p125FAK tyrosine phosphorylation and focal adhesion assembly: studies with phosphotyrosine phosphatase inhibitors.

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p125FAK Tyrosine Phosphorylation and Focal Adhesion Assembly: Studies with Phosphotyrosine Phosphatase Inhibitors

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p125FAK is a major tyrosine kinase phosphorylated in response to integrin-dependent adhesion. In this study we use vanadate and phenylarsine oxide (PAO), known inhibitors of phosphotyrosine phosphatases (PTPases), as a tool to artificially modulate p125FAK phosphorylation in human endothelial and in Chinese hamster ovary cells. Vanadate treatment strongly upregulates in a dose-dependent manner the level of tyrosine phosphorylation of several proteins in adherent cells. PAO induces a more restricted profile of tyrosine-phosphorylated proteins, increasing primarily a broad band of 120–140 kDa. Maximal stimulation of p125FAK tyrosine phosphorylation is reached at 10 μM PAO. In contrast, in vanadate-treated cells the p125FAK tyrosine phosphorylation shows a biphasic curve, being increased at high doses of vanadate (100 μM) and downregulated at low doses (25 μM). Immunofluorescence analysis of cells treated with PTPase inhibitors showed a direct correlation between the level of p125FAK tyrosine phosphorylation and the assembly of focal adhesions and actin stress fibers. Downregulation of p125FAK tyrosine phosphorylation is observed by treating cells with cytochalasin D (CD), a drug known to rapidly disrupt the actin cytoskeleton. When PTPase inhibitors are added in combination to CD, the level of tyrosine phosphorylation of p125FAK remains high and focal adhesions and actin stress fibers are preserved from the CD-mediated disruption. Based on these data we suggest that assembly of actin cytoskeleton plays an important role in inhibiting PTPases involved in p125FAK tyrosine phosphorylation. © 1995 Academic Press, Inc.

INTRODUCTION

Integrins are α and β heterodimeric proteins which span the plasma membrane and connect the extracellular matrix to the cytoplasmic cytoskeleton. There exist 9 β subunits and 15 α subunits that can associate in various combinations to form receptors with different ligand binding specificity [1]. It is now well established that the integrin-mediated physical connection between the outside and the inside of the cells is not purely mechanical but leads to induction of integrin-dependent signaling pathways. Gene expression can be critically affected by cell–matrix interactions [2, 3]. Adhesion-induced early events involve pH variations, Ca^{2+} influx, potassium channel activation [4–6], and tyrosine phosphorylation of cytoplasmic proteins [7–17]. Activation of protein kinase C [18], p21ras [19], and MAP kinases [20] has also been reported.

The increasing number of proteins whose tyrosine phosphorylation is induced by integrin ligand binding suggests an important role for tyrosine kinases in integrin-mediated signal transduction pathways. It has been reported that tyrosine kinase inhibitors block the assembly of focal adhesion structures [10, 11], actin stress fibers organization [11], and laminin-induced neurite elongation [21] and inhibit integrin-induced gene expression [16]. The p125FAK (focal adhesion kinase) has been shown to be a major tyrosine-phosphorylated protein in the adhesion-induced signaling pathway in several cell types [7–11, 22]. Tyrosine phosphorylation of p125FAK is an early event in adhesion: it occurs rapidly after adhesion and persists at high levels as long as the cells remain attached to the substratum [11]. p125FAK tyrosine phosphorylation may thus be crucial to the organization of the adhesive structures.

In this work we analyzed tyrosine phosphorylation of the p125FAK kinase during cell–matrix interactions by using vanadate and phenylarsine oxide (PAO), two PTPase inhibitors. Vanadate ions are inhibitors of PTPases known to activate or inactivate receptor and nonreceptor protein tyrosine kinases [23]. Vanadate treatment induces phenotypic changes in several cell types, such as contraction of smooth muscle cells [24] or inhibition of differentiation of PC12 cells in response to NGF [25]. It has also been reported that vanadate can mimic insulin action directly, activating insulin receptor β subunit autophosphorylation [26]. PAO is a trivalent arsenic derivative that has been shown to inhibit tyrosine phosphatase activity in insulin signal-

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ing [27, 28] and in the T cell receptor-mediated tyrosine phosphorylation pathway [29]. In this work we used these two PTPase inhibitors to artificially increase tyrosine phosphorylation in adhesion-dependent signaling. These compounds regulate p125FAK tyrosine phosphorylation, allowing us to demonstrate a direct correlation between p125FAK tyrosine phosphorylation and assembly of focal adhesions. Moreover, we show that inhibition of PTPases prevents stress fibers disruption and p125FAK tyrosine dephosphorylation induced by actin depolimerization. These data suggest that tyrosine phosphatases play a major role in regulating cytoskeletal organization and p125FAK tyrosine phosphorylation during adhesion.

MATERIALS AND METHODS

Cell cultures. Human endothelial cells (HEC) were prepared from umbilical vein as described previously [30] and cultured in Medium 199 supplemented with 10% fetal calf serum (FCS) (Hyclone), 100 μg/ml bovine brain extract and 100 μg/ml porcine heparin (Sigma), 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were used for experiments between passages two and six. Chinese hamster ovary (CHO) cells were grown in Ham's F-12 medium with 10% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Cell treatments. Purified sodium orthovanadate (Na_3VO_4) was obtained from Sigma. A 50 mM stock solution was prepared by dissolving solid Na_3VO_4 in water. The pH of the stock solution was approximately 10 and at this pH vanadate is predominantly monomeric as HVO_4^{2-}. When the use of H_2O_2–vanadate is indicated, a freshly prepared solution of 1 mM Na_3VO_4, 2 mM H_2O_2 was added to the cells. Stock solutions of cytochalasin D (Sigma) (1 mg/ml) and phenylarsine oxide (Sigma) (100 mM) were prepared in DMSO. Immediately before each experiment aliquots of the stock solutions were diluted in prewarmed culture medium in the presence of 20 mM Hepes, pH 7.4, and added to confluent cell cultures for the indicated times. The cells were then washed twice as indicated [11] and extracted in lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris–Cl, pH 8, 5 mM EDTA, 10 mM NaF, 10 mM $Na_4P_2O_7$, 0.4 mM Na_3VO_4, 10 μg/ml leupeptin, 4 μg/ml pepstatin, and 0.1 trypsin inhibitory unit/ml aprotinin) (all from Sigma).

Adhesion experiments. Tissue culture plates were coated with 10 μg/ml purified fibronectin (FN) by overnight incubation at 4°C and postcoated with bovine serum albumin (Sigma) for 1 h at 37°C. FN was purified from human plasma by affinity chromatography on gelatin–Sepharose as previously described [31]. Poly-L-lysine (PL) (Sigma) was used at 10 μg/ml. Cells at confluence were treated with 100 μM vanadate for 16 h, detached by EDTA treatment (5 mM) in PBS for 10 min, washed twice in PBS containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, resuspended in prewarmed DMEM medium containing 100 μM vanadate, and immediately plated on the tissue culture plates for the indicated times. At the end of the incubation, the cells were washed twice and extracted as indicated above.

Detection of phosphotyrosine containing proteins and p125FAK by Western blotting. Protein concentration was determined in each cell extract by the Bio-Rad protein assay method based on the Bradford dye-binding procedure (Bio-Rad, GmbH). Samples containing equal amounts of proteins were separated on 6% polyacrylamide gel electrophoresis in the presence of SDS (SDS–PAGE) under reducing conditions. Proteins were transferred to nitrocellulose using a semidry apparatus (Novablot, Pharmacia) according to manufacturer's instructions. The blots were incubated 1 h at 42°C in 5% BSA in TBS-T (150 mM NaCl, 20 mM Tris–Cl, pH 7.4, 0.3% Tween), washed with TBS-T, and incubated overnight in antiphosphotyrosine Mab PT66 (Sigma) or anti p125FAK Mab (see below) in TBS, 1% BSA. The blots were washed three times with TBS-T, incubated 2 h with anti-mouse IgG peroxidase conjugate (Sigma), and washed two times. Phosphotyrosil containing proteins were visualized by the ECL chemiluminescent detection method (Amersham, UK). Conditions of the development with the chemiluminescent substrate and exposure times were set in order to obtain a linear response.

Antibodies to p125FAK and immunoprecipitation. The antiserum FAK4 to p125FAK was prepared in our laboratory by immunizing a rabbit against a synthetic peptide reproducing an amino acid sequence from the carboxy terminus of mouse p125FAK [32]. The peptide KDVIDQARLKMLGQTRPH was synthesized by solid-phase methods by a LKB Biolynx synthetizer (Pharmacia Biotechnology) and coupled to keyhole limpet hemocyanin using glutaraldehyde. The rabbit was immunized with 500 μg of the conjugate in complete Freund's adjuvant. From both human and hamster cells the antiserum immunoprecipitates a tyrosine phosphorylated protein of 125 kDa comigrating with p125FAK detected with BC3 antiserum (gift of Dr. T. Parsons, University of Virginia). A monoclonal antibody to p125FAK was obtained by injecting Balb/c mice with a recombinant protein from amino acid residues 103 to 553 of chicken FAK expressed in the plasmid vector pQE32 of the QIAexpress vectors (QUI-AGEN, Ca). The chicken FAK cDNA used to prepare the recombinant protein was a kind gift of Dr. T. Parsons. In immunoprecipitation experiments equal amounts of cell extracts were immunoprecipitated, and immunocomplexes were bound to protein A–Sepharose beads and recovered by centrifugation. Bound material was eluted by boiling beads in 1% SDS and subjected to SDS–PAGE under reducing conditions as described above. After transfer to nitrocellulose, blots were incubated with MAb PT66 to phosphotyrosine as described above.

Fluorescence microscopy. For immunofluorescence microscopy, acid-washed glass coverslips were coated with 10 μg/ml FN overnight or left untreated. Cells were plated for 3 h in the appropriate medium without serum and subjected to the indicated treatment. When the cultures were performed over 16 h, 2% FCS was added to the medium. The cells were fixed in 3% paraformaldehyde, 60 mM sucrose in PBS for 10 min, permeabilized for 1 min at 4°C in TBS containing 0.5% Triton X-100, and washed twice with TBS. Cells were stained for 1 h at room temperature with primary antibodies, washed, and exposed to appropriate rhodamine-conjugated secondary antibodies. Actin fibers were visualized with fluorescein-labeled phalloidin (Sigma). The antibodies used were: Mab 7F9 to vinculin (gift of Dr. V. Kotelianski, CNRS-ENS, Paris, France), Mab LM534 to β1 integrin, and Mab PY20 to tyrosine phosphorylated molecules (Transduction Laboratories). The coverslips were mounted in PBS:glycerol, 1:1, and viewed on a Olimpus BH2-RFCA fluorescence microscope. Micrographs were taken on a Kodak 400 film.

RESULTS

Tyrosine Phosphorylation of p125FAK Is Upregulated by PTPase Inhibitors

We have previously shown that in endothelial cells adhesion to extracellular matrix induces tyrosine phosphorylation of the tyrosine kinase p125FAK [11]. Since induction of tyrosine phosphorylation is the result of the balance between activation of protein tyrosine kinases and protein tyrosine phosphatases, we used vanadate and PAO as potent inhibitors of tyrosine phosphatases to assess their potential role in p125FAK tyrosine phosphorylation. Conditions of treatment for
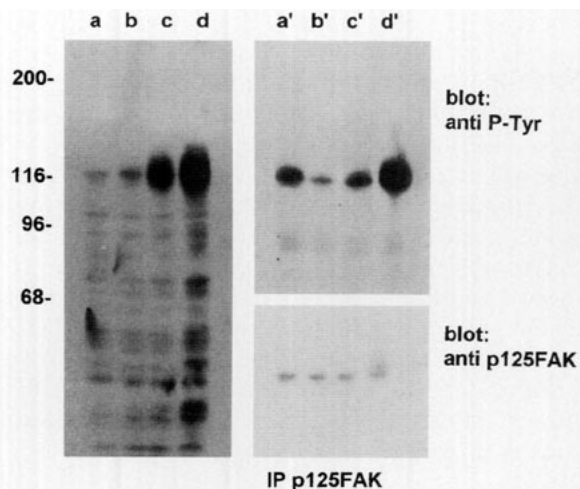
FIG. 1. p125FAK tyrosine phosphorylation in vanadate-treated HEC. Confluent cells were treated with 25, 50, or 100 μM orthovanadate for 16 h (b–d, b'–d') or left untreated (a and a'). Cells extracts (a–d) and molecules immunoprecipitated with FAK4 polyclonal antibody (a'–d') were separated on 6% SDS–PAGE and analyzed by Western blotting with antiphosphotyrosine Mab PT66 (left and top right) and anti-p125FAK Mab (lower right). In lane d' (lower right) the anti-p125FAK Mab reacts poorly with the protein which migrates with a slightly different molecular weight. This may reflect heavy tyrosine phosphorylation in these samples. The positions of molecular weight markers are indicated.

both of the inhibitors were defined in preliminary experiments. Confluent endothelial cells were treated with increasing doses of vanadate for 16 h. CHO cells were treated with doses ranging from 1 to 50 μM PAO for 15 min. Cell extracts, or immunoprecipitated p125FAK kinase, were run on SDS–PAGE and proteins were transferred to nitrocellulose to analyze phosphotyrosine contents. In vanadate-treated cells the level of tyrosine phosphorylated proteins on total cell extracts was increased in a dose-dependent manner and a consistent induction in tyrosine phosphorylation of proteins of molecular mass 100–130 kDa was observed (Fig. 1, lanes a–d). The analysis of p125FAK showed that tyrosine phosphorylation of this molecule was differently regulated by vanadate ions. Low doses of vanadate (25 μM) induced tyrosine dephosphorylation of p125FAK to levels lower than those found in cells adherent on the culture dish at confluence (Fig. 1, lanes a', b'). On the contrary, treatment with 100 μM vanadate strongly enhanced the p125FAK tyrosine phosphorylation (Fig. 1, lane d'). This bimodal regulation was specifically observed for p125FAK since tyrosine phosphorylation of 100- to 130-kDa proteins, comigrating with p125FAK, was linearly increased by vanadate treatment (Fig. 1 lanes a–d).

Treatment of CHO cells with 10 μM PAO induced a more restricted profile of tyrosine phosphorylated proteins in total cell extract, upregulating predominantly

a broad band migrating with apparent molecular weight of 120–140 kDa (Fig. 2, lanes a and b). In contrast, cells treated with 50 μM PAO did not show an increase of tyrosine phosphorylation (Fig. 2, lane c). This was not due to a toxic effect, since 50 μM PAO treatment did not affect cell viability (data not shown). Immunoprecipitation experiments showed that the band of 120–140 kDa contains the p125FAK kinase (Fig. 2, lanes a'–c'). Maximal induction of p125FAK tyrosine phosphorylation was reached at 10 μM PAO (Fig. 2, lane b'). Treatment of endothelial cells with 10 μM PAO gave similar results (data not shown).

These data show that PTPase inhibitors regulate tyrosine phosphorylation of p125FAK and suggest that the balance between PTPases and kinase activity can be differently modulated by their concentrations.

Vanadate Treatment Increases Integrin-Mediated p125FAK Tyrosine Phosphorylation

To further investigate the role of PTPases in adhesion-dependent tyrosine phosphorylation, we analyzed the phosphorylation of the p125FAK kinase in vanadate-treated cells plated either on FN or on PL as a nonspecific substrate. Endothelial cells were left untreated or treated with 100 μM orthovanadate for 16 h, detached from the dishes, and plated in the presence or absence of the same inhibitor for 30 min. Vanadate treatment caused an increase of the basal level of p125FAK tyrosine phosphorylation (Fig. 3, compare
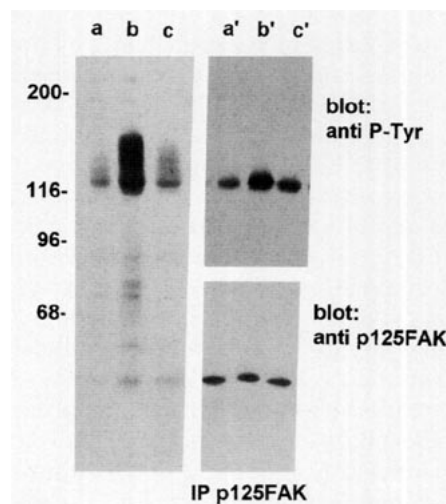
FIG. 2. p125FAK tyrosine phosphorylation in CHO cells treated with PAO. Confluent cells were left untreated (a and a') or treated for 15 min with 10 μM (b and b') or 50 μM PAO (c and c'). Cells extracts (a–c) and molecules immunoprecipitated with FAK4 polyclonal antibody (a'–c') were separated on 6% SDS–PAGE and analyzed by Western blotting with antiphosphotyrosine Mab PT66 (left and top right) and anti-p125FAK Mab (lower right). The positions of molecular weight markers are indicated.
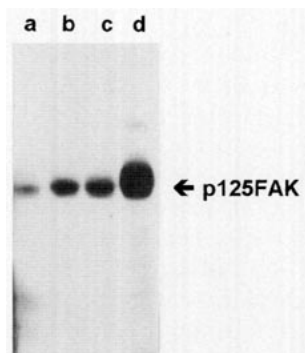
FIG. 3. Vanadate treatment increases FN-dependent p125FAK tyrosine phosphorylation. Untreated HEC (a and b) and HEC treated with 100 μM vanadate for 16 h (c and d) were detached and plated on PL (a and c)- or FN (b and d) -coated dishes for 30 min. In c and d 100 μM vanadate was also added during adhesion. Cell extracts immunoprecipitated with FAK4 polyclonal antibody were separated on 6% SDS–PAGE and the tyrosine phosphorylation of the p125FAK was visualized by Western blotting with Mab PT66. The position of the p125FAK is indicated by the arrow.

lanes a and c) as well as a stronger FN-dependent stimulation (Fig. 3, compare lanes b and d). Densitometric analysis showed that FN induces a 10-fold increase of p125FAK tyrosine phosphorylation in vanadate-treated cells, while only a 3-fold increase is observed in untreated cells. These data indicate that inhibition of PTPases by vanadate increases adhesion-dependent p125FAK tyrosine phosphorylation.

Focal Adhesion and Actin Stress Fibers Assembly Is Regulated by Vanadate Treatment

By immunofluorescence experiments we previously described colocalization of $\beta 1$ integrin, phosphotyrosil proteins, and p125FAK kinase at focal contacts [11]. We analyzed the effect of vanadate treatment during cell attachment to FN or on confluent monolayers. Endothelial cells treated for 16 h with 100 μM vanadate were plated on FN-coated coverslips in the presence of vanadate. Focal adhesion structures were analyzed 3 h after adhesion with antibodies directed to the $\beta 1$ integrin or the focal adhesion protein vinculin. Treated cells formed a higher number of focal adhesions compared to untreated cells (Fig. 4, compare a and g to c, e, and h). Vanadate-treated cells acquired a round shape and focal adhesions were radially distributed at the cell periphery. Actin also was preferentially polymerized in circumferential fibers (Fig. 4, compare b, d, and f). These results indicate that vanadate treatment

does not modify cell capacity to adhere to FN but it induces a distinct pattern of actin organization and enhanced ability to assemble adhesion plaques on FN.

We also evaluated the effect of vanadate treatment on endothelial cells grown on glass coverslips, which already organized their adhesive structure. The general morphology of the cells was not affected by the treatments, the cells remained flat and spread and organized focal contacts (Fig. 5). The focal adhesion structures were prominent in cells treated with 100 μM vanadate, but severely reduced in cells treated with 25 μM (Fig. 5 compare b, a, and d). Thus, low doses of vanadate, which lead to downregulation of p125FAK tyrosine phosphorylation (see Fig. 1, lane b'), induce a partial disassembly of focal adhesions.

*Cytochalasin D Downregulates p125FAK Tyrosine Phosphorylation*

Cytoskeletal integrity has been shown to affect p125FAK tyrosine phosphorylation induced in neuropeptide-treated fibroblasts [33] and during platelet aggregation [34, 35]. We treated confluent endothelial cells for 15 min with cytochalasin D (CD), a drug known to induce F-actin depolimerization. The tyrosine phosphorylation of p125FAK was reduced linearly in a dose-dependent manner (Fig. 6A), 0.6 μM CD being the lowest active concentration. CD was also capable of preventing induction of p125FAK tyrosine phosphorylation during adhesion to FN (not shown). We analyzed the cytoskeletal structures in CD-treated endothelial cells. The actin stress fibers were only partially disrupted at 0.3 μM CD and completely disorganized at concentrations higher than 1.25 μM (Fig. 6B).

These results indicated that in endothelial cells p125FAK tyrosine phosphorylation is impaired by actin depolimerization.

PTPase Inhibitors Counteract CD Action on Cytoskeletal Structures

To test whether PTPases may play a role in the CD-induced reduction of p125FAK tyrosine phosphorylation, endothelial cells were treated with 1 μM CD in the presence or absence of H_2O_2-vanadate. The combination of H_2O_2 and vanadate has been shown to lead to PTPase inactivation within 15 min compared to the 16 h required by vanadate alone [36 and this work]. This allows us to analyze vanadate effect at the time of CD action. The addition of H_2O_2-vanadate completely inhibited the downregulation of p125FAK tyrosine

FIG. 4. Vanadate treatment increases assembly of focal adhesion on FN. HEC were treated with 100 μM orthovanadate for 16 h (c, d, e, f, and h) or left untreated (a, b, and g), detached, and plated on FN-coated glass coverslips. At 3 h of adhesion cells were fixed, permeabilized, and stained with Mab LM534 to $\beta 1$ integrin (a, c, and e), double-labeled for F-actin (b, d, and f) or with Mab 7F9 to vinculin (g and h). Bar, 10 μM.
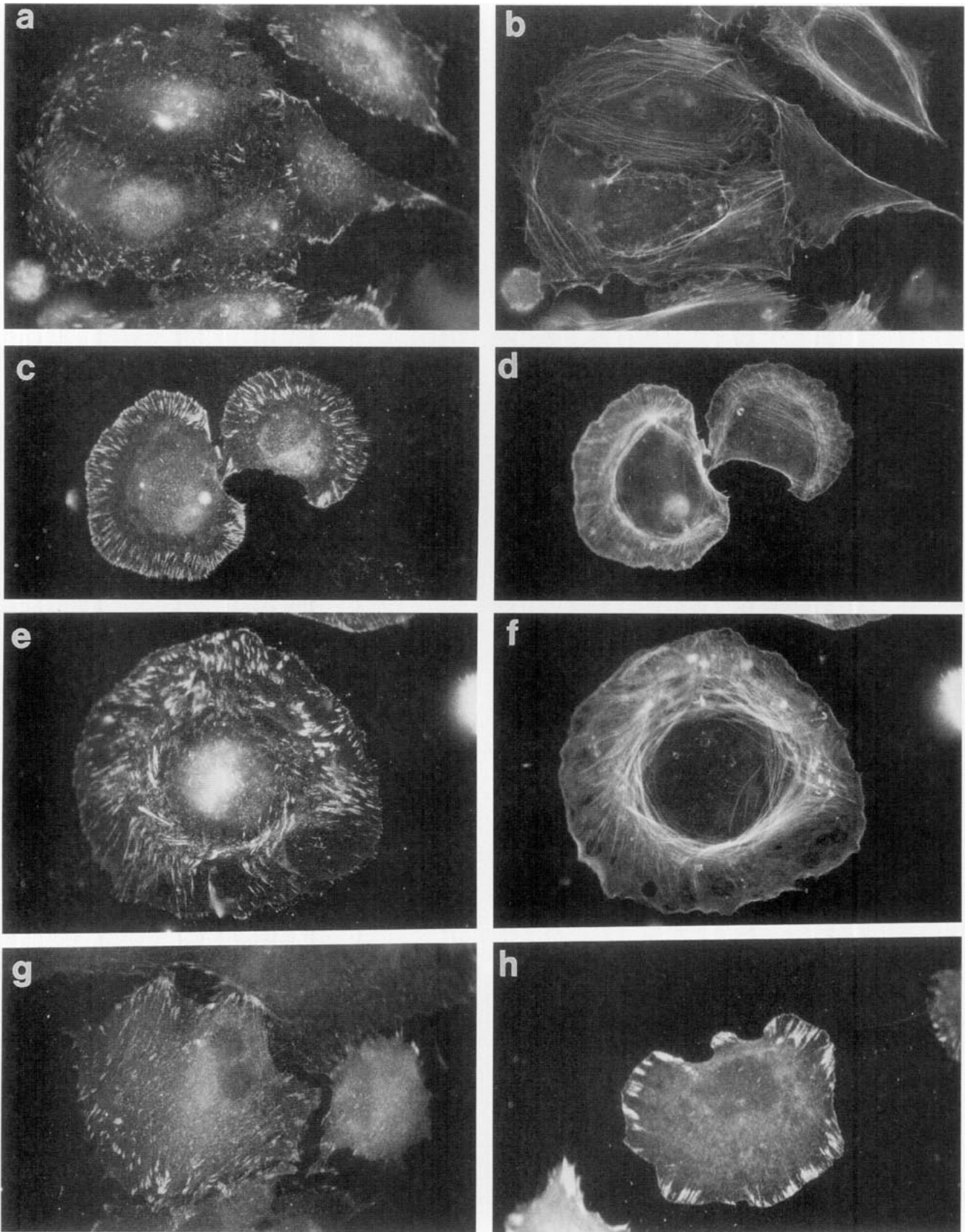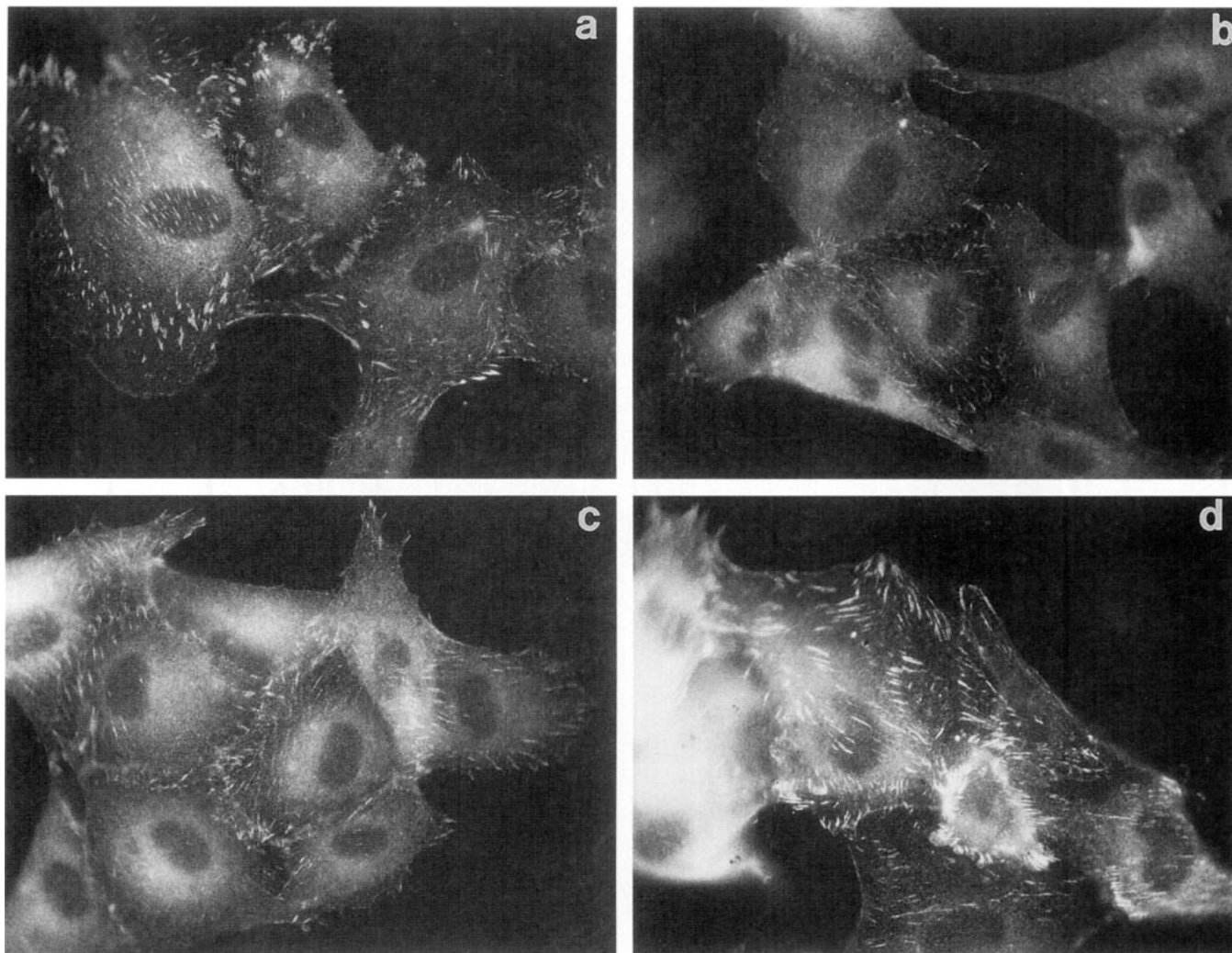
FIG. 5. Tyrosine phosphorylation of p125FAK correlates with focal adhesion formation in vanadate-treated HEC. HEC were grown on glass coverslips, treated with 25, 50, or 100 μM orthovanadate for 16 h (b, c, and d) or left untreated (a) fixed, permeabilized, and stained with Mab 7F9 to vinculin. Bar, 10 μM.

phosphorylation observed in cells treated with CD (Fig. 7A). The level of p125FAK tyrosine phosphorylation in cells treated with the combination of H_2O_2-vanadate and CD was comparable to that observed in H_2O_2-vanadate-treated cells (Fig. 7A, lanes c and e). CD-induced tyrosine dephosphorylation of p125FAK was also prevented by PAO treatment in CHO cells (Fig. 8A, lanes a and b). These data show that p125FAK tyrosine dephosphorylation induced by CD involves PTPase

activation rather than inhibition of tyrosine kinase activity.

Immunofluorescence experiments were performed to test the effects of PTPase inhibitors on CD-mediated actin stress fibers and focal adhesion disruption (Figs. 7B and 8B). As shown in Fig. 7B, endothelial cells treated with H_2O_2-vanadate and 1 μM CD retained organized stress fibers (compare c, d and e, f). In some cases actin organization was preserved at the cell pe-

FIG. 6. Cytochalasin D treatment depolimerizes F-actin and downregulates p125FAK tyrosine phosphorylation. (A) Confluent HEC were left untreated (a) or treated with 0.12 (b), 0.3 (c), 0.6 (d), 1.25 (e), and 2.5 (f) μM cytochalasin D (CD) for 15 min. Cell extracts, immunoprecipitated with FAK4 polyclonal antibody, were separated on 6% SDS–PAGE and the tyrosine phosphorylation of the p125FAK was visualized by Western blotting with Mab PT66. The position of p125FAK is indicated by the arrow. (B) HEC grown on FN-coated glass coverslips untreated (a) or treated with 0.12 (b), 0.3 (c), 1.25 (d), and 2.5 (e) μM CD for 15 min were fixed, permeabilized, and stained with phalloidin for F-actin. Bar, 10 μM.
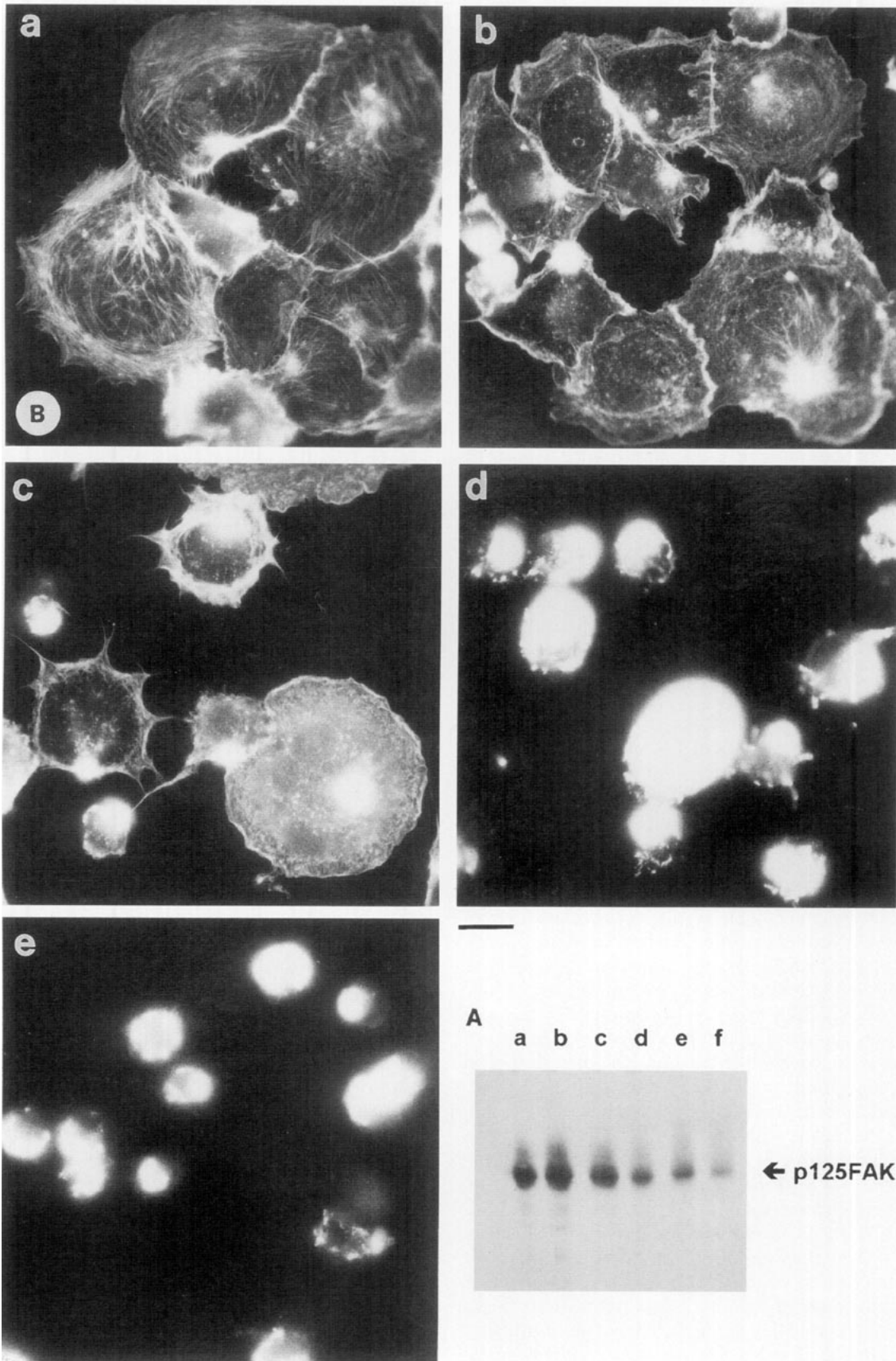
riphery, but a central area devoid of F-actin was present (Fig. 7Be). In CHO cells the combination of 10 μM PAO and 1 μM CD maintains prominent focal adhesion structures highly enriched in tyrosine phosphorylated proteins (Fig. 8B, a and b).

These data indicate that PTPase inhibitors are able to abolish CD-mediated actin depolimerization and focal adhesion disassembly.

DISCUSSION

In this paper we show that PTPase inhibitors such as vanadate and PAO are able to regulate tyrosine phosphorylation of the p125FAK kinase. Upregulation of p125FAK tyrosine phosphorylation correlates with increased organization of focal adhesion structures. Moreover, PTPase inhibitors prevent CD-mediated tyrosine dephosphorylation of p125FAK and disruption of actin stress fibers and of focal adhesions. These data suggest a prominent role for tyrosine phosphatases in the regulation of p125FAK tyrosine phosphorylation and cytoskeletal assembly during cell adhesion.

Focal adhesions are specialized regions of the cell where the cell surface is in tight contact to the substrate. The relevance of tyrosine phosphorylation to the assembly of focal adhesions has been studied by using phosphotyrosine kinase inhibitors. Herbimycin A and genistein, two inhibitors of tyrosine kinases, prevent cell spreading and focal adhesion formation in fibroblasts [10] and endothelial [11] and thyroid cells [37]. Genistein was shown to affect not only assembly but also maintenance of preformed focal adhesions [11]. Tyrosine phosphorylated proteins such as p125FAK or pp60src tyrosine kinases and structural proteins like paxillin and tensin have been described in the focal adhesions [38, 39]. Tyrosine phosphorylation of p125FAK, paxillin, and tensin is increased by integrin-dependent adhesion [7–12]. In this work we used vanadate and PAO, potent tyrosine phosphatases inhibitors, to modulate p125FAK tyrosine phosphorylation. The results demonstrate that these two inhibitors induce regulation of p125FAK tyrosine phosphorylation. A peculiar dose–response effect on p125FAK tyrosine phosphorylation was observed with vanadate in endothelial cells. Low concentrations of vanadate (25 μM) lead to downregulation of p125FAK tyrosine phosphorylation, while higher concentrations (100 μM) upregulate tyrosine phosphorylation of this molecule. This biphasic effect, specific for p125FAK tyrosine phosphorylation, correlates with assembly of focal adhesions. Focal adhesions are reduced in cells treated with low doses of vanadate, while they are prominent in cells treated with high vanadate concentrations. Vanadate-treated cells also show a strong integrin-dependent increase in focal adhesion assembly on FN. These data indicate that integrin-mediated tyrosine phosphorylation of
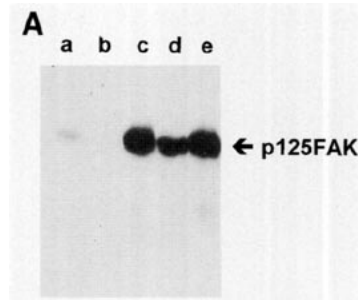
FIG. 7. Vanadate treatment restores p125FAK phosphorylation in CD-treated cells and prevents CD-mediated F-actin disruption. (A) Confluent HEC were left untreated (a), or treated with 1 μM CD for 15 min in the absence (b) or presence (d and e) of H_2O_2-vanadate (see Materials and Methods). In c only H_2O_2-vanadate was added. In d H_2O_2-vanadate was added for 15 min before CD treatment and in e H_2O_2-vanadate and CD were added in combination. Cell extracts, immunoprecipitated with FAK4 polyclonal antibody, were separated on 6% SDS–PAGE and the tyrosine phosphorylation of the p125FAK was visualized by Western blotting with Mab PT66. The position of p125FAK is indicated by the arrow. (B) HEC grown on glass coverslips left untreated (a and b), or treated with 1 μM CD for 15 min in the absence (c and d) or presence (e and f) of H_2O_2-vanadate, were fixed, permeabilized, and stained with phalloidin for F-actin. Bar, 10 μM.

p125FAK correlates with focal adhesions and actin stress fibers organization. Previous data on murine fibroblasts indicated a role for neuropeptides and growth factors in regulating p125FAK tyrosine phosphorylation and actin stress fibers formation [33, 40]. Platelet-derived growth factor (PDGF) is able to induce p125FAK tyrosine phosphorylation in these cells. The PDGF concentration which induces p125FAK tyrosine phosphorylation is the same as that which stimulates actin stress fibers formation. No induction of p125FAK phosphorylation was observed at doses of PDGF disrupting actin cytoskeleton [40]. RhoA-dependent assembly of focal adhesions has also been associated to p125FAK tyrosine phosphorylation in starved murine fibroblasts [41]. Thus, the regulation of p125FAK tyrosine phosphorylation by neuropeptides and growth factors or by integrin-mediated adhesion correlates with a high degree of assembly of actin cytoskeleton.

The p125FAK tyrosine phosphorylation is probably due to autophosphorylation or trans-phosphorylation of the molecule. A major site of tyrosine phosphorylation has been identified in tyr-397, which upon phosphorylation mediates associations with pp60src and pp59fyn [42]. Moreover, phosphorylation of tyr-925 has been shown to create a binding site for the SH2 domain of the adaptor protein GRB2 [43]. It is possible that different sites of tyrosine phosphorylation may also function as binding sites for proteins implicated in focal adhesion organization such as paxillin and tensin. Binding to phosphorylated tyrosine residues of p125FAK may thus represent a mechanism of recruitment of structural components to the focal adhesion.
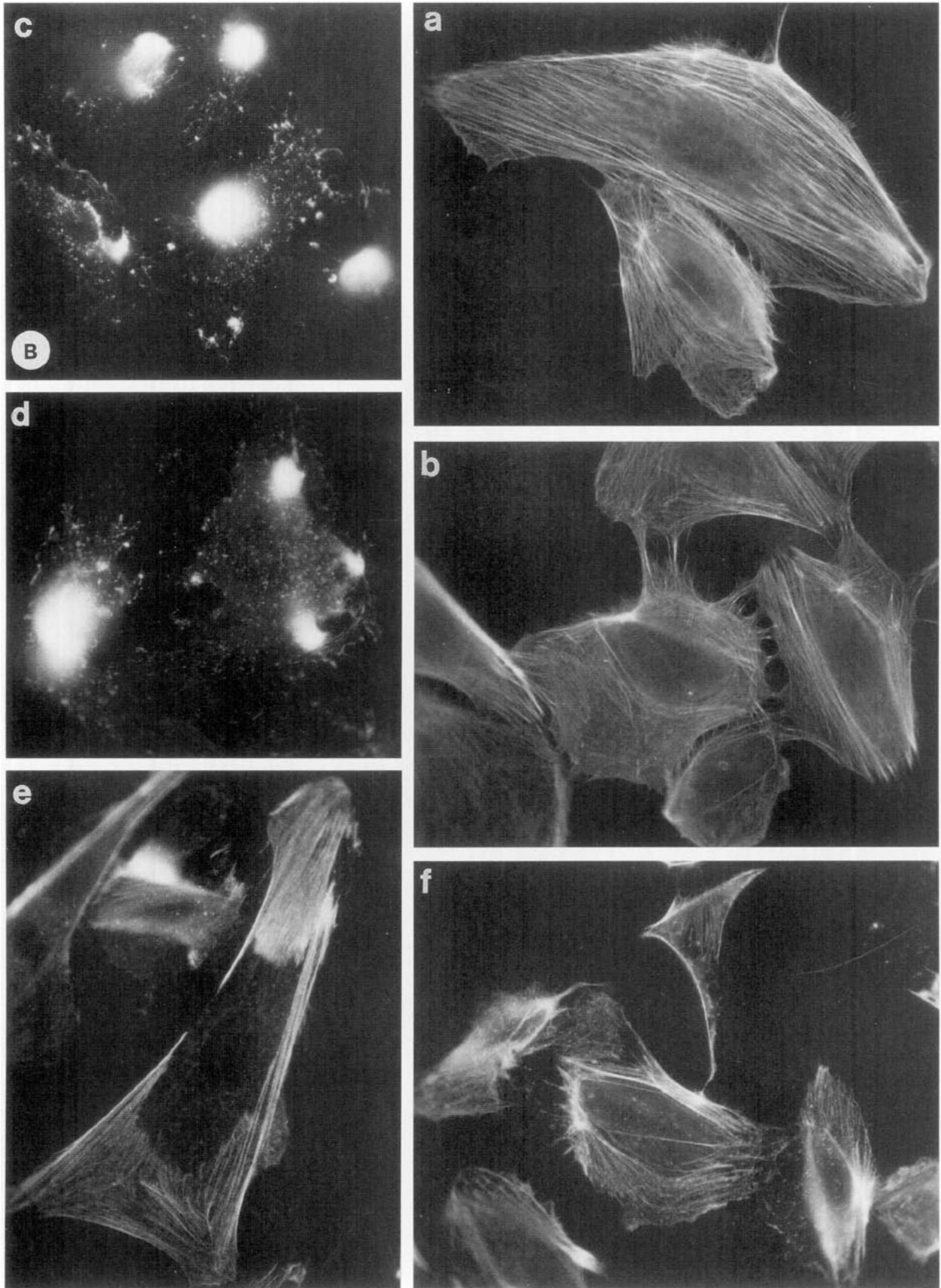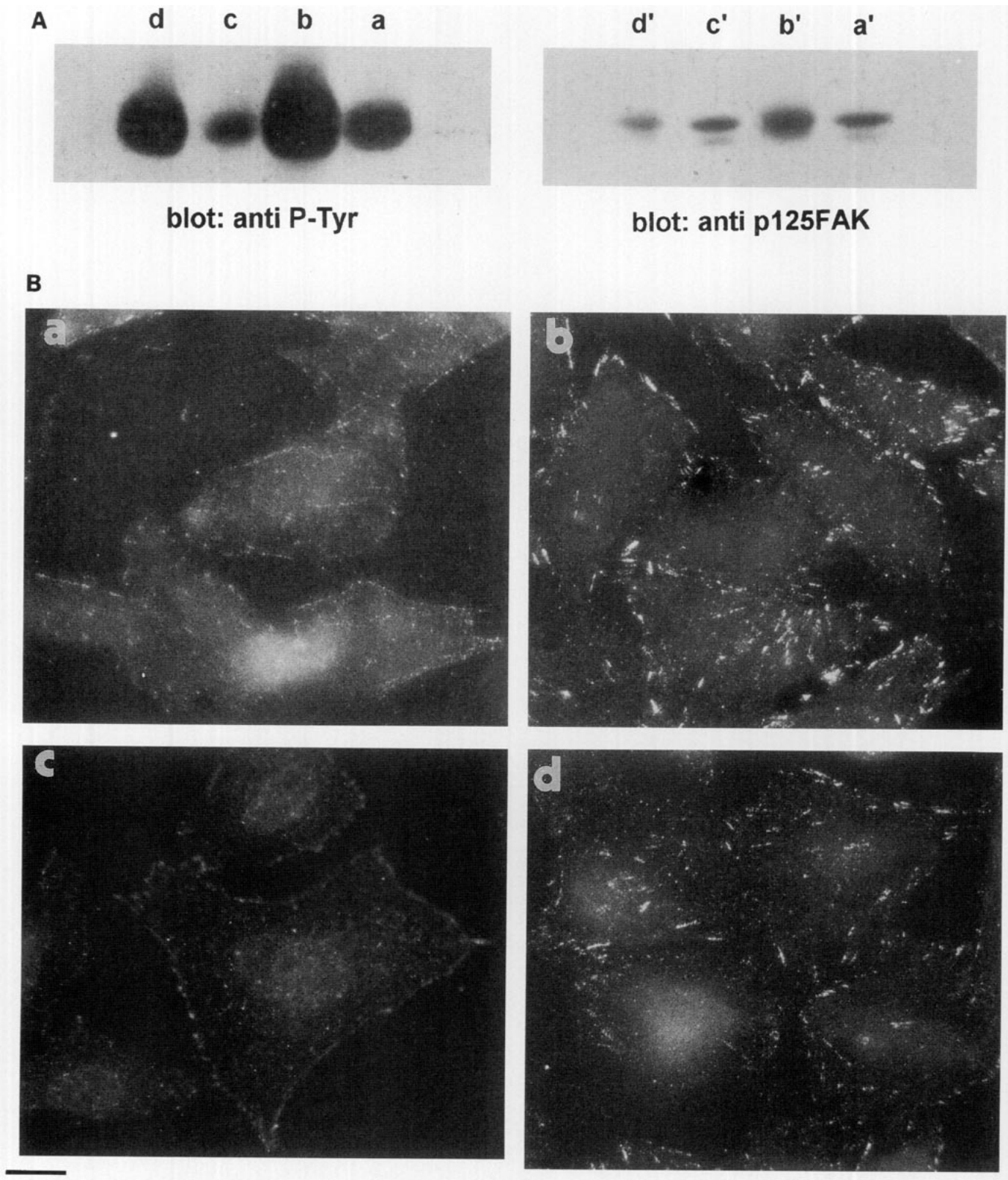
FIG. 7—*Continued*

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FIG. 8. PAO treatment restores p125FAK phosphorylation in CD-treated cells and maintains focal adhesion from CD-mediated disruption. (A) Confluent CHO were left untreated (a and a') or treated for 15 min with 10 μM PAO (b and b'), 1 μM CD (c and c'), or the combination of 1 μM CD with 10 μM PAO (d and d'). Cells extracts or molecules immunoprecipitated with FAK4 polyclonal antibody were separated on 6% SDS–PAGE and analyzed by Western blotting with anti-phosphotyrosine Mab PT66 (a–d) or anti-p125FAK Mab (a'–d'). (B) CHO grown on glass coverslips left untreated (a) or treated for 15 min with 10 μM PAO (b), 1 μM CD (c), or the combination of 1 μM CD and 10 μM PAO (d) were fixed, permeabilized, and stained with Mab PY20 to tyrosine phosphorylated proteins. Bar, 10 μM.
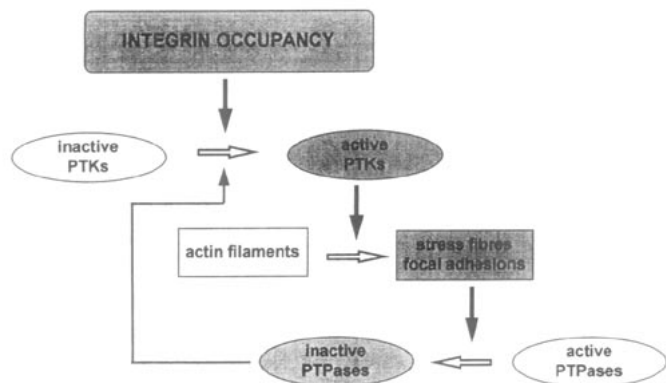
FIG. 9. Model of integrin-triggered signaling events. A primary event induced by integrin ligand occupancy is the increased tyrosine phosphorylation of p125FAK. Mechanisms leading to this event are still poorly understood. p125FAK tyrosine phosphorylation leads to actin stress fibers and focal adhesion organization as indicated by the effect of PTPase [this work] and tyrosine kinase (PTKs) [10, 11] inhibitors. Initial actin organization is likely to lead to inhibition of PTPases as indicated in the experiments performed with PTPase inhibitors and cytochalasin D [this work]. The inactivation of PTPases exerts a positive feedback regulation on p125FAK tyrosine phosphorylation and consequently on cytoskeleton organization.

It is interesting to note that downregulation of tyrosine phosphorylation by low vanadate concentrations is observed only on p125FAK, but not on other unidentified phosphotyrosine proteins of 100–130 kDa. In fact, the tyrosine phosphorylation of proteins that comigrate with p125FAK increased linearly by increasing vanadate concentration. The biphasic curve of p125FAK tyrosine phosphorylation may be explained assuming that low vanadate concentrations can block phosphatases responsible for triggering a tyrosine kinase activity that phosphorylates p125FAK, while high vanadate concentrations preferentially block phosphatases involved in p125FAK dephosphorylation.

We also tested PTPase inhibitors in cells treated with cytochalasin D, a drug known to lead to p125FAK tyrosine dephosphorylation [33]. We demonstrate that vanadate and PAO completely inhibit CD-induced p125FAK tyrosine dephosphorylation. In CD-treated cells these inhibitors also prevent CD-induced actin depolimerization and focal adhesion disruption. These results indicate that reduced tyrosine phosphorylation of p125FAK in CD-treated cells is due to activation of PTPases rather than inhibition of tyrosine kinase and suggest that tyrosine phosphatases may play an important role in actin cytoskeleton organization.

Based on the results presented here we might propose a model in which focal adhesion organization and PTPase activity are directly linked (Fig. 9). The initial event of integrin occupancy by its specific ligand triggers progressive organization of focal adhesions and actin stress fibers. This event requires increased p125FAK tyrosine phosphorylation as shown by the fact that tyrosine kinase inhibitors prevent this process. The mechanisms leading to p125FAK activation are still to be investigated. The initial assembly of the cytoskeleton in turn lead to inactivation of PTPases as suggested by the CD/PTPase inhibitor experiments. Phosphatase inactivation can have an important feedback effect leading to maximal p125FAK tyrosine phosphorylation and consequently to more extensive actin stress fibers and focal adhesion organization.

PTPases have recently been implicated in the regulation of integrin-dependent signal transduction. A direct role for CD45, a major lymphocytic transmembrane tyrosine phosphatase, in ICAM-3 or $\beta2$ integrin-mediated lymphocyte aggregation was also demonstrated [44]. More generally, reduction of cell adhesion by trypsin treatment has been reported to induce PTPase activity [45]. Analysis of the specific PTPases involved in $\beta1$ integrin-related signaling will be an important future line of research.

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