Serum and LPA (a bioactive lipid component of serum) are known to stimulate the formation of focal adhesion and actin stress fibers in Swiss 3T3 cells [46]. We thus tested their ability to affect PTPase activity. As shown in Fig. 7, LPA treatment caused significant inhibition of the PTPase activity on p125FAK in serum-starved cells, although a greater inhibition was observed upon addition of serum.

These results show that inhibition of PAO-sensitive PTPases leads to focal adhesion assembly and stress fiber formation.

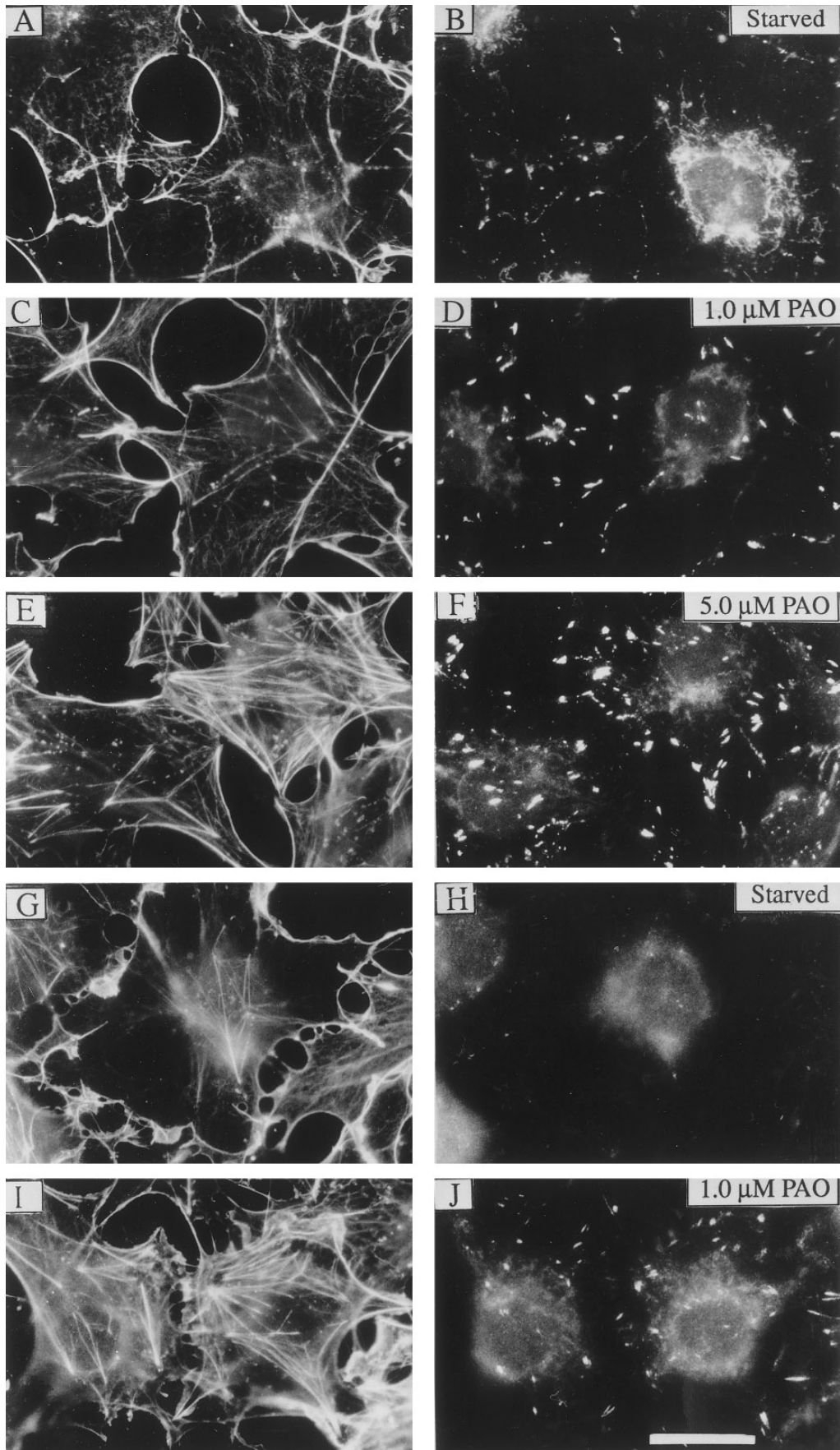
### DISCUSSION

In this report we describe an assay to measure PTPase activity on focal adhesion proteins p125FAK and paxillin and show that (i) PTPase activity is increased under conditions where actin stress fibers are disassembled; and (ii) inhibition of PTPases is a sufficient stimulus to drive focal adhesion and actin stress fiber organization.

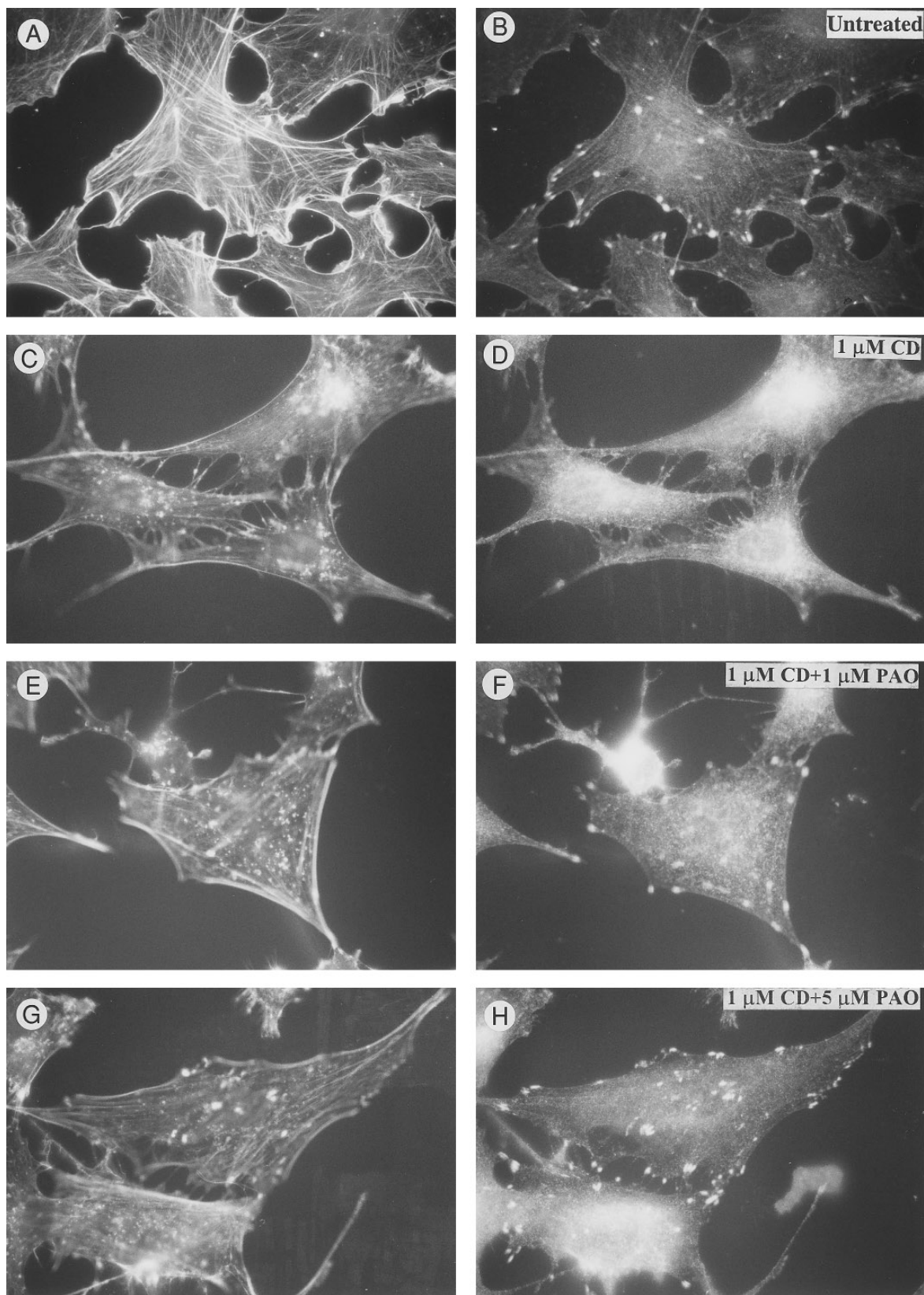
Most of the enzymology of PTPases has been deduced from measurements made with artificial substrates, either exogenous proteins or synthetic polypeptides. The PTPase activity on endogenous substrates is less well documented [38, 48]. In our studies, we chose p125FAK and paxillin as substrates for the assay of PTPase activity since these are major focal adhesion molecules that become tyrosine phosphorylated in response to different stimuli leading to focal adhesion and actin cytoskeleton assembly [11, 44, 49–51]. Moreover, these molecules are dephosphorylated when actin stress fibers are disassembled either by cell detachment from the substratum or by CD treatment [14, 47]. Tyrosine phosphorylation of p125FAK and paxillin is responsible for their interaction with molecules such as Src [12, 52], Grb2 [53], Csk [54], and Crk [55, 56]. Tyrosine phosphorylation of these molecules can thus trigger a cascade of molecular interactions leading to the organization of focal adhesion and actin fibers.

Based on the use of specific inhibitors, we previously suggested a prominent role for protein tyrosine phosphatases in the regulation of p125FAK tyrosine phosphorylation and cytoskeletal assembly during cell adhesion [14]. To further investigate this aspect, we have measured the PTPase activity under different conditions leading to the loss of actin stress fibers. Using

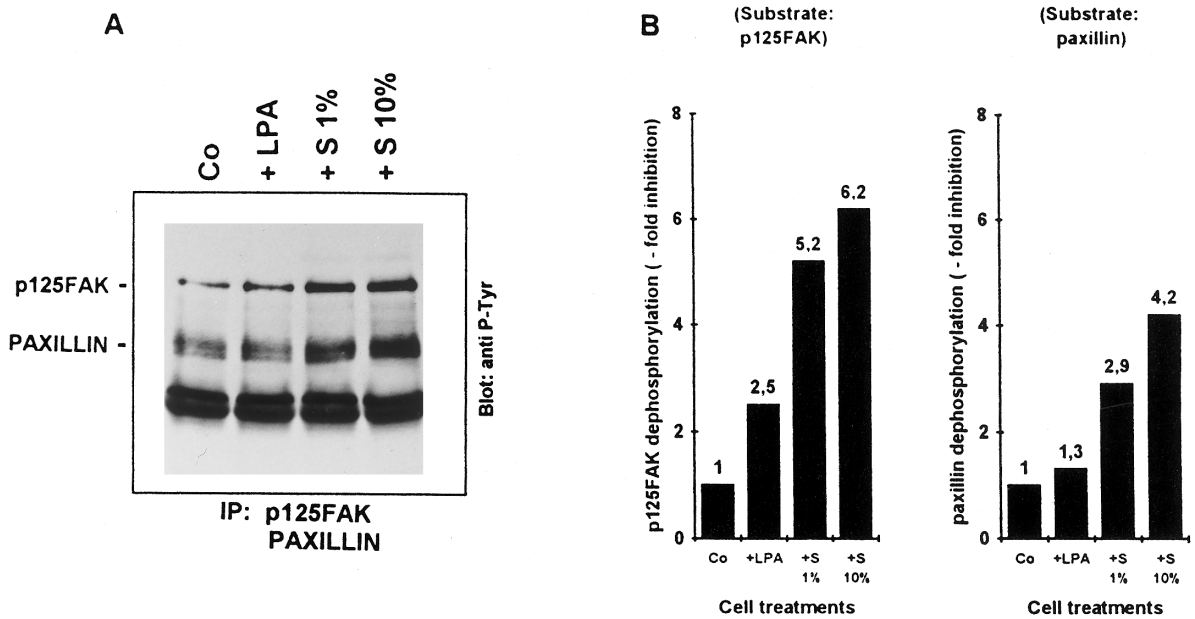
**FIG. 5.** PAO induces focal adhesion assembly and stress fiber formation. Swiss 3T3 cells cultured in serum-free medium for 16 h were treated for 10 min with fresh medium containing 1  $\mu$ M (C, D, I, J) or 5  $\mu$ M PAO (E, F) or the carrier (DMSO) (A, B, G, H). Cells were fixed, permeabilized, and then stained for F-actin (A, C, E, G, I), phosphotyrosine (B, D, F), or vinculin (H, J). Bar, 15  $\mu$ m.







**FIG. 6.** PAO protects focal adhesions from cytochalasin D-induced disassembly. Swiss 3T3 cells maintained in 10% serum were left untreated (A, B) or were treated for 10 min with 1  $\mu$ M cytochalasin D (C, D), 1  $\mu$ M cytochalasin D plus 1  $\mu$ M PAO (E, F), or 1  $\mu$ M cytochalasin D plus 5  $\mu$ M PAO (G, H). Cells were fixed, permeabilized, and then stained for F-actin (A, C, E, G) or paxillin (B, D, F, H).



**FIG. 7.** LPA or serum treatments lead to PTPase inhibition. (A) Lysates from serum-starved cells, untreated (Co) or treated with: 100 ng/ml LPA for 10 min (+LPA), 1% serum for 10 min (+S 1%), or 10% serum overnight (+S 10%), were assayed for PTPase activity on immunoprecipitated p125FAK and paxillin substrates as described. The blot has been immunostained with the anti-phosphotyrosine PY20. (B) Densitometric analysis of the blot reported in A. The values are represented as fold inhibition of p125FAK and paxillin dephosphorylation referred to serum-starved cells (Co).

the assay described above, we show that tyrosine phosphatase activity on p125FAK and paxillin is markedly increased after cell detachment from the substratum. In these cells the actin stress fibers are disassembled and cell-matrix interactions are lost. To determine which of these two events was responsible for PTPase activation we induced disassembly of actin stress fibers in adherent Swiss 3T3 by serum starvation. It was previously demonstrated that culturing Swiss 3T3 cells overnight in serum-free medium results in the loss of focal adhesions and actin stress fibers and in tyrosine dephosphorylation of p125FAK and paxillin [44, 46] without cell detachment from the substratum. In the present work we show that, in these cells, serum starvation also leads to an increase in cellular PTPase activity, indicating that the loss of actin stress fibers may be responsible for this event. It cannot be excluded, however, that withdrawal of growth factors during serum starvation might have contributed to this phenomenon. In fact, it was shown that some growth factors can induce tyrosine phosphorylation of p125FAK and paxillin and modulate actin cytoskeleton assembly [44, 57, 58]. To test this possibility we analyzed the PTPase activity in cells treated with cytochalasin D, a drug known to lead to cytoskeleton disassembly and p125FAK tyrosine dephosphorylation [47] without affecting cell attachment to the substratum. The fact that we found an increase in cellular PTPase activity in this system demonstrates that cell-matrix interaction and

growth factor deprivation are not directly involved in the increase in cellular PTPase activity. Rather, disruption of the actin cytoskeleton is a sufficient stimulus regulating cellular PTPase activity. These data add further insights to previous findings by Maher [59] showing increased PTPase activity in response to disruption of cell-substrate adhesion.

The PTPase activity detected in our system was sensitive to PAO, a relatively selective covalent inhibitor known to react with two thiol groups of closely spaced protein cystenyl residues to form stable dithioarsine rings [38, 60, 61]. The inhibition by PAO was shown to be specific since it can be competitively reverted by 1,4-dithiothreitol but not by monothiols. The PTPase activity on p125FAK and paxillin can also be inhibited by vanadate, a PTPase inhibitor with a much broader selectivity compared to PAO [14, 59, 60]. Thus, the PTPases implicated in the regulation of p125FAK and paxillin tyrosine phosphorylation belong to a restricted class of PTPases possessing vicinal cysteine residues at the active site.

We took advantage of the ability of PAO to block p125FAK and paxillin PTPases to test whether inhibition of this activity can affect cytoskeleton organization in cultured cells. When serum-starved Swiss 3T3 cells lacking actin stress fibers were treated with PAO, a dose-dependent increase in p125FAK tyrosine phosphorylation and assembly of focal adhesions and actin stress fibers were observed. Moreover, physiological

agents known to determine the increase in p125FAK and paxillin tyrosine phosphorylation and to stimulate the formation of focal adhesions and actin stress fibers, such as fetal calf serum and lysophosphatidic acid [44, 46], significantly reduced the cellular PTPase activity. We thus conclude that inhibition of one or more PAO-sensitive PTPases is sufficient to trigger focal adhesion and actin stress fiber formation in adherent cells. The identification of the nature of the PTPase(s) involved in this process will be a matter for future investigation. LAR phosphatase [62] is a possible candidate as suggested by its localization at focal adhesion sites.

Previous results suggest the involvement of tyrosine kinases in focal adhesion formation [8, 11, 45, 63–65]. The role of PTK has been mostly inferred by the use of specific inhibitors [8, 11, 46, 65] and only a modest increase in p125FAK or Src PTK activity has been detected in response to cell adhesion or stimuli leading to cytoskeleton assembly [66, 67]. Since tyrosine phosphorylation is a dynamic, reversible process, depending on the PTK and PTPase activities, the level of phosphate in target substrates depends on the balance between these enzyme activities. Our current data indicate that PTPases play a major role in regulating the tyrosine phosphorylation levels of p125FAK and paxillin during actin cytoskeleton assembly.

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