THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 7, Issue of February 18, pp. 4640–4646, 2000

Printed in U.S.A.

The Low M_r Protein-tyrosine Phosphatase Is Involved in Rho-mediated Cytoskeleton Rearrangement after Integrin and Platelet-derived Growth Factor Stimulation*

(Received for publication, August 4, 1999, and in revised form, November 10, 1999)

Paola Chiarugi, Paolo Cirri, Letizia Taddei, Elisa Giannoni, Guido Camici, Giampaolo Manao, Giovanni Raugei, and Giampietro Ramponi‡

From the Dipartimento di Scienze Biochimiche, Università di Firenze, 50134 Firenze, Italy

The low molecular weight protein-tyrosine phosphatase (LMW-PTP) is an enzyme that is involved in the early events of platelet-derived growth factor (PDGF) receptor signal transduction. In fact, LMW-PTP is able to specifically bind and dephosphorylate activated PDGF receptor, thus modulating PDGF-induced mitogenesis. In particular, LMW-PTP is involved in pathways that regulate the transcription of the immediately early genes *myc* and *fos* in response to growth factor stimulation. Recently, we have found that LMW-PTP exists constitutively in cytosolic and cytoskeleton-associated localization and that, after PDGF stimulation, c-Src is able to bind and phosphorylate LMW-PTP only in the cytoskeleton-associated fraction. As a consequence of its phosphorylation, LMW-PTP increases its catalytic activity about 20-fold. In this study, our interest was to investigate the role of LMW-PTP phosphorylation in cellular response to PDGF stimulation. To address this issue, we have transfected in NIH-3T3 cells a mutant form of LMW-PTP in which the c-Src phosphorylation sites (Tyr^{131} and Tyr^{132}) were mutated to alanine. We have established that LMW-PTP phosphorylation by c-Src after PDGF treatment strongly influences both cell adhesion and migration. In addition, we have discovered a new LMW-PTP substrate localized in the cytoskeleton that becomes tyrosine-phosphorylated after PDGF treatment: p190Rho-GAP. Hence, LMW-PTP plays multiple roles in PDGF receptor-mediated mitogenesis, since it can bind and dephosphorylate PDGF receptor, and, at the same time, the cytoskeleton-associated LMW-PTP, through the regulation of the p190Rho-GAP phosphorylation state, controls the cytoskeleton rearrangement in response to PDGF stimulation.

Many cellular processes such as cell migration, adhesion, and proliferation require the collaborative interaction between growth factors and extracellular matrix (ECM)[1] stimuli (1–3). Cell adhesion on ECM results in clustering of integrins in focal adhesions that contain both cytoskeletal and signaling proteins. Formation of focal adhesions as well as the closely associated actin stress fibers requires activation of the small GTP-binding protein Rho (4). Rho, a member of the Ras superfamily of GTP-binding proteins, cycles between a GDP-bound inactive form and a GTP-bound active state. Rho is regulated primarily by two groups of proteins: guanine nucleotide exchange factors that catalyze exchange of GDP for GTP and GTPase-activating proteins (GAPs) that stimulate the hydrolysis of GTP to GDP. Upon binding to GTP, Rho interacts with and activates proteins such as Rho kinase and phosphatidylinositol 4-phosphate 5-kinase (5).

p190Rho-GAP is a GTPase-activating protein for Rho. During growth factor stimulation p190Rho-GAP becomes tyrosine-phosphorylated by c-Src in Tyr^{1105} (6, 7) and undergoes transient redistribution into perinuclear concentric arcs that coincide with epidermal growth factor-mediated focal adhesion assembly and reassembly (6). p190Rho-GAP tyrosine phosphorylation correlates with rapid disassembly of actin stress fiber, suggesting that this phosphorylation may increase its Rho-GAP activity (6). Another function proposed for p190Rho-GAP phosphorylation is to form a binding site for the Src homology 2 domain of p120Ras-GAP even if a significant portion of p190Rho-GAP/p120Ras-GAP binding is Tyr(P)-independent (7). In addition to p190Rho-GAP, many other proteins present in focal adhesions, such as tensin, paxillin, p130Cas, and focal adhesion kinase, become tyrosine-phosphorylated during integrin and/or growth factor stimulation. This evidence would predict the requirement for protein-tyrosine phosphatases (PTPs) in the integrin downstream signaling.

The PTP superfamily is composed of over 70 enzymes that, despite very limited sequence similarity, share a common active site motif CX_5R and an identical catalytic mechanism. On the basis of their function, structure, and sequence, PTPs can be classified in four main families: 1) tyrosine-specific phosphatases; 2) VH1-like dual specificity PTPs; 3) the Cdc25; and 4) the low molecular weight phosphatase (8).

The low molecular weight protein-tyrosine phosphatase (LMW-PTP) is an 18-kDa enzyme that is expressed in many mammalian tissues (9). Our previous studies on the molecular biology of LMW-PTP in NIH-3T3 cells evidenced a well defined role of this enzyme in PDGF-induced mitogenesis. The most relevant phenotypic effect of LMW-PTP overexpression was the

* This work was supported by Consiglio Nazionale delle Ricerche (CNR) Grant 97.03810.CT14 and target project on Biotechnology and by the Italian Association for Cancer Research (AIRC) in part by European Community Grant ERB BI04-CT96-0517, in part by the Ministero della Università e Ricerca Scientifica e Tecnologica (Meccanismi biochimici di controllo delle funzioni cellulari, 1997), and in part by a CNR strategic project (Controlli post-trascrizionali dell'espressione genica). 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.

‡ To whom correspondence should be addressed: Dipartimento di Scienze Biochimiche, Viale Morgagni 50, 50134 Firenze, Italy. Tel.: 39-055-413765; Fax: 39-055-4222725; E-mail: raugei@cesit1.unifi.it.

[1] The abbreviations used are: ECM, extracellular matrix; dtmLMW-PTP, double tyrosine mutant (Y131A/Y132A) LMW-PTP; dnLMW-PTP, dominant negative (C12S) LMW-PTP; GAP, GTPase-activating protein; LMW-PTP, low molecular weight protein-tyrosine phosphatase; PDGF, platelet-derived growth factor; PDGF-R, PDGF receptor; PTP, protein-tyrosine phosphatase; wtLMW-PTP, wild type LMW-PTP; STAT, signal transducers and activators of transcription; DMEM, Dulbecco's modified Eagle's medium; RIPA, radioimmune precipitation assay; PBS, phosphate-buffered saline; Ip, immunoprecipitation; Wb, Western blot.

strong reduction of cell growth rate in response to PDGF stimulation. We have shown that activated PDGF-R is a LMW-PTP substrate (10) and that LMW-PTP is involved in the control of specific pathways triggered by PDGF-R activation. In particular, LMW-PTP is able to modulate both *myc* expression, interfering with the Src pathway, and *fos* expression through an extracellular signal regulated kinase-independent pathway mediated by the STAT proteins (11). More recently, we have found that in NIH-3T3 cells LMW-PTP is localized constitutively in both cytoplasmic and cytoskeleton-associated fraction. These two different LMW-PTP pools are differentially regulated, since only the cytoskeleton-associated LMW-PTP fraction is specifically phosphorylated by c-Src after PDGF stimulation (12). As a consequence of its phosphorylation, LMW-PTP shows an average 20-fold increase in its *in vitro* catalytic activity (13) instead of the 2-fold activation that was previously reported (14, 15).

In the present study, we have investigated the physiological role of cytoskeleton-associated LMW-PTP in cell adhesion, migration, and spreading in relationship to the phosphatase phosphorylation state using a LMW-PTP mutant in which tyrosines in position 131 and 132 were replaced by alanine (double tyrosine mutant, dtmLMW-PTP). Hence, dtmLMW-PTP cannot be phosphorylated and, consequently, activated by c-Src. Here we have shown that cytoskeleton-associated LMW-PTP influences cell adhesion, spreading, and migration controlling the phosphorylation state of p190Rho-GAP, a protein that is able to regulate Rho activity and, hence, cytoskeleton rearrangement in response to PDGF stimulation. In addition, in this study we have demonstrated that, *in vivo*, LMW-PTP itself is regulated by c-Src phosphorylation, since phosphorylated LMW-PTP presents an increased activity on a physiologic substrate such as p190Rho-GAP. In conclusion, our findings strongly support the notion that LMW-PTP is able to perform multiple roles in PDGF-induced mitogenesis. In fact, cytosolic LMW-PTP binds and dephosphorylates PDGF-R (10), thus modulating part of its signaling cascade, whereas cytoskeleton-associated LMW-PTP acts on phosphorylated p190Rho-GAP, consequently playing a role in PDGF-mediated cytoskeleton rearrangement.

EXPERIMENTAL PROCEDURES

Materials—Unless otherwise specified, all reagents were obtained from Sigma. NIH-3T3 cells were purchased from ATCC; human recombinant platelet-derived growth factor BB (PDGF-BB) was from Peprotech; the ECL kit was from Amersham Pharmacia Biotech; and all antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), except those against tensin (gift of Dr. Su Hao Lo).

Site-specific Mutagenesis and Cloning of dtmLMW-PTP in Eukaryotic Expression Vector—Oligonucleotide-directed mutagenesis was performed using the Unique Restriction Elimination Site kit (Amersham Pharmacia Biotech). The 26-base-long target mutagenesis primer contains two sequential ACA codons (alanine) substituting the original TAT codon (tyrosine). The mutated LMW-PTP coding sequence was completely sequenced by the Sanger method and subcloned in the *Hin*dIII and *Xba*I restriction sites of pRcCMV eukaryotic expression vector, harboring the neomycin resistance gene.

Cell Culture and Transfections—NIH-3T3 cells were routinely cultured in DMEM supplemented with 10% fetal calf serum in a 5% CO_2 humidified atmosphere. 10 μg of pRcCMV-wtLMW-PTP, pRcCMV-dnLMW-PTP, or pRcCMV-dtmLMW-PTP, conferring neomycin resistance, were transfected in NIH-3T3 cells using the calcium phosphate method. Stable transfected clonal cell lines were isolated by selection with G418 (400 μg/ml). Control cell lines were obtained by transfecting 2 μg of pRcCMVneo alone. The clonal lines were screened for expression of the transfected genes by (*a*) Northern blot analysis and (*b*) enzyme-linked immunosorbent assay using polyclonal anti-LMW-PTP rabbit antibodies, which do not cross-react with murine endogenous LMW-PTP.

Immunoprecipitations and Western Blot Analysis—1×10^6 cells were seeded in 10-cm plates in DMEM supplemented with 10% fetal calf serum. Cells were serum-starved for 24 h before receiving 30 ng/ml PDGF-BB. Cells were then lysed for 20 min on ice in 500 μl of RIPA lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 2 mM EGTA, 1 mM sodium orthovanadate, 1 mM phenylmethanesulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin). Lysates were clarified by centrifugation and immunoprecipitated for 4 h at 4 °C with 0.1 μg of the specific antibodies. Immune complexes were collected on protein A-Sepharose (Amersham Pharmacia Biotech), separated by SDS-polyacrylamide gel electrophoresis, and transferred onto nitrocellulose (Sartorius). Immunoblots were incubated in 3% bovine serum albumin, 10 mM Tris/HCl, pH 7.5, 1 mM EDTA, and 0.1% Tween 20, for 1 h at room temperature, probed first with specific antibodies and then with secondary antibodies conjugated with horseradish peroxidase, washed, and developed with the ECL kit (Amersham Pharmacia Biotech).

Cell Lysate Fractionations—Cell lysate fractions were obtained as already described (12). Briefly, PDGF-stimulated NIH-3T3 cells were lysed in RIPA buffer, and the lysates were clarified by centrifugation for 30 min at 20,000 $\times$ *g*. Pellets were washed twice with 1 ml of RIPA and then resuspended in complete RIPA buffer, which is RIPA buffer plus 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate, by shaking for 1 h at room temperature and newly clarified by centrifugation at 20,000 $\times$ *g* for 30 min. RIPA or complete RIPA fractions were then used for immunoprecipitation analysis.

Cell Adhesion on Different Substrata—Cell adhesion was assessed as described elsewhere (16). Briefly, 1×10^6 cells were seeded in 10-cm plates in DMEM supplemented with 10% fetal calf serum. Cells were serum-starved for 24 h before detaching with 0.25% trypsin for 1 min. Trypsin was blocked with 0.2 mg/ml soybean trypsin inhibitor, centrifuged at 1200 rpm for 10 min, and then resuspended in 2 ml/10-cm dish of DMEM containing 0.2% bovine serum albumin. Resuspended cells were maintained in suspension with gentle agitation for 30 min at 37 °C and then directly seeded in precoated dishes treated overnight with 10 μg/ml human fibronectin or 10 μg/ml poly-D-lysine in PBS and then washed twice in PBS and blocked for 2 h with 2% bovine serum albumin in PBS.

Cell Adhesion Assay—Cell adhesion was assessed as described elsewhere (16). Briefly, 3×10^4 cells were seeded for the indicated time in a 96-well dish precoated for 2 h with 10 μg/ml human fibronectin and washed twice with PBS. Cell adhesion was stopped by removing the medium and by the addition of a 0.5% crystal violet solution in 20% methanol. After 5 min of staining, the fixed cells were washed with PBS and solubilized with 200 μl/well of 0.1 M sodium citrate, pH 4.2. The absorbance at 595 nm was evaluated using a microplate reader. The adhesion assay was performed either in complete medium or after 24 h of serum starvation followed by the addition of 10 ng/ml PDGF. All cell adhesion assays were performed in triplicate.

Cell Spreading Assay—Cell spreading was assessed as described elsewhere (16). Briefly, 5×10^5 cells were seeded in complete medium for the indicated time in a 24-well dish precoated for 2 h with 10 μg/ml human fibronectin and washed twice with PBS containing 0.2% soybean trypsin inhibitor. After removal of the medium, the cells were fixed in 1 ml of 0.25% *p*-formaldehyde for at least 1 h. Photographs were taken with Kodak 100 ASA film.

Cell Motility Assay—Cell migration was assessed as described elsewhere with minor modifications (11). Migration of NIH-3T3 cells was assayed with the Transwell system of Costar, equipped with 8-μm pore polyvinylpyrrolidone-free polycarbonate filters (6-mm diameter) precoated with human type I collagen (20 μg/ml) and placed between the chemoattractant (lower chamber) and the upper chamber. The lower chamber was filled with medium supplemented with different concentrations of PDGF-BB. Serum-free DMEM cultured cells were suspended by trypsinization, and 3×10^4 cells in 200 μl were added to the top wells and incubated at 37 °C in 5% CO_2 for 6 h. After incubation, the cells attached to the upper side but not migrated through the filter were mechanically removed using cotton swabs. The filters were fixed in 96% methanol and stained with Diff Quick staining solutions. Chemotaxis was evaluated by counting the cells that had migrated to the lower surface of the polycarbonate filters. For each filter, the number of cells in six randomly chosen fields was determined, and the counts were averaged (mean $\pm$ S.D.).

RESULTS

LMW-PTP Is Involved in Integrin-mediated Adhesion—In a previous paper (12), we demonstrated that LMW-PTP exists in two spatially and functionally separated pools. The cytosolic LMW-PTP is not phosphorylated and is able to bind and dephosphorylate the activated PDGF receptor. In contrast, the cytoskeleton-associated LMW-PTP becomes phosphorylated by

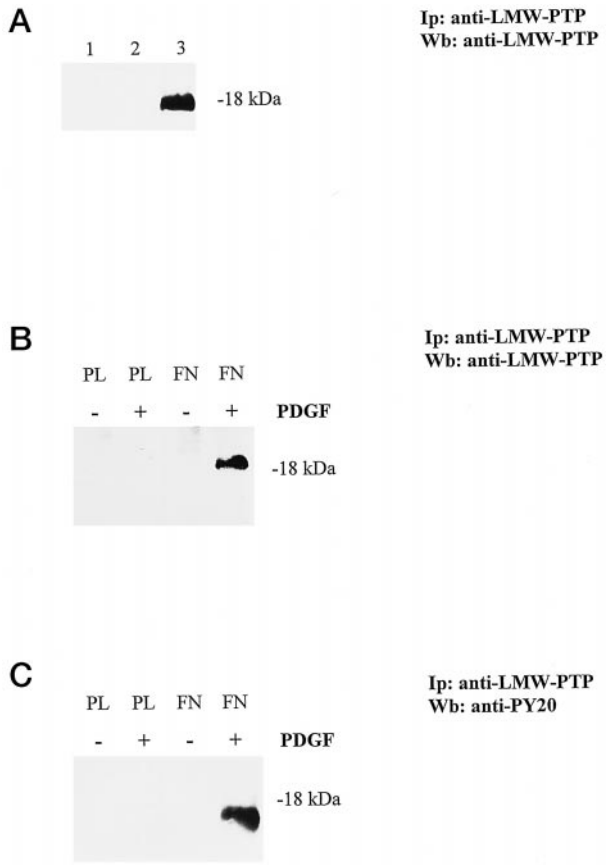

FIG. 1. **Localization and phosphorylation of LMW-PTP in integrin-mediated cell adhesion structures.** 1×10^6 NIH-3T3 cells overexpressing wtLMW-PTP were serum-starved for 24 h and then treated as indicated. After cell fractionation, the cytoskeleton fractions were immunoprecipitated with anti-LMW-PTP antibodies, and an anti-LMW-PTP or anti-PY20 immunoblot was performed. *A*, the cells were left on the untreated dish (*lane 1*) or kept in suspension for 30 min (*lane 2*) and for 1 h (*lane 3*) in serum-free medium. *B*, cells were kept in suspension for 30 min and then seeded, in serum-free medium, on polylysine (*PL*)-coated, or on fibronectin (*FN*)-coated dishes for 15 min, and then stimulated or not with 30 ng/ml PDGF-BB for 15 min. *C*, cells were kept in suspension for 30 min and then seeded, in serum-free medium, on fibronectin or polylysine-coated dishes for the indicated times and then stimulated or not with 30 ng/ml PDGF-BB for 15 min.

c-Src during PDGF stimulation, and its substrate, in this subcellular localization, is a protein of about 190 kDa (12). In order to study the properties of the cytoskeleton-associated LMW-PTP fraction, we analyzed if the disruption of the cytoskeleton architecture affects the localization of this phosphatase. NIH-3T3 cells overexpressing the active LMW-PTP (wtLMW-PTP) were kept in suspension for 30 min and then stimulated with 30 ng/ml PDGF-BB in parallel with control adherent cells. After cell fractionation by differential detergent treatment, we analyzed the cytoskeleton fractions by anti-LMW-PTP immunoblot. The result (Fig. 1*A*) indicates that LMW-PTP is not present in the cytoskeleton-associated fraction of the suspended cells, thus suggesting a requirement of cytoskeleton integrity for LMW-PTP localization in this subcellular district. Then we analyzed the dependence of LMW-PTP localization in the cell adhesion structures that are formed upon reseeding cells on different substrata. We seeded wtLMW-PTP-overexpressing cells on dishes coated with polylysine, a substratum that is unable to induce integrin clustering, or with fibronectin, a specific integrin receptor agonist. After 15 min, cells were stimulated or not with PDGF-BB, and the cytoskeletal fractions were assayed for LMW-PTP content. Fig. 1*B* reports that LMW-PTP accumulates in the cytoskeleton-associated fraction

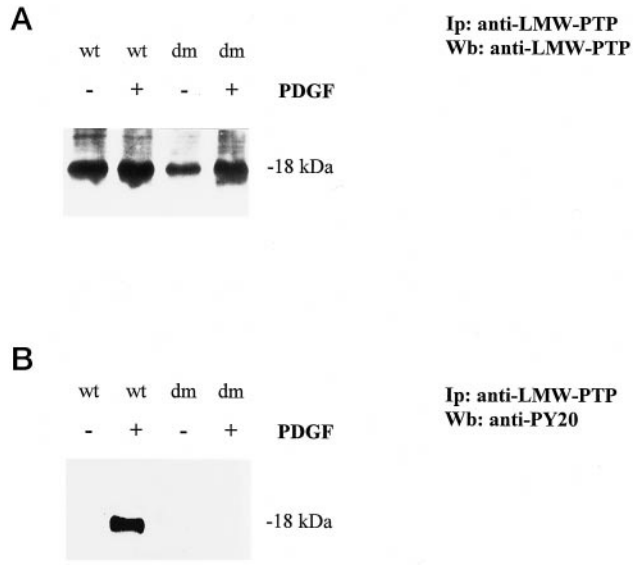

FIG. 2. **Localization and tyrosine phosphorylation analysis of dtmLMW-PTP in NIH-3T3 cells.** 1×10^6 NIH-3T3 cells overexpressing either wtLMW-PTP or dtmLMW-PTP were serum-starved for 24 h on untreated dishes and then stimulated or not with 30 ng/ml PDGF-BB for 15 min. After cell fractionation, the cytoskeleton fractions were immunoprecipitated with anti-LMW-PTP antibodies, and an anti-LMW-PTP immunoblot was performed (*A*). The same blot was stripped and reprobed with anti-phosphotyrosine antibodies (*B*).

only in fibronectin and PDGF-treated cells, suggesting that the targeting of LMW-PTP in this subcellular district is driven by the formation of integrin-mediated cell adhesion structures. Similar results were obtained treating cells with collagen type I (data not shown). In addition, only in cells seeded on fibronectin, LMW-PTP undergoes phosphorylation after PDGF treatment, suggesting that LMW-PTP phosphorylation is a phenomenon that requires both integrin-mediated cell adhesion and PDGF receptor stimulation (Fig. 1*C*). Similar results were obtained with collagen type I (data not shown).

LMW-PTP Phosphorylation and Its Role in the Integrin-mediated Cytoskeleton Rearrangement—Taylor *et al.* (15) have reported that LMW-PTP tyrosine 131 and 132 are phosphorylated by Src family kinases in Jurkat T-cells. In order to study the physiological role of LMW-PTP phosphorylation, we generated by site-specific mutagenesis a double mutant (dtmLMW-PTP) in which both tyrosine 131 and 132 were substituted with an alanine. The resulting mutated enzyme, which maintains about 40% of the wtLMW-PTP specific activity on *p*-nitrophenyl phosphate, cannot be subjected to c-Src phosphorylation (13). We have subcloned the dtmLMW-PTP coding region in pRcCMV eukaryotic expression vector, and we have stably transfected NIH-3T3 cells. Overexpressing clones were isolated by neomycin selection and anti-LMW-PTP Western blot screening. In all of the following experiments, we have used clones that overexpress wtLMW-PTP and dtmLMW-PTP at a similar level. Fig. 2*A* shows that dtmLMW-PTP is targeted to the cytoskeleton structures in response to PDGF treatment as well as wtLMW-PTP. As expected, the dtmLMW-PTP is not tyrosine-phosphorylated in response to PDGF treatment and fibronectin adhesion (Fig. 2*B*). These findings indicate that LMW-PTP targeting to cytoskeleton is independent from its tyrosine phosphorylation and that, also *in vivo*, residues 131 and 132 are the only LMW-PTP tyrosines that are phosphorylated in response to PDGF stimulation.

Furthermore, we were interested in determining the role of LMW-PTP phosphorylation in integrin-mediated signaling, especially for what concerns cytoskeleton rearrangements following the mitotic stimulus, such as cell adhesion, spreading, and

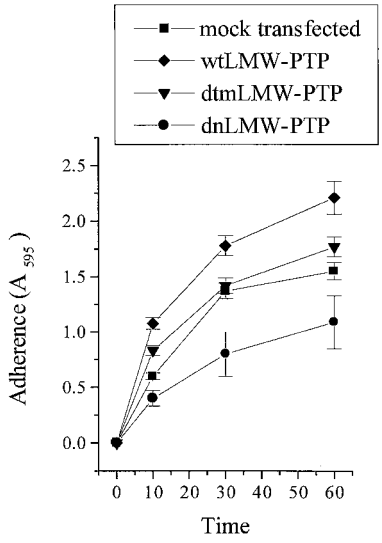

FIG. 3. **Integrin-mediated cell adhesion evaluation in NIH-3T3 cells.** 3×10^4 cells of each indicated type were seeded in a 96-well plate precoated with fibronectin, serum-starved for 24 h, and then treated with 30 ng/ml PDGF-BB. The cells were allowed to attach for the indicated times, and the adhesion was evaluated with crystal violet staining. The result is representative of three independent experiments with similar results. S.D. is indicated.

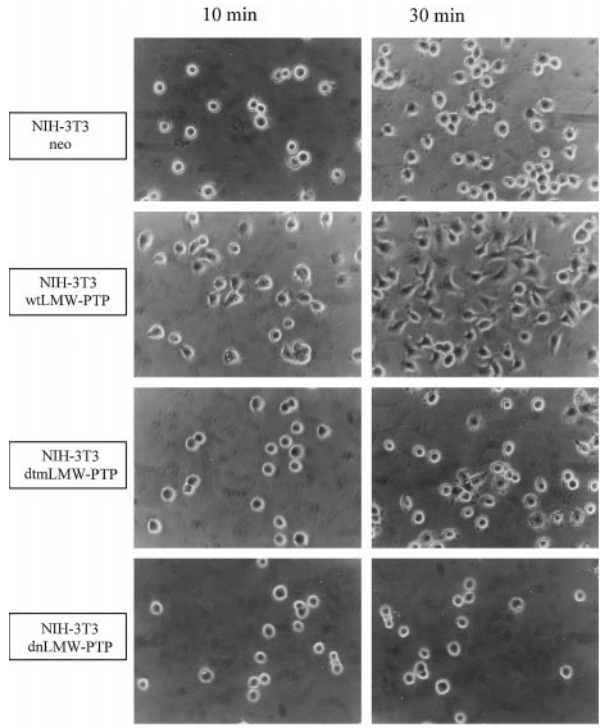

FIG. 4. **Cell spreading on fibronectin treated dishes of NIH-3T3 cells.** 1×10^5 cells of each indicated type were seeded in a 24-well plate precoated with fibronectin. Cells were allowed to spread on the substratum for 10 min or 30 min and immediately fixed in *p*-formaldehyde. The photographs are shown. The result is representative of three independent experiments with similar results.

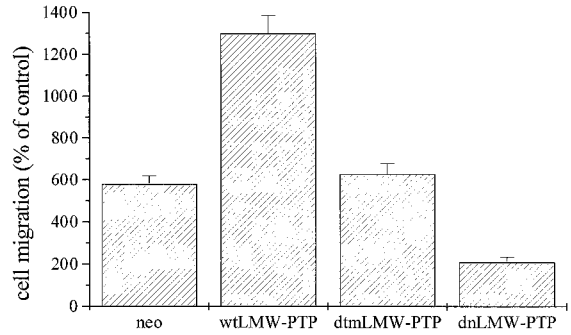

FIG. 5. **PDGF-induced chemotaxis in NIH-3T3 cells.** 1.5×10^5 cells of the indicated type were seeded into a 2.5-cm Transwell. 10 ng/ml of PDGF-BB was added to the lower chamber, and the cell migration was evaluated after Diff-Quick staining and reported in the histogram as a percentage of the control unstimulated cells. The result is representative of three independent experiments with similar results. S.D. is indicated.

migration. Integrin-mediated cell adhesion was tested by seeding previously suspended cells on fibronectin-coated dishes for 10, 30, and 60 min. The adherence of cells in the presence of 10 ng/ml PDGF-BB was evaluated with crystal violet staining, and absorbance at 595 nm was plotted against time (Fig. 3). After PDGF stimulation, wtLMW-PTP-expressing cells showed a significant increase of cell adhesion in comparison with mock-transfected cells, whereas the dtmLMW-PTP overexpressing cells behave more similar to mock-transfected cells. We do not observe any effect of the overexpression of LMW-PTP in unstimulated cells. In agreement with this observation, dnLMW-PTP-expressing cells exhibit an opposite phenotype showing a clear decrease in cell adhesion with respect to wtLMW-PTP cells. The dominant negative LMW-PTP is a protein in which the mutation of the cysteine residue to serine in the signature motif (C12S) causes the complete loss of catalytic activity. Nevertheless, this dominant negative mutant (dnLMW-PTP) is still able to bind specific substrates (10, 17). These data indicate a role of LMW-PTP phosphorylation in integrin-mediated cell adhesion upon PDGF stimulation, since overexpression of an unphosphorylatable enzyme (dtmLMW-PTP) has a little effect on cell adhesion. Hence, we evaluated the effect of LMW-PTP overexpression in the cell spreading on the extracellular matrix protein fibronectin. Cells expressing the wild type, dnLMW-PTP, or the dtmLMW-PTP were seeded on fibronectin-treated six-well dishes and fixed by *p*-formaldehyde treatment after 10 and 30 min from seeding. Fig. 4 shows that the wtLMW-PTP overexpression leads to an increase in cell spreading with respect to control cells both at 10 and 30 min, while the overexpression of the dtmLMW-PTP has only a marginal effect, suggesting a specific role of LMW-PTP phosphorylation in mediating cell spreading. In addition, dnLMW-PTP overexpression inhibits cell spreading on fibronectin. Finally, we analyzed the chemotactic response to PDGF-BB in cells overexpressing either wild type or dtmLMW-PTP in comparison with mock-transfected cells. The results, shown in Fig. 5, show that the wtLMW-PTP overexpression leads to an increase of the PDGF-induced chemotaxis with respect to mock-transfected cells, whereas the dtmLMW-PTP overexpression has little effect on the chemotactic response, indicating a possible regulative role of LMW-PTP in PDGF-induced cell migration. As previously reported (11), dnLMW-PTP overexpression leads to an inhibition of PDGF-induced chemotaxis.

Altogether, these results provide evidence for a critical role of LMW-PTP phosphorylation in modulating PDGF- and ECM-mediated signaling that leads to cytoskeleton rearrangements.

p190Rho-GAP Is a LMW-PTP Substrate in the Cytoskeleton Fraction—We previously reported that a protein of about 190 kDa, distinct from PDGF-R, could be an LMW-PTP substrate in the cytoskeleton-associated fraction. In fact, this protein is differentially phosphorylated in wtLMW-PTP-overexpressing cells with respect to control cells after PDGF stimulation (12). In order to obtain evidence of a direct association between the phosphorylated p190 and LMW-PTP, we performed an anti-LMW-PTP immunoprecipitation of the cytoskeleton fraction followed by a Western blot analysis with anti-phosphotyrosine

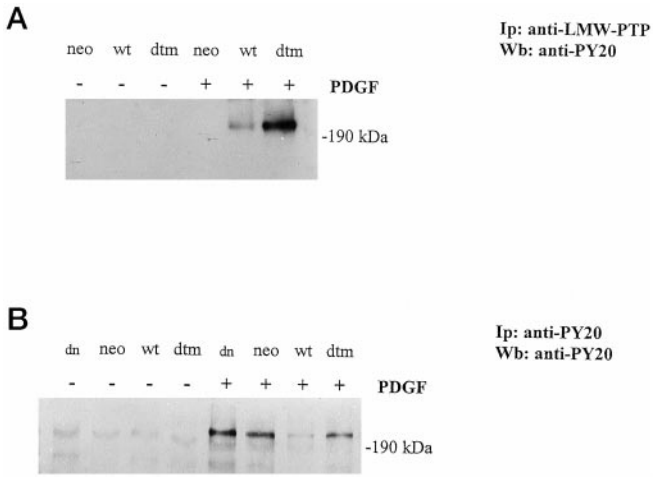

FIG. 6. **Tyrosine-phosphorylated LMW-PTP directly interacts with p190 in the cytoskeleton fraction.** 1×10^6 cells of the indicated type were serum-starved for 24 h and then stimulated or not with 30 ng/ml PDGF-BB for 10 min. After cell fractionation, the cytoskeleton fractions were immunoprecipitated either with anti-LMW-PTP (*A*) or anti-phosphotyrosine (*B*) antibodies, and an anti-phosphotyrosine immunoblot was performed. *A* shows the direct association between LMW-PTP and p190; *B* shows the tyrosine phosphorylation level of p190 in each cell type. The result is representative of three independent experiments with similar results.

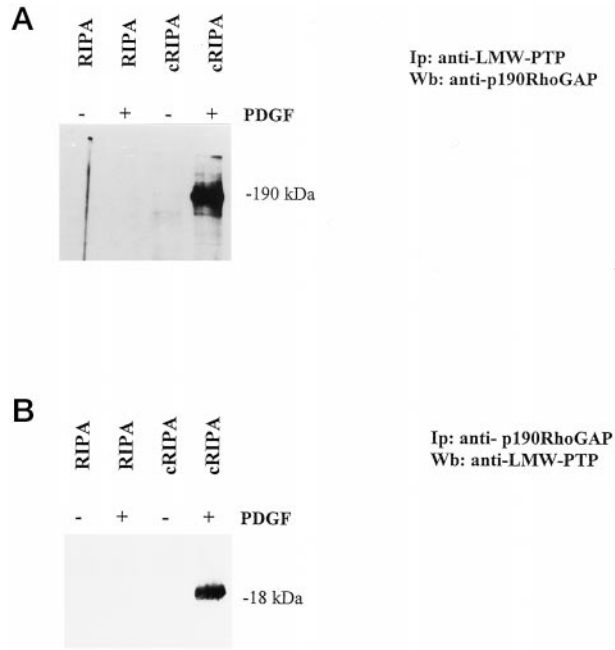

FIG. 7. **LMW-PTP interacts with p190Rho-GAP.** 1×10^6 wtLMW-PTP-overexpressing cells were serum-starved for 24 h and then stimulated or not with 30 ng/ml PDGF-BB for 10 min. After cell fractionation, the cytosolic or the cytoskeleton fraction was either immunoprecipitated with antibodies and revealed with an anti-LMW-PTP immunoblot (*A*) or immunoprecipitated with anti-LMW-PTP and revealed with anti-Rho-GAP immunoblot (*B*). The result is representative of three independent experiments with similar results. *cRIPA*, complete RIPA buffer.

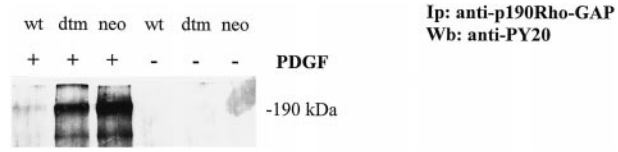

FIG. 8. **Tyrosine phosphorylation level of p190Rho-GAP in LMW-PTP-expressing cells.** 1×10^6 cells of the indicated type were serum-starved for 24 h and then stimulated or not with 30 ng/ml PDGF-BB for 10 min. After cell fractionation, the cytoskeleton fraction was immunoprecipitated with anti-p190Rho-GAP antibodies and recognized with anti-phosphotyrosine antibody. The result is representative of three independent experiments with similar results.

antibodies. Fig. 6*A* shows that a protein of about 190 kDa coimmunoprecipitates with LMW-PTP both in wtLMW-PTP- and dtmLMW-PTP-overexpressing cells. We have recently shown that, *in vitro*, LMW-PTP phosphorylation by c-Src leads to an approximately 20-fold increase in LMW-PTP enzymatic activity (13). In order to establish if also *in vivo* LMW-PTP phosphorylation induces LMW-PTP activation, we analyzed the tyrosine phosphorylation level of p190 in cells overexpressing wild type, double tyrosine mutant, or dominant negative LMW-PTP. Cells serum-starved for 24 h were stimulated with PDGF, and the cytoskeleton fractions were immunoprecipitated with anti-phosphotyrosine antibodies. The anti-phosphotyrosine immunoblot (Fig. 6*B*) reveals that the wtLMW-PTP overexpression leads to a decrease in the p190 tyrosine phosphorylation, whereas this effect is much less evident in dtm-LMW-PTP cells. Maximum phosphorylation of p190 was achieved, as expected, in the dominant negative-overexpressing cells, since this mutant is completely inactive. These data demonstrate that, also *in vivo*, LMW-PTP tyrosine phosphorylation in response to PDGF leads to an increase of the catalytic activity of the phosphatase on a physiological substrate like p190.

As far as the identity of p190 is concerned, we have tested various antibodies against cytoskeleton-associated proteins that are tyrosine-phosphorylated in response to PDGF stimulation, such as talin, tensin, p190Rho-GAP, and PDGF-R itself, and we have found that p190 is p190Rho-GAP, the GTPase-activating protein for the small G-protein Rho. In fact, as shown in Fig. 7, *a* and *b*, LMW-PTP and p190Rho-GAP coimmunoprecipitate in LMW-PTP-expressing cells after PDGF treatment. In addition, as expected, LMW-PTP/p190Rho-GAP interaction is restricted to the cytoskeleton-associated fraction (Fig. 7). In addition, we have verified that not only p190Rho-GAP and LMW-PTP interact in the cytoskeleton-associated fraction but that p190Rho-GAP is indeed a LMW-PTP substrate. The results shown in Fig. 8 clearly demonstrate that p190Rho-GAP is a LMW-PTP substrate, since the p190Rho-GAP phosphorylation level in cells expressing wtLMW-PTP is lower than in mock-transfected cells. The dtmLMW-PTP is not phosphorylated upon PDGF treatment, and hence its catalytic activity is not increased (13). In fact, p190Rho-GAP phosphorylation level in dtmLMW-PTP expressing cells, although lower with respect to mock-transfected cells, is clearly higher than in wtLMW-PTP-expressing cells. On the basis of these findings, we suggest that p190Rho-GAP is a substrate of LMW-PTP in the cytoskeleton-associated fraction, in agreement with the observed effects of the LMW-PTP overexpression in cell adhesion, spreading, and migration, events that are all regulated by Rho-mediated cytoskeleton reorganization. In addition, LMW-PTP itself is regulated, *in vivo*, by means of phosphorylation on tyrosine 131 and/or 132, since this modification influences LMW-PTP activity toward p190Rho-GAP.

DISCUSSION

Over the past few years, accumulating evidence indicates that signal transduction and cell cycle events are regulated coordinately by growth factors, cell anchorage, and cytoskeletal structure (18). For example, it has been shown that cyclin D1 mRNA is poorly induced if quiescent cells are stimulated with mitogens in the absence of cell adhesion (19, 20). In addition, cyclin D1 translation from preexisting cyclin D1 mRNA is blocked when cells are incubated in the absence of substratum (20). Cell adhesion also plays an important role in cyclin

E/Cdk2 activity, since it is involved in the down-regulation of the Cip/Kip family of cyclin-dependent kinase inhibitor proteins (21, 22). Therefore, growth factor stimulation of cyclin D and E, and consequently G_1 progression, requires cell adhesion to substratum.

On the other hand, growth factor stimulation induces profound modifications of cell adhesion structures and cytoskeleton rearrangement. For example, serum and lysophosphatidic acid induce rapid (within 5 min) stress fiber formation but little ruffling, whereas PDGF and epidermal growth factor are slow to induce stress fiber formation (about 30 min) but stimulate rapid membrane ruffling (4). The reorganization of actin cytoskeletal structures after mitogenic stimulation is mainly due to the Rac/Rho family of small GTP-binding proteins. It is well established that Rac is involved in the formation of filopodia, whereas Rho is implicated in stress fiber and focal adhesion assembly (23). Rho activity is regulated both by integrins (24) and soluble factors (25). In a recent paper, Ren *et al.* (26) showed that Rho activation by lysophosphatidic acid is independent of adhesion to ECM, but Rho is down-regulated within 10 min in adherent cells, whereas its activity remains high in suspended cells. p190Rho-GAP is one of the candidates for this negative feedback that has been shown to inhibit Rho-induced stress fiber formation (27).

Growing evidence indicates the involvement of PTPs members in the control of cell adhesion, migration, and spreading (16, 28, 29). In our previous studies, we have investigated the role of LMW-PTP in PDGF-induced mitogenesis. LMW-PTP is a key intermediate in the early steps of PDGF-R signal transduction, since it is able to bind and dephosphorylate the activated receptor, thus specifically modulating the Src and the STAT pathways. The consequence is the regulation of *myc* and *fos* expression, two protooncogenes crucial for G_1 progression (11). Recently, we have shown that LMW-PTP is present in both cytosolic and cytoskeleton-associated subcellular fractions. These two distinct LMW-PTP pools are subjected to different regulation and are able to play different cellular functions (12). The cytosolic LMW-PTP pool, the only one that interacts with the activated PDGF-R, is always present in the unphosphorylated form, whereas the cytoskeleton-associated LMW-PTP fraction becomes tyrosine-phosphorylated in response to PDGF stimulation (12). This phosphorylation is performed *in vivo* by c-Src on residues 131 and 132 of LMW-PTP (12, 15) and leads, at least *in vitro*, to a strong increase of LMW-PTP activity (13).

In the present study, we have further investigated the physiological role of cytoskeleton-associated LMW-PTP and the regulative meaning of its tyrosine phosphorylation by c-Src. First, we have established that LMW-PTP localized in the cytoskeleton-associated fraction is involved in the integrin-mediated cell adhesion. In fact, LMW-PTP is present in the cytoskeleton-associated fraction in cells seeded on fibronectin (Fig. 1*B*) or collagen type I, which are ECM proteins able to induce integrin receptor clustering. Instead, in cells seeded on polylysine, which promotes cell adhesion in an integrin-independent manner, or in cells kept in suspension, LMW-PTP is not localized in the cytoskeleton-associated fraction (Fig. 1, *A* and *B*).

In order to investigate the relationship between LMW-PTP phosphorylation and its functionality, we have expressed in NIH-3T3 a protein mutated in the c-Src phosphorylation sites (dtmLMW-PTP), and we have studied some parameters related to cytoskeleton rearrangement in comparison with wtLMW-PTP-overexpressing cells and mock-transfected cells. We emphasize that the double mutant dtmLMW-PTP is a functional phosphatase retaining about 40% of the activity of the wild type enzyme (13) and that it is able to bind *in vivo* both

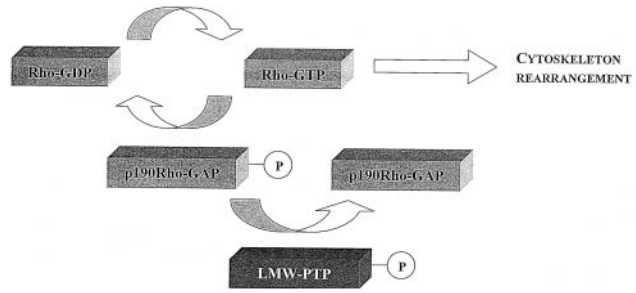

FIG. 9. **Proposed model for LMW-PTP-mediated effect on cytoskeleton rearrangement after PDGF stimulation.**

PDGF-R (data not shown) and p190Rho-GAP (Fig. 7). The decrease of the basal catalytic activity of the dtmLMW-PTP with respect to the wild type enzyme (2.5-fold) is about 1 order of magnitude lower with respect to the difference observed between the unphosphorylated and the phosphorylated wild type enzyme (25-fold). For this reason, it is likely that the phenotypic differences observed in cells expressing either wtLMW-PTP or dtmLMW-PTP are essentially due to the lack of phosphorylation of the double mutant enzyme and not to its reduced basal catalytic activity, although we could not completely exclude this possibility. First, we established that LMW-PTP tyrosine phosphorylation, which is consequent to PDGF administration, is not essential for LMW-PTP translocation from cytoplasm to cytoskeleton, since dtmLMW-PTP, which cannot be phosphorylated, is still present in the cytoskeleton-associated fraction (Fig. 2*A*). In addition, we find that, *in vivo*, the position 131 and 132 residues are the only LMW-PTP tyrosines that are phosphorylated in response to PDGF stimulation (Fig. 2*B*).

Further, we have found that LMW-PTP phosphorylation is a phenomenon that has a profound effect in integrin- and PDGF-mediated signaling, especially for what concerns cytoskeleton rearrangements following the mitotic stimulus, such as cell adhesion, spreading, and migration. In fact, wtLMW-PTP overexpression in NIH-3T3 cells leads to a strong increase of cell adhesion, spreading, and migration, while the expression of the dominant negative LMW-PTP leads to the opposite phenotypic effect. On the other hand, dtmLMW-PTP-expressing cells have only a slight variation of these parameters with respect to control cells, indicating that LMW-PTP phosphorylation is required for regulating all of these aspects of cytoskeleton rearrangement (Figs. 3–5).

To clarify the LMW-PTP role in integrin-mediated cell adhesion following PDGF stimulation, it was necessary to find a specific substrate of our enzyme within the cytoskeleton component. In our previous report (12), we indicated a protein of about 190 kDa, differentially phosphorylated after PDGF stimulation in wtLMW-PTP-overexpressing cells with respect to control cells, that could represent a LMW-PTP substrate in this subcellular district. Here we have shown that p190 coimmunoprecipitates with LMW-PTP in the cytoskeleton-associated fraction after PDGF stimulation (Fig. 6*A*) and that the LMW-PTP phosphorylation increases its phosphatase activity toward phosphorylated p190 (Fig. 6*B*). In fact, cells overexpressing dtmLMW-PTP, a mutant that cannot be phosphorylated by c-Src, have a higher p190 phosphorylation level with respect to wtLMW-PTP-expressing cells. Hence, we observe a correlation between the phosphorylation level of p190, which is under LMW-PTP control, and the phenotypic effect on cell adhesion, spreading, and migration. Finally, we find that the p190 tyrosine-phosphorylated protein, which is the major target of LMW-PTP in the cytoskeleton-associated fraction after PDGF stimulation, is p190Rho-GAP and that the overexpression of

LMW-PTP influences its tyrosine phosphorylation level. In fact, LMW-PTP and p190Rho-GAP associate in the cytoskeleton-associated fraction after PDGF stimulation, and this leads to a dephosphorylation of p190Rho-GAP (Figs. 7 and 8).

Very recently, Ren and co-workers (26) have shown that Rho activity, in response to lysophosphatidic acid, is triggered equally well in adherent and detached cells, but Rho is downregulated in adherent cells, whereas it remains elevated in suspended cells. The authors (26) hypothesized the existence of a negative feedback loop that might prevent excessive contraction under physiological conditions. One of the candidates that could mediate this regulative effect on Rho activity is p190Rho-GAP. The inhibition of p190Rho-GAP activity is sufficient for the induction of Rho-mediated actin reorganization (30). p190Rho-GAP itself becomes tyrosine-phosphorylated by c-Src after epidermal growth factor stimulation, and this correlates with rapid disassembly of actin stress fibers (6, 7). It has been proposed that p190Rho-GAP is a strong candidate effector of v-Src-induced cytoskeletal disruption, most likely mediated by antagonism of the cellular function of Rho (31). Till now, the exact role of the tyrosine phosphorylation of p190Rho-GAP has not been elucidated. p190Rho-GAP phosphorylation may modulate its own enzymatic (GTP binding/hydrolysis of Rho-GAP) activities through phosphorylation-induced conformational changes. Alternatively, p190Rho-GAP phosphorylation on tyrosine 1105 (7) could serve as a docking site for Src homology 2-containing signaling proteins. In fact, it has been proposed that the p190Rho-GAP tyrosine-dependent binding to p120Ras-GAP may link the Ras-mediated mitogenic signaling with signaling through the actin cytoskeleton (7).

The LMW-PTP is able to bind and to dephosphorylate p190Rho-GAP upon PDGF stimulation and, therefore, could play an important role in Rho-mediated cytoskeleton rearrangement following growth factor stimulation. In particular, LMW-PTP acting on p190Rho-GAP leads to a potentiation of Rho action. In fact, LMW-PTP dephosphorylates and hence inhibits p190Rho-GAP, leading ultimately to a potentiation of Rho action (Fig. 9). This hypothesis of LMW-PTP regulation of Rho functionality is confirmed by our data on wtLMW-PTP-transfected cells in which we observe an increased cell adhesion, spreading, and migration with respect to dtmLMW-PTP-transfected and mock-transfected cells. In addition, we have demonstrated that LMW-PTP itself is, *in vivo*, regulated by phosphorylation, since cytoskeleton-associated LMW-PTP is phosphorylated by c-Src and increases its catalytic activity toward phosphorylated p190Rho-GAP.

The emerging picture of LMW-PTP's physiologic role in the PDGF-induced mitogenesis reveals the existence of two distinct LMW-PTP intracellular pools that act on different substrates and are differentially regulated. The LMW-PTP cytosolic pool, which does not undergo tyrosine phosphorylation, binds to and dephosphorylates the activated PDGF-R. At the same time, the cytoskeleton-associated LMW-PTP pool is phosphorylated by c-Src and consequently increases its catalytic activity toward p190Rho-GAP, thus modulating the p190Rho-GAP effect on Rho. Hence, we propose that LMW-PTP function in this subcellular compartment is to control Rho-mediated cytoskeleton rearrangements in response to PDGF stimulation, through the regulation of p190Rho-GAP phosphorylation. In conclusion, LMW-PTP acts as a negative regulator of PDGF-induced mitosis both by turning off the receptor and by promoting cytoskeleton assembly.

REFERENCES

1. Miyamoto, S., Teramoto, H., Gutkind, J. S., and Yamada, K. M. (1996) *J. Cell Biol.* **135,** 1633–1642
2. Giancotti, F. G. (1997) *Curr. Opin. Cell Biol.* **9,** 691–700
3. Howe, A., Aplin, A. E., Alahari, S. K and Juliano, R. L. (1998) *Curr. Opin. Cell Biol.* **10,** 220–231
4. Ridley, A. J., and Hall, A., (1992) *Cell* **70,** 389–399
5. Narumiya, S. (1996) *J. Biochem.* (*Tokyo*) **120,** 215–228
6. Chang, J. H., Gill, S., Settleman, J., and Parsons, S. J. (1995) *J. Cell Biol.* **130,** 335–368
7. Roof, R. W., Haskell, M. D., Dukes, B. D., Sherman, N., Kinter, M., and Parsons, S. J. (1998) *Mol. Cell. Biol.* **18,** 7052–7063
8. Fauman, E. B., and Saper, M. A., (1996) *Trends Biochem. Sci.* **21,** 413–417
9. Ramponi, G. (1994) *Adv. Protein Phosphatases* **8,** 1–25
10. Chiarugi, P., Cirri, P., Raugei, G., Camici, G., Dolfi, F., Berti, A., and Ramponi, G. (1995) *FEBS Lett.* **372,** 49–53
11. Chiarugi, P., Cirri, P., Marra, F., Raugei, G., Fiaschi, T., Camici, G., Manao, G., Romanelli, R. G., and Ramponi, G., (1998) *J. Biol. Chem.* **273,** 6776–6785
12. Cirri, P., Chiarugi, P., Taddei, L., Raugei, G., Camici, G., Manao, G., and Ramponi, G. (1998) *J. Biol. Chem.* **273,** 32522–32527
13. Bucciantini, M., Chiarugi, P., Cirri, P., Taddei, L., Stefani, M., Raugei, G., Nordlund, P., and Ramponi, G. (1999) *FEBS Lett.* **456,** 73–78
14. Rigacci, S., Degl'Innocenti, D., Bucciantini, M., Cirri, P., Berti, A., and Ramponi, G. (1996) *J. Biol. Chem.* **271,** 1278–1281
15. Tailor, P., Gilman, J., Williams, S., Couture, C., and Mustelin, T. (1997) *J. Biol. Chem.* **272,** 5371–5374
16. Yu, D. H., Qu, C. K., Henegariu, O., Lu, X., and Feng, G. S. (1998) *J. Biol. Chem.* **273,** 21125–21131
17. Cirri, P., Chiarugi, P., Camici, G., Manao, G., Pazzagli, L., Caselli, A., Barghini, I., Cappugi, G., Raugei, G., and Ramponi, G. (1993) *Biochim. Biophys. Acta* **1161,** 216–222
18. Assoian, R. K., and Zhu, X. (1997) *Curr. Opin. Cell Biol.* **9,** 93–98
19. Bohmer, R. M., Scharf, E., and Assoian, R. K. (1996) *Mol. Biol. Cell* **7,** 101–111
20. Zhu, X., Ohtsubo, M., Bohmer, R. M., Roberts, J. M., and Assoian, R. K. (1996) *J. Cell Biol.* **133,** 391–403
21. Zerfass-Thome, K., Schulze, A., Zwerschke, W., Vogt, B., Helin, K., Bartek, J., Henglein, B., and Jansen-Durr, P. (1997) *Mol. Cell. Biol.* **17,** 407–415
22. Fang, F., Orend, G., Watanabe, N., Hunter, T., and Ruoslahti, E. (1996) *Science* **271,** 499–502
23. Tapon, N., and Hall, A., (1997) *Curr. Opin. Cell Biol.* **9,** 86–92
24. Barry, S. T., Flinn, H. M., Humphries, M. J., Critchley, D. R., and Ridley, A. J. (1997) *Cell. Adhes. Commun.* **4,** 387–398
25. Seufferlein, T., and Rozengurt E., (1995) *J. Biol. Chem.* **270,** 24343–24351
26. Ren, X-D., Kiosses, W. B., and Schwarts, M. A. (1999) *EMBO J.* **18,** 578–585
27. McGlade, J., Brunkhorst, B., Anderson, D., Mbamalu, G., Settleman, J., Dedhar, S., Rozakis-Adcock, M., Chen, L. B., and Pawson, T. (1993) *EMBO J.* **12,** 3073–3081
28. Garton, A. J., and Tonks, N. K. (1999) *J. Biol. Chem.* **274,** 3811–3818
29. Arregui, C. O., Balsamo, J., and Lilien, J. (1998) *J. Cell Biol.* **143,** 861–873
30. Vincent, S., and Settleman, J., (1999) *Eur. J. Cell Biol.* **78,** 539–548
31. Fincam, V. J., Cudleigh, A., and Frame, M. C., (1999) *J. Cell Sci.* **112,** 947–956

The Low M_r Protein-tyrosine Phosphatase Is Involved in Rho-mediated Cytoskeleton Rearrangement after Integrin and Platelet-derived Growth Factor Stimulation

Paola Chiarugi, Paolo Cirri, Letizia Taddei, Elisa Giannoni, Guido Camici, Giampaolo Manao, Giovanni Raugei and Giampietro Ramponi

J. Biol. Chem. 2000, 275:4640-4646.
doi: 10.1074/jbc.275.7.4640