THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 44, Issue of November 1, pp. 41631–41636, 2002

Printed in U.S.A.

Low M_r Phosphotyrosine Protein Phosphatase Associates and Dephosphorylates p125 Focal Adhesion Kinase, Interfering with Cell Motility and Spreading*

Received for publication, February 20, 2002, and in revised form, May 23, 2002
Published, JBC Papers in Press, June 7, 2002, DOI 10.1074/jbc.M201709200

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Low M_r phosphotyrosine protein phosphatase interferes *in vivo* with the activation of several growth factor receptors and is transiently redistributed, following cell stimulation with platelet-derived growth factor, from the cytosol to the cytoskeleton. We demonstrate here that this phosphatase also participates in the regulation of cell spreading and migration, pointing to its involvement in cytoskeleton organization. Low M_r phosphotyrosine protein phosphatase-overexpressing fibroblasts are, indeed, less spread than controls and display a significantly decreased number of focal adhesions and increased cell motility. Furthermore, p125 focal adhesion kinase is associated to, and dephosphorylated by, low M_r phosphotyrosine protein phosphatase both *in vitro* and *in vivo*. This event is consistent with an altered association of pp60^{src} with focal adhesion kinase. The activation of extracellular signal-regulated kinase, another well known event downstream of the focal adhesion kinase, is also affected. On the other hand, cells overexpressing the dominant-negative form of low M_r phosphotyrosine protein phosphatase exhibit hyperphosphorylated focal adhesion kinase, reduced motility, and an increased number of focal adhesions, which are distributed all over the ventral cell surface. Taken together, the results reported here are in keeping with low M_r phosphotyrosine protein phosphatase participation in FAK-mediated focal adhesion remodeling.

Cell adhesion and motility are based on the organization and remodeling of macromolecular complexes called focal adhesions. Following integrin engagement and clustering, several proteins, such as tensin, paxillin, p190Rho-GAP, and p125 focal adhesion kinase (FAK),[1] accumulate in these specialized sites at the cytoplasmic face of plasma membrane (1–3). Autophosphorylation of FAK on Tyr-397 creates a binding site for pp60^{src}, which phosphorylates FAK further (4). This enables the recruitment of other structural or signaling proteins, including pp130^{CAS}, Csk, talin, PI3-kinase, α-actinin, and Grb2, which triggers the activation of the extracellular signal-regulated kinase (ERK) cascade (5–7). FAK is implicated in the control of cell migration by modulating the turnover of focal adhesions and in the transmission of cell survival signals from the extracellular matrix (1–3). Cell motility and spreading are clearly affected by FAK expression as fibroblasts isolated from FAK knockout mice exhibit reduced motility and a more rounded shape with abundant focal contacts that cover much of the ventral surface (8–10). *Vice versa*, overexpression of FAK stimulates cell migration in Chinese hamster ovary cells (11).

The role of FAK phosphorylation in the above processes is not completely clarified yet; the catalytic activity of FAK and its autophosphorylation site are required to restore cell migration in FAK-deficient cells (12). Indeed, PTEN and PTP1B tyrosine phosphatases are negative regulators of FAK tyrosine phosphorylation, cell spreading, and migration (13, 14). However, in contrast to the above findings, fibroblasts expressing an inactive form of the SHP2 tyrosine phosphatase (15) or deficient in PTP-PEST (16) exhibit elevated tyrosine phosphorylation of FAK, an increase in number and size of focal adhesions, and decreased rates of cell migration. Expression of a dominant-negative SHP-2 in breast cancer cells blocks heregulin-mediated FAK dephosphorylation, leading to persistent accumulation of focal adhesions, decreased cell motility (17), and a phenotype not very dissimilar from that described above for FAK knockout fibroblasts. Moreover, epidermal growth factor receptor-induced dephosphorylation of FAK correlates with cell detachment and increased motility in various human carcinoma cells (18). Thus, the role of protein tyrosine phosphatases in regulating FAK-mediated focal adhesion remodeling is evident and likely to be important, although not fully clarified yet.

Low M_r phosphotyrosine protein phosphatase (LMW-PTP) is an 18-kDa enzyme that is able to interfere *in vivo* with the activation of several growth factor receptors, including those for platelet-derived growth factor (PDGF), fibroblast growth factor, macrophage colony-stimulating factor, and insulin (19–22). We showed previously that LMW-PTP is constitutively distributed in both the cytosol and the Triton X-100-insoluble fraction (23). Following PDGF stimulation, LMW-PTP level transiently increases in the Triton-insoluble fraction, at the expense of the cytosol. This redistribution does not seem critical for LMW-PTP activity on growth factor receptors as neither fibroblast growth factor nor macrophage colony-stimulating factor induces enzyme redistribution, although their activated receptors are substrates of LMW-PTP (20). As the Triton-insoluble fraction contains cytoskeletal components, LMW-PTP re-

* This work was supported by Associazione Italiana per la Ricerca sul Cancro (AIRC), Ministero Istruzione, Università e Ricerca (MIUR), and Ente Cassa di Risparmio di Firenze. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Supported by a Federazione Italiana per la Ricerca sul Cancro (FIRC) fellowship.

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[1] The abbreviations used are: FAK, p125 focal adhesion kinase; ERK, extracellular signal-regulated kinase; LMW-PTP, low *M*r phosphotyrosine protein phosphatase; PDGF, platelet-derived growth factor; FCS, fetal calf serum; NIH/3T3-dnPTP, NIH/3T3 cells overexpressing dominant-negative LMW-PTP; NIH/3T3-PTP, NIH/3T3 cells overexpressing LMW-PTP; PBS, phosphate-buffered saline; T-PBS, PBS containing 1% Triton X-100; DMEM, Dulbecco's modified Eagle's medium; RIPA, radioimmune precipitation buffer; dn, dominant-negative.

distribution could interfere with the organization of cytoskeleton. Accordingly, it was recently demonstrated that, following PDGF stimulation, LMW-PTP is tyrosine-phosphorylated and interacts with p190Rho-GAP (24, 25). With the experiments reported in this study, we investigated the role of LMW-PTP in cytoskeleton dynamics under adhesion stimuli only, in the absence of acute growth factor stimulation, and demonstrated that unphosphorylated LMW-PTP dephosphorylates FAK, thus participating in focal adhesion remodeling.

MATERIALS AND METHODS

Cell Culture and Culture Surface Coating—NIH/3T3 murine fibroblasts were routinely cultured in DMEM (Sigma D7777) containing 4.5 g/liter of glucose supplemented with 10% (v/v) fetal calf serum (FCS) (complete medium). BAC1.2F5 macrophages overexpressing v-Fes obtained as already described (26) were cultured in DMEM supplemented with 10% FCS and 3 ng/ml murine recombinant macrophage colony-stimulating factor. Cells overexpressing active LMW-PTP (NIH/3T3-PTP), dominant-negative LMW-PTP (NIH/3T3-dnPTP), as well as mock-transfected controls (NIH/3T3) had been obtained and characterized previously (24, 27). Stable overexpression was maintained by periodically culturing cells in selective medium containing 400 μg/ml G418 (Sigma) and verified by Western blotting. Experiments were performed using three clones of PTP-overexpressing cells.

Petri dishes were incubated overnight at room temperature with 25 μg/ml fibronectin (Sigma) or 20 μg/ml poly-D-lysine (Sigma) dissolved in PBS (10 mM Na_2HPO_4, 150 mM NaCl, pH 7.4). Dishes were then washed twice with PBS, treated with 2% heat-inactivated bovine serum albumin (Sigma) for 2 h at room temperature, and washed twice with PBS prior to use.

Phase-contrast Microscopy and Cell Surface Area Evaluation—Cells were serum-starved for 24 h and seeded in the presence of 10% FCS or in culture dishes coated with 25 μg/ml fibronectin in the presence of 0.25 mg/ml heat-inactivated bovine serum albumin. Cells were observed after 24 h, and cell surface area was calculated using QuantiScan software (Bio-Rad).

Cell Motility Assay—Cells were serum-starved for 24 h, detached by trypsin treatment followed by inhibition with soybean trypsin inhibitor, and suspended in DMEM containing 0.25 mg/ml heat-inactivated bovine serum albumin. 3×10^4 cells were seeded in the upper compartment of a Boyden chamber equipped with 8-μm pore polycarbonate filters, whereas the lower compartment was filled with DMEM containing 10 μg/ml fibronectin. Incubation was performed at 37 °C in 5% CO_2 for different times. Filters were fixed with methanol overnight at 4 °C and stained with the DIFF-QUICK dye (Dade Diagnostics). Cells attached to the upper surface of the filter were removed with a cotton swab, whereas cells that were migrated to the lower surface of the filter were counted.

Wound-healing Assay—Cells were serum-starved for 24 h and seeded in the absence of FCS in culture dishes coated with 25 μg/ml fibronectin. After 4 h of incubation, dishes were scored with a sterile 200-μl micropipette tip and photographed. After 18 h, the wounds were photographed again to visualize incoming cells.

Immunofluorescence Analysis—Cells were plated on glass coverslips in complete medium. After 24 h, cells were washed once with PBS and fixed with 3% paraformaldehyde for 20 min at 4 °C. Coverslips were then washed three times with T-PBS (PBS containing 0.1% Triton X-100) and saturated with 5.5% horse serum in T-PBS for 45 min at room temperature. After an overnight incubation at 4 °C with mouse anti-vinculin V 9131 (Sigma) or rabbit anti-FAK C-20 (Santa Cruz Biotechnology), coverslips were washed in T-PBS, and immunocomplexes were revealed with rhodamine-labeled secondary antibodies (Chemicon; anti-mouse AP192C, anti-rabbit AP182C). Following prolonged washes in T-PBS, the coverslips were mounted with propylthiogallate and observed using a confocal microscope (Bio-Rad MRC 1024ES).

LMW-PTP and FAK Subcellular Distribution during Cell Suspension and Adhesion—Non-confluent cells were serum-starved for 24 h, washed twice with PBS, and collected as a single cell suspension in DMEM plus 0.25 mg/ml bovine serum albumin. Cells were kept in suspension for 60 min at 37 °C on an oscillating platform and then seeded in fibronectin- or polylysine-coated culture dishes for 1 h before being harvested in hypotonic buffer (10 mM Hepes, pH 7.5, 10 mM NaCl, 1 mM KH_2PO_4, 5 mM $NaHCO_3$, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM EDTA, 1 mM sodium vanadate, 20 mM NaF, 10 mM sodium pyrophosphate, 10 μg/ml leupeptin, 20 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride) and sonicated twice for 15 s in ice. Alternatively, to evaluate conditions prior to cell suspension, adherent cells were washed with PBS and lysed *in situ* by the direct addition of hypotonic buffer to culture dishes ("off-dish" lysate).

The separation of cytosol and Triton X-100-soluble and -insoluble fractions was performed as described previously (23). Briefly, the lysate was centrifuged at 100,000 $\times$ *g* for 30 min at 4 °C, the resulting supernatant corresponding to the cytosol fraction. The pellet was then extracted for 30 min in the presence of 1% (v/v) Triton X-100 and centrifuged again at 100,000 $\times$ *g* for 30 min. The Triton-insoluble fraction was further solubilized in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% (v/v) Nonidet P-40, 0.1% (w/v) SDS, 0.5% (w/v) sodium deoxycholate, 1 mM sodium vanadate, 20 mM NaF, 10 mM sodium pyrophosphate, 10 μg/ml leupeptin, 20 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride) for 30 min in ice, and then it was supplemented with Laemmli buffer (66 mM Tris-HCl, pH 6.8, containing 2% (w/v) SDS, 10 mM EDTA, 10% (w/v) glycerol, and bromphenol blue) and boiled for 10 min. Protein concentration was determined through the bicinchoninic acid assay (Pierce).

The distribution of LMW-PTP or FAK was determined in samples analyzed by 15 or 8% SDS-PAGE, respectively, and immunoblotting with a polyclonal anti LMW-PTP antibody (28) or C-20 anti-FAK antibody. Protein bands were revealed by means of peroxidase-conjugated anti-rabbit immunoglobulin secondary antibody (Calbiochem). The immunocomplexes were then evidenced by the Supersignal chemiluminescent method (Pierce).

Protein Phosphorylation Analysis—Cells were plated in complete medium, and 24 h later, they were washed twice with PBS and lysed in Laemmli buffer. Cell lysates were boiled for 10 min and clarified at 10,000 $\times$ *g* for 10 min. Equal protein amounts were subjected to SDS-PAGE and electrotransferred onto a nitrocellulose membrane (Sartorius).

The membrane was then treated with PY20 anti-phosphotyrosine antibody (Affiniti Research Products Ltd.) followed by a peroxidase-labeled anti-mouse immunoglobulin secondary antibody (Calbiochem). Alternatively, membranes were incubated with phosphotyrosine-specific anti-FAK (BIOSOURCE International) or anti-phosphoERK antibodies (New England Biolabs). The immunorecognition of FAK or ERK was carried out on the same membranes after stripping by using C-20 anti-FAK or anti-ERK antibodies (Santa Cruz Biotechnology), according to the procedure cited above.

In Vitro Tyrosine Phosphatase Assay—NIH/3T3 fibroblasts or BAC1.2F5 macrophages overexpressing v-Fes were treated for 30 min with 20 μM phenylarsine oxide and for 10 min with 2 mM sodium vanadate to maximize protein tyrosine phosphorylation. Cells were lysed with 50 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EGTA, 1% Nonidet P-40, 2 mM sodium vanadate, 20 μM phenylarsine oxide, 20 mM NaF, 10 mM sodium pyrophosphate, 10 μg/ml leupeptin, 20 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride for 1 h on ice. The lysate was then clarified at 10,000 $\times$ *g* for 10 min, and 1 mg of total proteins from fibroblasts or macrophages was subjected to immunoprecipitation with either 5 μg of a mixture of C-20 and C-903 anti-FAK (Santa Cruz Biotechnology) antibodies or 5 μg of monoclonal anti-Fes (LP167; Quality Biotech) for 2 h on ice. The immunocomplexes were collected on protein G-Sepharose (Amersham Biosciences) overnight at 4 °C, washed twice with 50 mM Tris, pH 7.5, 150 mM NaCl supplemented with 1 mM sodium vanadate and twice with the same buffer without sodium vanadate, and then divided in aliquots, which were resuspended in 0.1 M sodium acetate, pH 5.5, 1 mM EDTA, with or without 100 milliunits recombinant LMW-PTP. The enzyme was obtained as a fusion protein with glutathione *S*-transferase (29), cleaved by thrombin, and purified. Following 10 or 20 min of incubation at room temperature, the reaction mixtures were supplemented with 4$\times$ Laemmli buffer, boiled for 10 min, and analyzed through 8% SDS-PAGE and immunoblotting with anti-phosphotyrosine antibodies. The presence of an equal amount of FAK or Fes in the reaction mixtures was verified by stripping and reprobing the nitrocellulose membrane with specific antibodies.

Co-immunoprecipitation Experiments—Adherent cells not fed with fresh medium during the last 24 h were washed twice with PBS and then lysed in RIPA buffer. The lysate was then clarified at 10,000 $\times$ *g* for 10 min and divided in aliquots containing 0.25 mg of total proteins, which were subjected to immunoprecipitation with 3 μg of anti-LMW-PTP or anti-FAK antibodies for 2 h on ice. Immunocomplexes were collected on protein G-Sepharose overnight at 4 °C, washed, eluted with boiling Laemmli buffer, and analyzed through 15 or 8% SDS-PAGE and immunoblotting with specific antibodies to evidence LMW-PTP, FAK, and Src (MAB4030; Chemicon).

LMW-PTP Tyrosine Phosphorylation Analysis—Adherent cells were not fed with fresh medium during the last 24 h or serum-starved for

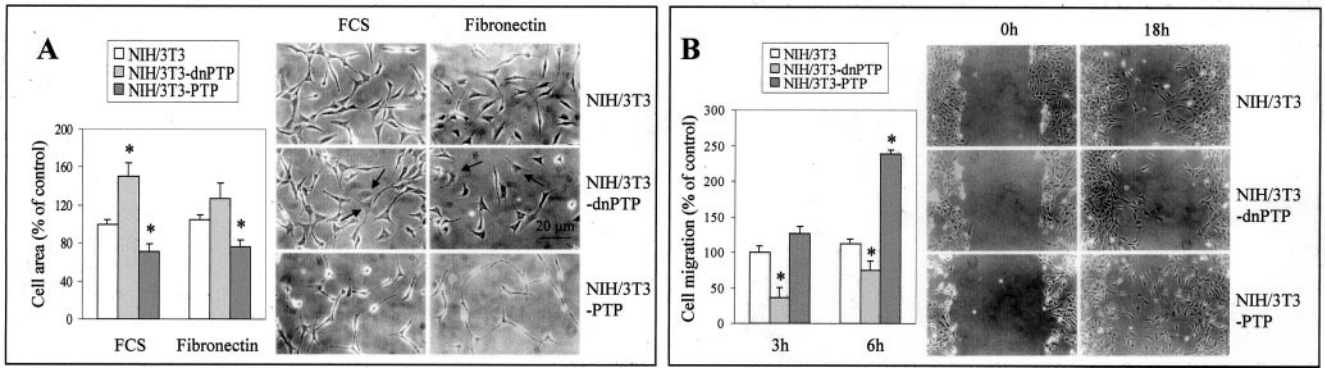

FIG. 1. **Analysis of cell morphology and motility.** As shown in *A*, serum-starved cells were seeded on cell culture plastics in the presence of 10% FCS or onto fibronectin-coated dishes and observed after 24 h (*pictures*). Images were acquired with a digital camera, and the cell area was calculated with the QuantiScan software (Bio-Rad) and expressed as a percentage of the NIH/3T3 cell area in every experimental condition (*graph*). Data represent the average ± S.E. of three independent experiments in which four randomly chosen fields for the dish were examined. As shown in *B*, cell migration was assayed, following serum starvation, in a Boyden chamber whose lower compartment was filled with 10 μg/ml fibronectin. Migrated cells were counted after 3 or 6 h of incubation. For each filter, four randomly chosen fields were counted. The value obtained for NIH/3T3 cells at 3 h was taken as 100%. Data represent the average ± S.E. of three independent experiments (*graph*). Cell migration was also determined by a wound-healing assay (*pictures*). Serum-starved cells were allowed to adhere on dishes, scored with a micropipette tip, and photographed either immediately or after 18 h to visualize incoming cells. The statistical significance of differences between transfected cells and controls was determined by the Student's *t* test. Significant differences are indicated by an *asterisk* ($p < 0.05$).

24 h. Serum-starved cells were stimulated with 30 ng/ml PDGF for 10 min. Cells were placed on ice, washed with PBS, and lysed in RIPA buffer. LMW-PTP was immunoprecipitated from aliquots containing 0.25 mg of total proteins and analyzed through 15% SDS-PAGE and immunoblotting with anti-LMW-PTP or anti-phosphotyrosine antibodies.

Software Applications—Digital images were scanned using Vista Scan 32 software. Confocal images were analyzed using Confocal Assistant (Bio-Rad). Images were processed with Adobe PhotoShop.

RESULTS

Effects of LMW-PTP Overexpression on Cell Morphology and on Fibronectin-promoted Cell Motility—Cells were serum-starved and then plated and cultured for 24 h in the presence of 10% FCS or on fibronectin-coated dishes in the absence of serum (Fig. 1*A*). In the presence of FCS, NIH/3T3-PTP cells were less spread, more refrangent, and more spindle-shaped than NIH/3T3 controls (Fig. 1*A*, *left pictures*). These effects were even more pronounced on fibronectin (Fig. 1*A*, *right pictures*). NIH/3T3-dnPTP cells, on the contrary, were more spread and less refrangent, showing in some cases an extreme degree of spreading (*arrows*). The histograms of Fig. 1*A* (*graph*) indicate the average cell area.

To investigate the involvement of LMW-PTP in cell motility in the absence of mitogenic stimulation, cells were serum-starved, suspended in serum-free medium, and assayed for their migration toward fibronectin in a Boyden chamber for 3 or 6 h (Fig. 1*B*, *graph*). NIH/3T3-PTP cells were more motile than control NIH/3T3 cells, whereas the motility of NIH/3T3-dnPTP cells was reduced. These results were supported by those of a wound-healing assay (Fig. 1*B*, *pictures*). NIH/3T3-PTP cells migrated more rapidly into the groove than control cells, whereas NIH/3T3-dnPTP cells migrated more slowly.

Immunofluorescence of Focal Adhesions—As the above results pointed to a possible effect of LMW-PTP overexpression on the organization of cytoskeletal structures involved in cell spreading and motility, we verified whether the formation of focal adhesions was somehow altered in LMW-PTP-overexpressing cells with respect to controls. The immunofluorescence of adherent cells stained with an anti-vinculin antibody was performed in cultures that had not been fed with fresh medium during the last 24 h. This protocol was chosen, for this and other experiments of the present study, to prevent suffering of NIH/3T3-PTP cells, which occurs when serum-starved cells are incubated for prolonged time periods. This experiment showed that the number of focal adhesions in LMW-PTP-overexpressing cells was significantly lower than in control cells (Fig. 2, *C versus A*); a diffused cytoplasmic staining was also evident in NIH/3T3-PTP cells (*C*). On the other hand, NIH/3T3-dnPTP cells displayed a marked increase in the number of focal adhesions, which were distributed all over the ventral cell surface (*E versus A*). Similar results were obtained by staining cells with anti-FAK antibodies (*B*, *D*, and *F*), including a particularly evident diffused cytoplasmic staining of NIH/3T3-PTP cells (*D*). Immunoblotting of whole cell lysates with anti-vinculin or anti-FAK antibodies revealed that either protein was similarly expressed in the three cell lines (not shown). The experiment was also performed on serum-starved cells, plated for 24 h on fibronectin-coated coverslips, giving qualitatively identical results.

LMW-PTP and FAK Distribution during Cell Adhesion—We showed previously that PDGF stimulation induces a redistribution of LMW-PTP from the cytosol to the Triton-insoluble fraction, which contains cytoskeletal components (23). The association of LMW-PTP with this fraction seems very intimate as harsh conditions (2.1% SDS, 100 °C, 10 min) are necessary to completely solubilize the Triton-insoluble fraction of the enzyme. We therefore decided to investigate the possible involvement of LMW-PTP in cytoskeleton dynamics, starting from the analysis of its distribution in the cytosol or the Triton-insoluble fraction with respect to the adhesion-suspension state of cells in the absence of growth factor stimulation (Fig. 3, *left panel*). Serum-starved NIH/3T3-PTP cells were lysed while adhering onto the culture dish (off-dish samples, *lanes 1* and *5*), a condition that represents the basal LMW-PTP distribution, when cells are attached to the extracellular matrix. In parallel, cells were detached, kept in suspension for 1 h, and then plated onto fibronectin- or polylysine-coated dishes (for 1 h). During cell suspension, LMW-PTP was partly redistributed from the Triton-insoluble fraction to the cytosol (*lanes 2* and *6*) in keeping with what was shown previously (24). The subsequent cell adhesion produced a backward movement of LMW-PTP, which tended to restore the basal off-dish condition (*lanes 3* and *4* and *lanes 7* and *8*). In this respect, fibronectin seemed to be more effective (*lane 7 versus lane 3*) than polylysine (*lane 8 versus lane 4*). Thus, the sole adhesion to either fibronectin or polylysine, in the absence of growth factor stimulation, was able to influence LMW-PTP distribution among subcellular fractions. FAK redistribution with respect to the adhesion-suspension state of cells was also investigated (Fig. 3, *right panel*). In

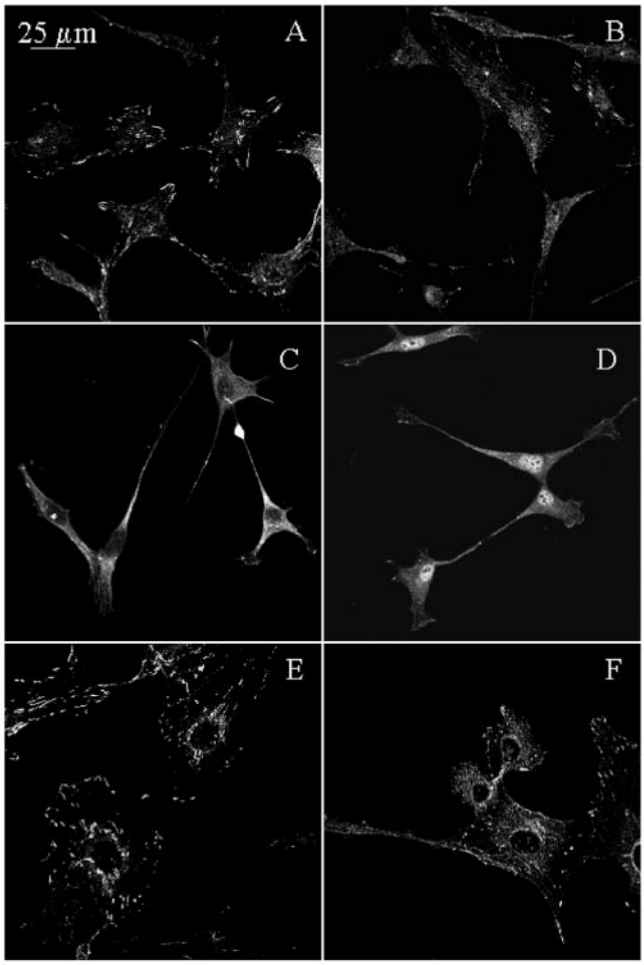

FIG. 2. **Immunofluorescence of focal adhesions.** Cells were plated on glass coverslips in complete medium and stained with the V 9131 anti-vinculin antibody (*A*, *C*, and *E*) or C-20 anti-FAK (*B*, *D*, and *F*) and rhodamine-conjugated secondary antibodies. Pictures were obtained with a confocal microscope. *A* and *B*, NIH/3T3; *C* and *D*, NIH/3T3-PTP; *E* and *F*, NIH/3T3-dnPTP.

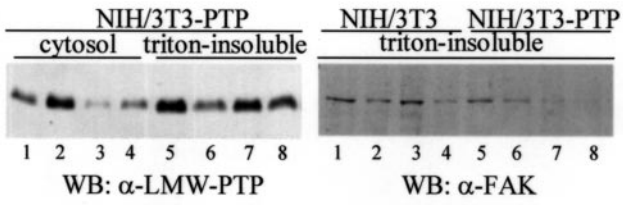

FIG. 3. **LMW-PTP and FAK distribution during cell suspension or adhesion.** Cells were serum-starved, detached from culture dishes, and maintained in suspension for 1 h before being seeded onto fibronectin- or polylysine-coated dishes for 1 h. Lysates from suspended or adherent cells were fractionated, and 10 μg of protein for each sample was subjected to SDS-PAGE and immunoblotting with anti-LMW-PTP (*left panel*) or anti-FAK (*right panel*) antibodies. *Lanes 1* and *5*, off-dish; *lanes 2* and *6*, suspension; *lanes 3* and *7*, adhesion onto fibronectin; *lanes 4* and *8*, adhesion onto polylysine. *WB*, Western blot.

NIH/3T3 cells, as well established, cell suspension determined a reduction of FAK within the Triton-insoluble fraction, whereas plating onto fibronectin determined a backward movement to this fraction. In NIH/3T3-PTP cells, on the contrary, FAK redistribution to the Triton-insoluble fraction following adhesion onto fibronectin was inhibited (*lane 7 versus lane 3*).

Effects of LMW-PTP Overexpression on FAK Phosphorylation—The above results suggested looking for a possible target

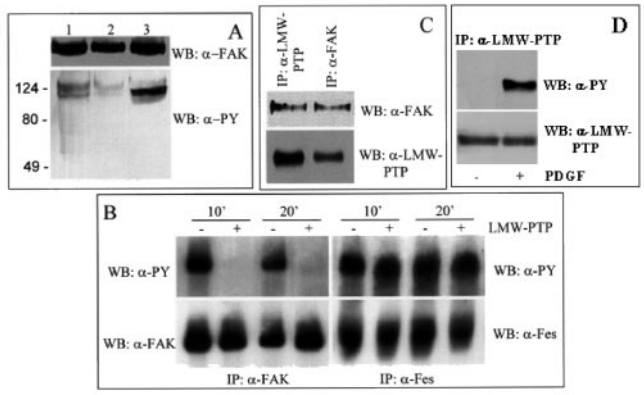

FIG. 4. **Effects of LMW-PTP overexpression on FAK phosphorylation.** Cells were not fed with fresh medium during the last 24 h prior to lysis. *A*, FAK tyrosine phosphorylation *in vivo*. Total cell lysates were subjected to SDS-PAGE and immunoblotting with anti-phosphotyrosine (*α-PY*) or anti-FAK antibodies. *1*, NIH/3T3; *2*, NIH/3T3-PTP; *3*, NIH/3T3-dnPTP. *WB*, Western blot. *B*, FAK dephosphorylation *in vitro*. FAK was immunoprecipitated (*IP*) from NIH/3T3 cells pretreated with 20 μM phenylarsine oxide and 2 mM sodium vanadate and incubated for the indicated times with or without 100 milliunits recombinant LMW-PTP. The reaction mixtures were subjected to SDS-PAGE and immunoblotting with anti-phosphotyrosine antibodies (*upper panels*). The presence of an equal amount of FAK in the reaction mixtures was verified reprobing with anti-FAK antibodies (*left panels*). The same assay was carried out with Fes immunoprecipitated from macrophages (*right panels*) *C*, LMW-PTP and FAK co-immunoprecipitation. NIH/3T3-PTP cells were lysed and subjected to immunoprecipitation with either anti-LMW-PTP or anti-FAK antibodies. Immunoprecipitates were analyzed by SDS-PAGE and immunoblotting with either anti-LMW-PTP or anti-FAK antibodies. *D*, LMW-PTP tyrosine phosphorylation analysis. NIH/3T3-PTP cells were serum-starved and stimulated with 30 ng/ml PDGF-BB for 10 min; alternatively, cells were cultured in complete medium, not changed during the last 24 h prior to cell lysis. Immunoprecipitates obtained with anti-LMW-PTP antibodies were analyzed by SDS-PAGE and immunoblotting with either anti-phosphotyrosine or anti-LMW-PTP antibodies.

of LMW-PTP phosphatase activity among focal adhesion components. To this purpose, the tyrosine phosphorylation pattern was determined in lysates of control cells and of cells overexpressing either the active or the inactive dominant-negative form of LMW-PTP, not fed with fresh medium during the last 24 h. Under these conditions, the difference in tyrosine phosphorylation between control and LMW-PTP-overexpressing cells pertained to a ~125-kDa protein, which was dephosphorylated in NIH/3T3-PTP and hyperphosphorylated in NIH/3T3-dnPTP as compared with control cells (Fig. 4*A*, *lower panel*). A prominent tyrosine-phosphorylated protein involved in the organization of focal adhesions is p125FAK, which is tyrosine-phosphorylated upon integrin-mediated cell adhesion and retains its phosphorylation as long as cells remain attached to the extracellular matrix (30). The use of polyclonal anti-FAK antibodies allowed identifying the 125-kDa protein as FAK (Fig. 3*A*, *upper panel*).

The activity of LMW-PTP on phosphorylated FAK was confirmed in an *in vitro* phosphatase assay (Fig. 3*B*) in which immunoprecipitated FAK was incubated with or without recombinant LMW-PTP for 10 or 20 min at room temperature. Immunoblotting with anti-phosphotyrosine antibodies showed the extensive dephosphorylation of FAK operated by LMW-PTP, already complete after 10 min of incubation (*left panels*). To confirm the specificity of LMW-PTP activity *in vitro*, Fes was immunoprecipitated from Fes-overexpressing macrophages and incubated under the same conditions as above. LMW-PTP did not affect Fes phosphorylation (*right panels*).

The possibility of a direct interaction between LMW-PTP and FAK was explored *in vivo*. Either protein was immunopre-

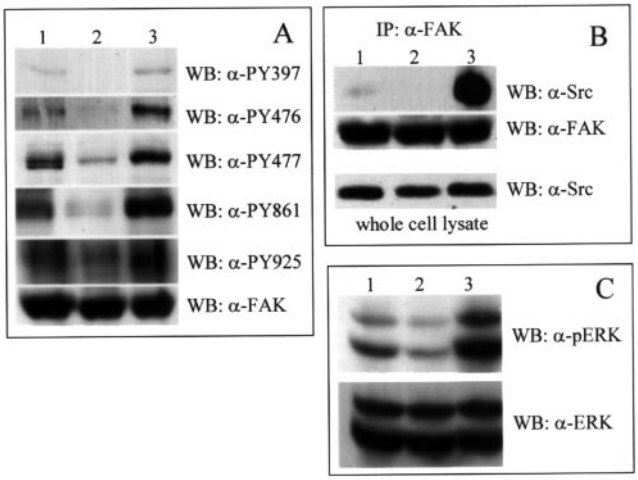

FIG. 5. **LMW-PTP activity on FAK affects downstream signaling pathways.** Cells were not fed with fresh medium during the last 24 h prior to lysis. As shown in *A*, total cell lysates were subjected to SDS-PAGE and immunoblotting with antibodies directed against the indicated phosphotyrosine residues of FAK. *1*, NIH/3T3; *2*, NIH/3T3-PTP; *3*, NIH/3T3-dnPTP. *WB*, Western blot. *B*, FAK and Src co-immunoprecipitation. Immunoprecipitates obtained with anti-FAK antibodies were analyzed by SDS-PAGE and immunoblotting with anti-Src or anti-FAK antibodies. Total Src content was verified by subjecting whole cell lysates to SDS-PAGE and immunoblotting with anti-Src antibodies (*lowest panel*). *C*, ERK activation. Total cell lysates were analyzed by SDS-PAGE and immunoblotting with anti-phosphoERK (*α-pERK*) or anti-ERK antibodies (*α-ERK*).

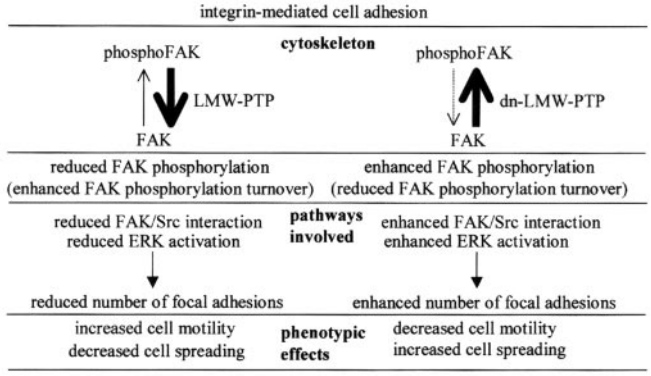

FIG. 6. **Effects of LMW-PTP overexpression on FAK phosphorylation and signaling pathways downstream of FAK (for details see "Discussion").**

cipitated from NIH/3T3-PTP adherent cells, which had not been fed with fresh medium during the last 24 h. Immunoblotting with either anti-FAK or anti-LMW-PTP antibodies showed that LMW-PTP and FAK co-immunoprecipitated (Fig. 3*C*). The co-immunoprecipitation was also obtained with NIH/3T3-dnPTP cells (not shown). LMW-PTP and FAK were found to co-immunoprecipitate irrespectively of cell suspension or adhesion, at least within the RIPA-soluble fraction (not shown). The harsh conditions necessary to extract LMW-PTP from the insoluble fraction did not allow investigating such an association in the cytoskeleton. We also verified that LMW-PTP is not tyrosine-phosphorylated under our experimental conditions, *i.e.* in the absence of acute growth factor stimulation (Fig. 4*D*), whereas it is markedly phosphorylated in response to PDGF, as demonstrated previously (25). Thus, the LMW-PTP/FAK interaction is independent of LMW-PTP tyrosine phosphorylation.

LMW-PTP Activity on FAK Affects Downstream Signaling Pathways—To investigate the effects of LMW-PTP activity on signaling pathways downstream of FAK, we determined the phosphorylation level of specific tyrosine residues of FAK in adherent cells 24 h after plating without medium change. FAK phosphorylation was affected in all residues in NIH/3T3-PTP as well as NIH/-3T3-dnPTP cells (Fig. 5*A*). The homogeneity of this pattern is consistent with an altered association of Src with FAK at PY397, which is likely to affect the phosphorylation level of the other residues. We found, indeed, that the amount of Src co-immunoprecipitated with FAK was undetectable in NIH/3T3-PTP cells, whereas the expression of dnLMW-PTP was associated with a very abundant recovery of Src in anti-FAK immunoprecipitates (Fig. 5*B*). Since ERK activation is related to the phosphorylation of Tyr-925 by Src via Grb2 recruitment, we tested whether ERK phosphorylation was affected. Fig. 5*C* shows that both ERK1 and ERK2 were dephosphorylated in NIH/3T3-PTP cells and strongly hyperphosphorylated in NIH/3T3-dnPTP cells as compared with controls.

DISCUSSION

We show in this study that, when adhesion stimuli are isolated (that is, in the absence of acute growth factor stimulation), NIH/3T3-PTP fibroblasts display impaired cell spreading, increased motility, and a concomitant reduction in the number of focal adhesions. All these phenotypic effects correlate with LMW-PTP redistribution in the cytoskeleton following cell adhesion and with impaired FAK phosphorylation. The co-immunoprecipitation of LMW-PTP and FAK demonstrates their association, whereas FAK hyperphosphorylation in NIH/3T3-dnPTP and *in vitro* dephosphorylation of FAK by LMW-PTP reveal that FAK is a substrate of LMW-PTP. It is worth pointing out here that, in the presence of adhesion stimuli only, the sole electrophoretic band whose phosphorylation is significantly altered by LMW-PTP overexpression corresponds to FAK. These data indicate that the main target of LMW-PTP is FAK, whose dephosphorylation then results, via downstream events involving Src and ERK, in the observed changes of cell morphology and motility. The involvement of both Src and ERK, indeed, in the regulation of cell motility has been extensively characterized (1, 2).

The hypothesis that LMW-PTP might be involved in cytoskeleton dynamics was suggested by the discovery of a cytosol and a Triton-insoluble pools of LMW-PTP and of the transient redistribution of the enzyme from the former to the latter pool following PDGF stimulation (23). The study reported here was focused on the characterization of the involvement of LMW-PTP in cell spreading and motility under conditions discriminating the effects induced by adhesion from those induced by acute growth factor stimulation. In the absence of growth factor stimulation, we found that the intracellular distribution of LMW-PTP was sensitive to the suspension/adhesion state of cells. Indeed, the enzyme level increased in cytosol at the expenses of cytoskeleton during suspension, whereas a backward redistribution from cytosol to cytoskeleton was observed when cells were made to adhere onto either fibronectin or polylysine.

FAK moves to the cytoskeleton following cell adhesion onto fibronectin, thus undergoing the same type of redistribution as LMW-PTP. Accordingly, we found that in adherent NIH/3T3-PTP cells, FAK is mainly distributed in the cytosol at the expense of focal adhesions and that the redistribution of FAK to the cytoskeleton following adhesion onto fibronectin is partially compromised. Thus, LMW-PTP overexpression affects FAK status in two ways: by causing its dephosphorylation and by impairing its adhesion-dependent redistribution to the cytoskeleton. We can hypothesize that this impairment is a consequence of dephosphorylation as a correlation was found between the level of tyrosine phosphorylation of FAK and its

presence in the cytoskeleton, which possibly occurs via the stabilization of FAK·CAS complexes (31). These pieces of evidence prompted us to verify whether LMW-PTP and FAK associate. We found that the two proteins co-immunoprecipitate and that their association, at least within their RIPA-soluble pools, does not depend on cell adhesion. Due to the harsh conditions necessary to extract LMW-PTP from the cytoskeleton, nothing could be said concerning LMW-PTP association with FAK in this compartment. The fact that we could co-immunoprecipitate FAK and LMW-PTP (the active form) irrespectively of cell suspension or adhesion strongly suggests that the interaction between the two proteins does not depend on the phosphorylation status of FAK nor does it involve the active site of LMW-PTP.

Based on all the above data, we can hypothesize that, following integrin-mediated cell adhesion, LMW-PTP and FAK co-distribute in the cytoskeleton, where FAK undergoes tyrosine phosphorylation. LMW-PTP activity would then determine FAK dephosphorylation, thus resulting in focal adhesion remodeling (Fig. 6). It is worth pointing out here that LMW-PTP is also redistributed to the cytoskeleton following cell adhesion onto polylysine, whereas FAK is not, indicating that LMW-PTP responds to different types of adhesion-related stimuli and possibly acts on different cytoskeletal targets. Our results contribute to clarify the phenotypes deriving from alterations of FAK phosphorylation or distribution. As discussed by several authors (1, 3, 32), cell migration seems to be controlled by an increased rate of phosphorylation/dephosphorylation cycles rather than by FAK phosphorylation level *per se*. Our findings that LMW-PTP dephosphorylates FAK and that cells overexpressing LMW-PTP are more motile, are less spread, and display a reduced number of focal adhesions are in keeping with an increased turnover of focal contacts and a shift of the equilibrium toward their disassembly. Accordingly, dnLMW-PTP, once overexpressed in NIH/3T3, binds FAK but is unable to dephosphorylate it, possibly altering the equilibrium of phosphorylation/dephosphorylation cycles toward FAK hyperphosphorylation (Fig. 6). These features are in keeping with the fact that the NIH/3T3-dnPTP phenotype is similar to that of FAK knockout fibroblasts (8, 9), which are less motile cells with a high number of focal adhesions all over the ventral surface. The latter feature is determined by the fact that, although spreading proceeds and new adhesions form at the leading edge, the disassembling of older adhesions is deficient (10).

It was demonstrated previously that the cytoskeleton-associated fraction of LMW-PTP is phosphorylated by Src under PDGF stimulation in adherent cells (33). Such a phosphorylation seems fundamental for LMW-PTP association with p190Rho-GAP, which is phosphorylated only following PDGF stimulation (25). In this study, we show that the sole adhesion, in the absence of PDGF stimulation, allowed the distribution of the enzyme in the cytoskeleton. Under this condition, LMW-PTP is not tyrosine-phosphorylated, indicating that this is not a prerequisite for enzyme localization in the cytoskeleton and for its activity on FAK. This result is in agreement with previous observations showing that an LMW-PTP mutant lacking the two tyrosine phosphorylation sites (Tyr-131 and Tyr-132) still localizes in the cytoskeleton (24). Thus, LMW-PTP during cell adhesion behaves differently depending on the absence or the presence of PDGF, integrating the effects of adhesive and mitogenic stimuli on focal adhesion remodeling.

Acknowledgment—We are grateful to Prof. Giampietro Ramponi for helpful discussion.

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Low M_r Phosphotyrosine Protein Phosphatase Associates and Dephosphorylates p125 Focal Adhesion Kinase, Interfering with Cell Motility and Spreading

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J. Biol. Chem. 2002, 277:41631-41636.
doi: 10.1074/jbc.M201709200 originally published online June 7, 2002
