Low Molecular Weight Protein-tyrosine Phosphatase Controls the Rate and the Strength of NIH-3T3 Cells Adhesion through Its Phosphorylation on Tyrosine 131 or 132*

Paola Chiarugi, Maria Letizia Taddei, Paolo Cirri, Doriana Talini, Francesca Buricchi, Guido Camici, Giampaolo Manao, Giovanni Raugei, and Giampietro Ramponi‡

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

Received for publication, July 18, 2000, and in revised form, September 5, 2000
Published, JBC Papers in Press, September 8, 2000, DOI 10.1074/jbc.M006375200

The low molecular weight protein-tyrosine phosphatase (LMW-PTP) is an enzyme involved in platelet-derived growth factor (PDGF)-induced mitogenesis and cytoskeleton rearrangement. Our previous results demonstrated that LMW-PTP is able to bind and dephosphorylate activated PDGF receptor, thus inhibiting cell proliferation. Recently we have shown that LMW-PTP is specifically phosphorylated by c-Src in a cytoskeleton-associated fraction in response to PDGF, and this phosphorylation increases LMW-PTP activity about 20-fold. LMW-PTP strongly influences cell adhesion, spreading, and chemotaxis induced by PDGF stimulation, by regulating the phosphorylation level of p190Rho-GAP, a protein that is able to regulate Rho activity and hence cytoskeleton rearrangement. In the present study we investigate the physiological role of the two LMW-PTP tyrosine phosphorylation sites, using LMW-PTP mutants on tyrosine 131 or 132. We demonstrate that each tyrosine residue is involved in specific LMW-PTP functions. Both of them are phosphorylated during PDG signaling. Phosphorylation on tyrosine 131 influences mitogenesis, dephosphorylating activated PDGF-R and cytoskeleton rearrangement, acting on p190RhoGAP. Phosphorylation on tyrosine 132 leads to an increase in the strength of cell substrate adhesion, down-regulating matrix metalloproteases expression, through the inhibition of Grb2/MAPK pathway. In conclusion, LMW-PTP strongly influences cell adhesion, spreading, and chemotaxis induced by PDGF stimulation. In addition, we have demonstrated that, cytoskeleton-associated LMW-PTP influences the phosphorylation state of p190Rho-GAP, a protein that is able to regulate Rho activity and, hence, cytoskeleton rearrangement in response to PDGF stimulation. We have previously shown that activated PDGF-R is a LMW-PTP substrate (4) 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 Src pathway, and fos expression through a MAPK-independent pathway mediated by the STAT proteins (5). More recently, we have found that in NIH-3T3 cells LMW-PTP is constitutively localized in both cytoplasmic and cytoskeleton-associated fractions. These two LMW-PTP pools are differentially regulated because only the cytoskeleton-associated LMW-PTP fraction is specifically phosphorylated by c-Src after PDGF stimulation (6). As a consequence of its phosphorylation, LMW-PTP shows an average 20-fold increase in its in vitro catalytic activity (7) instead of the 2-fold activation that was previously reported (8, 9).

Furthermore, we have shown that the cytoskeleton-associated LMW-PTP influences cell adhesion, migration, and spreading and that these effects are dependent on LMW-PTP tyrosine phosphorylation (10). We have shown that cytoskeleton-associated LMW-PTP influences 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, we have demonstrated that, in vivo, LMW-PTP is regulated by c-Src phosphorylation, because phosphorylated LMW-PTP presents an increased activity on a physiologic substrate such as p190Rho-GAP (10). We suggest that LMW-PTP is able to perform multiple roles in PDGF-induced mitogenesis. In fact, cytosolic LMW-PTP binds and dephosphorylates the control of cell phosphorylation state is as relevant as that of phosphotyrosine protein kinases. The PTPs superfamily is composed of over 70 enzymes that, despite very limited sequence similarity, share a common active site motif CX6R 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 (2).

The low molecular weight protein-tyrosine phosphatase (LMW-PTP) is an 18-kDa enzyme that is expressed in many mammalian tissues (3). 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 strong reduction of cell growth rate in response to PDGF stimulation. 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, vialle Morgagni 50, 50134 Firenze, Italy. Tel.: 39-055-413765; Fax: 39-055-4222725; E-mail: ramponi@scibio.unifi.it.

1 The abbreviations used are: PTP, protein-tyrosine phosphatase; ECM, extracellular matrix; MAPK, mitogen activated protein kinase; LMW-PTP, low molecular weight protein-tyrosine phosphatase; MMP, matrix metalloprotease; PDGF, platelet-derived growth factor; PDGF-R, PDGF receptor; wtLMW-PTP, wild type LMW-PTP; FCS, fetal calf serum; PBS, phosphate-buffered saline; RIP, radioimmune precipitation buffer.
rylates PDGF-R (4), 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 (10).

We have previously demonstrated that, in vitro, the tyrosine phosphorylation of LMW-PTP by c-Src is directed to both tyrosine 131 and 132, although they appear to have different effects: tyrosine 131 phosphorylation determines a strong increase in LMW-PTP specific activity, whereas phosphorylation of tyrosine 132 leads to in vitro Grb2 binding (7). The relative in vivo contribution of each tyrosine phosphorylation remained to be determined. In this paper we analyze the role of Tyr131 and Tyr132 LMW-PTP mutants in cell growth, migration, and adhesion to determine the specific function of the two phosphorylation sites. Our findings suggest that, in vivo, both LMW-PTP tyrosines are efficiently phosphorylated during PDGF signaling, leading to different effects. Phosphorylation on Tyr131, leads to the regulation of Rho-mediated cell adhesion rate through the dephosphorylation of p190Rho-GAP. On the other side, the phosphorylation on Tyr132 leads to an increase in the strength of cell adhesion through the down-regulation of MMPs expression, probably through the inhibition of the Grb2/MAPK pathway.

**EXPERIMENTAL PROCEDURES**

**Materials**—Unless specified all reagents were obtained from Sigma. NIH-3T3 cells were purchased from American Type Culture Collection; human recombinant platelet-derived growth factor (PDGF) BB was from Peprotech; the enhanced chemiluminescence kit was from ECL, New England Biolabs. Phospho-specific antibodies were from Cell Signaling Technologies, anti-LMW-PTP rabbit antibodies, which do not cross-react with mouse endogenous LMW-PTP, were isolated by selection with G418 (400 μg/ml). Anti-LMW-PTP rabbit antibodies were transfected in NIH-3T3 cells by lipofection using 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 EDTA, 1 mM sodium orthovanadate, 1 mM sodium deoxycholate, and 0.1% sodium dodecylsulfate, by shaking for 20 min at 4°C). The lysates were clarified by centrifugation at 20,000 × g for 30 min. RIPA or complete RIPA buffer fractions were then used for immunoprecipitation analysis.

**Cell Lysate Fractionation**—Cells were lysed with 500 μl of RIPA buffer, and the lysates were clarified by centrifugation for 30 min at 20000 × g. Pellets were washed twice with 1 ml RIPA and then resuspended in complete RIPA buffer, which is RIPA buffer plus 0.5% sodium deoxycholate and 0.1% sodium dodecylsulfate, by shaking for 20 min at 4°C. The lysates were centrifuged at 20,000 × g for 30 min. RIPA or complete RIPA buffer fractions were then used for immunoprecipitation analysis.

**Cell Adhesion Assay**—Cell adhesion was assessed as described elsewhere (11). Briefly, 3 × 10⁶ cells are seeded for the indicated time in a 96-well dish precoated for 2 h with 10 μg/ml of 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 in complete medium. All cell adhesion assays were performed in triplicate.

**Cell Motility Assay**—Cell migration was assessed as described elsewhere with minor modifications (5). Migration of NIH-3T3 cells was assayed with the Transwell system of Costar, equipped with 8-μm pore polycarbonate filters (diameter, 13 mm) precoated with human type I collagen (20 μg/ml) and placed between the chemotactrant (lower chamber) and the upper chamber. The lower chamber was filled with medium supplemented with 10 ng/ml of PDGF-BB. Serum-free Dulbecco’s modified Eagle’s medium cultured cells were suspended by trypsinization, and 1.5 × 10⁵ cells in 200 μl were added to the top wells and incubated at 37 °C in 5% CO₂ 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 ± S.D.).

**Cell Detachment Assay**—The strength of cell adhesion was measured as reported elsewhere (12). Briefly, confluent monolayers of cells treated or not with 0.5 μM of PDGF-BB. Serum-free Dulbecco’s modified Eagle’s medium cultured cells were suspended by trypsinization, and 1.5 × 10⁵ cells in 200 μl were added to the top wells and incubated at 37 °C for 5 min. The dishes were washed twice with PBS, and the attached cells were detached by crystal violet staining. 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 value of the control (A文化旅游) is the ratio between the cells attached after and before the treatment. Zymography—Metalloprotease zymography was assessed as described elsewhere with minor modifications (13). Briefly, culture medium from confluent cell monolayers treated or not with 0.5 μM of PDGF-BB source family kinase inhibitor was harvested with PBS containing 0.05% sodium deoxycholate and 0.1% sodium dodecylsulfate, by shaking for 20 min at 4°C. Calcium and magnesium free PBS containing 0.05% trypsin, 0.5 mg/ml soybean trypsin inhibitor was added, and the cells were incubated at 37 °C for 5 min. The sample was run on a 8% SDS gel containing 0.1% gelatin. After electrophoresis the gel was washed twice with 2.5% Triton X-100 and once with reaction buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5 mM CaCl₂). The gel was incubated overnight at 37 °C with freshly added reaction buffer and stained with Laemmli Comassie blue solution.

**MAPK Activation**—3 × 10⁴ cells were plated on a 6-well dish in complete medium. After 48 h the cells were washed twice in PBS, lysed in RIP buffer, and centrifuged to remove the insoluble debris. The total protein content was detected with the BCA protein assay and 20 μg of lysates were resolved on a 12% SDS-polyacrylamide gel electrophoresis. The resolved proteins were transferred to nitrocellulose and probed overnight with anti-phospho-p44/42MAPK monoclonal antibodies.

**MAPK Activation Assay**—The PTP activity was measured as previously reported (7). Briefly, 1.5 × 10⁵ cells were collected in 300 μl of 0.1 M sodium acetate, pH 5.5, 10 mM EDTA, 1 mM β-mercaptoethanol and sonicated for 10 s. The lysates were centrifuged by clarification, and 50 μl were used in the PTP activity assay with 50 μl of 10 mM p-nitrophenylphosphate at 37 °C for 30 min. The production of p-nitrophenol was measured colorimetrically at 410 nm. The results were normalized on
The expression of Y131A and Y132A LMW-PTP mutants in NIH-3T3 cells—To study the role of LMW-PTP phosphorylation on Tyr131 and Tyr132, we generated by site specific mutagenesis two LMW-PTP tyrosine to alanine single mutants (Y131A and Y132A). We overexpressed these LMW-PTP mutants in NIH-3T3 cells, selecting two different clones for each mutant for further experiments (Y131A, clones P and M; Y132A, clones X and XII). Because we have previously demonstrated that LMW-PTP exists in cytoplasmic and cytoskeleton associated distinct pools, we analyzed the subcellular distribution and the tyrosine phosphorylation of the mutant forms of LMW-PTP. As far as the double mutant Y131A/Y132A-LMW-PTP is concerned, we have already reported that it is targeted to cytoskeleton but not tyrosine phosphorylated upon PDGF treatment (10). The results show that both mutants are efficiently targeted to cytoskeleton (Fig. 1A) and differently phosphorylated on tyrosine (Fig. 1B). This finding indicates that, in vivo, both tyrosine 131 and 132 are efficiently phosphorylated in NIH-3T3 cells after PDGF stimulation. As we reported previously, the activity on p-nitrophenylphosphate of Y131A and Y132A LMW-PTP mutants was 40 and 70%, respectively, with respect to wtLMW-PTP. The LMW-PTP, which is a C12A mutant, is totally inactive (7). Because the tyrosine phosphorylation level of cytoskeleton-associated LMW-PTP seems to be dependent on LMW-PTP catalytic activity, we suggest that LMW-PTP could undergo autodephosphorylation in vivo.

Furthermore, to exclude the possibility that the LMW-PTP mutants are inactive in vivo, we quantitated their catalytic activity in NIH-3T3 cells. The results are very similar to those obtained in vitro; taken as 100% the catalytic activity of the wtLMW-PTP, we obtained about 40 and 90% for Y131A-LMW-PTP and Y132A-LMW-PTP, respectively. These findings confirm that in vivo both LMW-PTP single tyrosine mutants are functional phosphatases, at least on synthetic substrates such as p-nitrophenylphosphate.

Effect of Single Tyrosine Mutants of LMW-PTP in PDGF-induced Mitogenesis—We analyzed the serum-induced cell growth rate of Y131A-LMW-PTP and Y132A-LMW-PTP transfected cells in comparison with wtLMW-PTP and mock transfected cells (Fig. 2A). The results reveal that the Y132A-LMW-PTP, which maintains the possibility of phosphorylation on tyrosine 131, induces a decrease in cell growth rate, similar to the wtLMW-PTP, with respect to mock transfected cells. On the contrary, the Y131A-LMW-PTP, which maintains only the tyrosine in position 132, determines a slight increase in the cell growth rate with respect to mock transfected cells. Phosphorylation on Tyr131 but not on Tyr132 gives rise to a behavior similar to the wtLMW-PTP, suggesting that only the phosphorylation of LMW-PTP in position 131 mediates the inhibitory effect of this enzyme on serum-induced mitosis.

We analyzed the tyrosine phosphorylation level of PDGF-R in Y131A-NIH-3T3 and Y132A-NIH-3T3 cells in comparison with wtLMW-PTP and mock transfected cells. The result, shown in Fig. 2B, demonstrates that although the Y132A mutant behaves as wtLMW-PTP, dephosphorylating the PDGF-R even more efficiently than the wild type enzyme, the Y131A mutant appears to be inefficient in this action. To determine whether the mutation of tyrosine 132 impairs the PDGF-R binding of LMW-PTP or only its ability to dephosphorylate the receptor, we coinmunoprecipitated LMW-PTP and the PDGF-R in all cell lines. We were not able to detect any interaction between the activated PDGF-R and Y131A-LMW-PTP (data not shown). Taken together, these findings suggest that both tyrosine 131 and 132 are phosphorylated in vivo upon PDGF administration, but these events lead to different LMW-PTP effects on mitosis regulation and PDGF receptor activation.

Role of LMW-PTP Tyr(P)131 in the Regulation of Rho-dependent Cytoskeleton Rearrangement—wtLMW-PTP overexpression induces a marked increase in integrin-mediated cell adhesion rate and PDGF-induced migration (10). To analyze the role of tyrosine phosphorylation on Tyr131 or Tyr132 in this phenomenon, we evaluated cell adhesion on fibronectin of cells expressing wtLMW-PTP or the two single tyrosine mutants in comparison with mock transfected cells. Fig. 3A shows that Y131A mutant behaves similarly to mock transfected cells. On the contrary, the Y132A mutant leads to a marked potentiation of fibronectin-mediated cell adhesion rate. This effect is even stronger than in wtLMW-PTP. In addition we evaluated the chemotaxis induced by PDGF-BB in all cell lines. Fig. 3B shows that again the phosphorylation in Tyr131 mainly mediates the increase of cell chemotaxis toward PDGF induced by LMW-PTP overexpression.

LMW-PTP is involved in Rho-mediated cytoskeleton rearrangement such as chemotaxis and cell adhesion, as it specifically dephosphorylates the GTPase activating protein of Rho, called p190Rho-GAP, thus leading to an up-regulation of Rho activity (14, 15). To analyze the activity of Y131A and Y132A LMW-PTP mutants on p190Rho-GAP, we tested the tyrosine phosphorylation level of p190Rho-GAP in all cell lines upon PDGF treatment. Fig. 4 shows that the Y132A-LMW-PTP mutant is able to dephosphorylate p190Rho-GAP even better than wtLMW-PTP, but the Y131A-LMW-PTP mutant is almost ineffective on this substrate. These findings indicate that only the phosphorylation of tyrosine 131 is essential for the function of LMW-PTP in Rho-mediated cytoskeleton rearrangement leading to cell migration toward PDGF-BB and adhesion to extracellular matrix, through the regulation of p190Rho-GAP tyrosine phosphorylation.

Role of LMW-PTP Tyr(P)132 in the Regulation of Cell Detachment—The transfection of Y131A-LMW-PTP mutant leads to the expression of a protein that is efficiently targeted to the cytoskeleton architecture (Fig. 1A) and phosphorylated upon PDGF treatment (Fig. 1B). Nevertheless, in Y131A-NIH-3T3 cells we failed to observe any variations in cell growth rate, PDGF-induced migration, and cell adhesion, with respect to mock transfected cells. We routinely observed, in all clones
expressing Y131A-LMW-PTP mutant a resistance to trypsin detachment during cell culturing. Hence, we analyzed the strength of cell substratum adhesion by a detachment assay (12) in exponentially growing cells treated or not with the PP1 Src family kinase inhibitor (16) (Fig. 5). The overexpression of wtLMW-PTP leads to a slight decrease of the strength of cell substratum adhesion in 10% FCS growing cells (Fig. 5), as indicated by the decrease in the resistance to detachment reported in the ordinate of the plot. Although the Y132A-NIH-3T3 cells showed a behavior similar to wtLMW-PTP-NIH-3T3 cells, the Y131A-NIH-3T3 cells exhibited a dramatic increase in the resistance to detachment (Fig. 5). The NIH-3T3 cells overexpressing the LMW-PTP Y131A/Y132A double mutant, that is not phosphorylated upon PDGF treatment (10), show a phenotype similar to mock-transfected cells. Hence, this increase in the resistance to cell detachment is likely due to the phosphorylation of LMW-PTP on tyrosine 132. In fact, the increased adhesion strength of Y131A-NIH-3T3 is abolished in serum-starved cells (data not shown), where the LMW-PTP is not phosphorylated by c-Src (6), and in PP1 treated cells (Fig. 5), suggesting a role for LMW-PTP phosphorylation on tyrosine 132 in the modulation of cell adhesion strength.

As reported in literature, the adhesion of cells to the extracellular matrix is controlled by an equilibrium between integrin receptors interaction with ECM components and the activity of membrane protease acting on them. Matrix metalloprotease expression increases when remodelling of ECM is required (17, 18) and is primarily regulated at a transcriptional level (19, 20). Recently it has been demonstrated that there is a modulation of the expression of MMPs in re-
response to many growth factors such as PDGF or epidermal growth factor (21, 22). To assess whether the increased resistance to cell detachment of Y131A-NIH-3T3 cells could be due to variations in MMPs production in response to growth factors, we analyzed by gelatin zymography the MMPs activity of Y131A, Y132A, and Y131A/Y132A-NIH-3T3 cells in comparison with mock transfected and wtLMW-PTP-expressing cells. We observed in growing cells (Fig. 6A) a general down-regulation of MMPs activity in Y131A-NIH-3T3 cells with respect to mock transfected cells, whereas both Y132A-NIH-3T3 cells and wtLMW-PTP-expressing cells showed an increased MMPs production. In the same condition MMPs activity of Y131A/Y132A-NIH-3T3 cells is similar to mock transfected cells (Fig. 6A). In both serum-starved cells (data not shown) and PPI1-treated cells, where LMW-PTP is not phosphorylated (6, 10), all cell lines show a similar MMPs activity (Fig. 6B). These findings correlate with the cell detachment phenotypes observed in all cell lines. Hence, the data obtained by cell detachment and MMPs activity experiment indicate a role for LMW-PTP phosphorylation on tyrosine 132 in regulating cell adhesion.

Role of LMW-PTP Tyr(P) 132 in Grb2/APK Pathway Regulation—We have previously reported that, in vitro, the phosphorylation of Tyr132 (but not Tyr131) creates a docking site for Grb2 binding (7). To assess whether Grb2 adapter protein really binds LMW-PTP in vivo, we analyzed by coimmunoprecipitation the interaction between Grb2 and the phosphatase, in wtLMW-PTP, Y131A and Y132A mutant-expressing cells. The result of the anti-LMW-PTP immunoblot of Grb2 immunoprecipitates is shown in Fig. 7; we detect the interaction between LMW-PTP and Grb2 mainly in PDGF-treated Y131A-NIH-3T3 cells, although there is a detectable association also in wtLMW-PTP-NIH-3T3 cells. In any case the interaction between Grb2 and LMW-PTP is dependent on the activation of PDGF signaling, in agreement with the dependence of LMW-PTP tyrosine phosphorylation by c-Src upon PDGF treatment (6). This result indicates that the phosphorylation of tyrosine 132 really represents in vivo a Grb2 binding site, as we hypothesized on the basis of structural data and in vitro binding assay (7).

It has been recently reported that many MMPs are under transcriptional control by growth factors, cytokines, and other environmental factors such as contact with ECM (23–25). The Grb2/Ras/MAPK pathway is implicated in the transduction of the signal starting from tyrosine kinase receptors (26–29), leading to the induction of membrane proteases. LMW-PTP could be involved in the regulation of this pathway through the recruitment of the Grb2 adapter protein by its phosphotyrosine 132 docking site. We analyze the p44/p42 MAPK activation in NIH-3T3 cells expressing wtLMW-PTP or the tyrosine mutants Y131A and Y132A, during exponential cell growth. Fig. 8 shows that p44/p42 MAPK activation is down-regulated in the Y131A-LMW-PTP mutant-expressing cells in comparison with wtLMW-PTP-expressing cells. The data reported in Fig. 8 suggest that the phosphorylation of tyrosine 132 of LMW-PTP leads to a MAPK down-regulation, and this is probably due to its binding to the SH2 domain of Grb2. In fact, the Grb2/Sos complex is able to activate the Ras/MAPK pathway when the SH2 domain of Grb2 binds the phosphorylated tyrosines of the activated tyrosine kinase receptors. The Grb2/LMW-PTP association through phosphorysine 132 could prevent the Grb2/Sos complex binding to the receptor, thus determining a down-regulation of the MAPK pathway. The p44/p42 MAPK activation levels (Fig. 8) in the analyzed cell clones are in agreement with the MMPs activities shown in Fig. 7. Taken together these data suggest that the phosphorylation of Tyr132 leads to a regulation of the strength of the cell substratum adhesion through the control of MMPs expression via a Grb2/MAPK regulated pathway.

FIG. 3. Effect of wtLMW-PTP and single tyrosine mutants on integrin mediated cell adhesion and PDGF-induced migration. A, 3 × 10^3 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 of PDGF-BB. The cells were allowed to attach for the indicated times, and the adhesion was evaluated with crystal violet staining. B. 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. S.D. is indicated. wt, wild type.
DISCUSSION

Many cellular processes such as cell migration, adhesion, and proliferation require the collaborative interaction between growth factors and ECM stimuli (17, 30). In addition, cell adhesion to ECM is a balance between focal adhesion formation and proteolysis of extracellular matrix. The addition of a growth factor that induces cell migration has a double role in promoting cell substratum adhesion through the regulation of Rho-mediated cytoskeleton rearrangement and the induction of MMPs expression, which promotes the degradation of basement membranes and stromal extracellular matrix. These concurrent events allow cells to adopt an adhesive state permissive to migration (17).

Our previous results on LMW-PTP function in PDGF-induced mitogenesis indicate that the tyrosine phosphorylation of LMW-PTP is essential in Rho-mediated cytoskeleton rearrangement (10). Herein, we have investigated the specific role of the single LMW-PTP tyrosine phosphorylation sites in this phenomenon. In this report, we show that the LMW-PTP tyrosines in positions 131 and 132 are independently phosphorylated in response to PDGF. These effects lead to differential cell behavior. It is possible that in the wtLMW-PTP, where both 131 and 132 sites are accessible and thus phosphorylatable by the c-Src tyrosine kinase, the amount of Tyr(P)131 is counterbalanced by the phosphorylation of Tyr 132. On the basis of structural data (7), it is likely that only one tyrosine is phosphorylated on a LMW-PTP molecule by c-Src, because of the steric block of the phosphorylated residue. In this light, it is possible that the LMW-PTP mutant, which does not contain the tyrosine 132 (which is mutated to alanine), lacking any competition between the two phosphorylation sites, is fully phosphorylated in the remaining tyrosine 131. The reverse condition leads to the full phosphorylation of tyrosine in position 132 in the Y131A-LMW-PTP mutant. This situation gives us a tool to study the differential role of the two tyrosines in LMW-PTP regulation of PDGF signaling, because both LMW-PTP mutants give rise to an enhancement of the native phosphorylation in each tyrosine of the phosphatase.

Firstly, the phosphorylation on tyrosine 131 seems to mainly mediate the inhibition of wtLMW-PTP in growth factor-induced mitosis (Fig. 1). In fact, the Y132A-LMW-PTP-expressing cells show a decreased cell growth rate with respect to wtLMW-PTP cells. The effect of the tyrosine phosphorylation in position 131 on PDGF-induced mitosis is in agreement with the activation of PDGF receptor (Fig. 2). In fact, the tyrosine phosphorylation level of PDGF receptor is down-regulated in Y132A-LMW-PTP-expressing cells with respect to wtLMW-PTP transfected cells.
We observed that the Y132A-LMW-PTP mutant overexpressing cells show an increased fibronectin mediated cell adhesion (Fig. 3A) and PDGF-induced cell migration (Fig. 3B), with respect to wtLMW-PTP-expressing cells. These phenotypic effects are in agreement with the increased activity of Y132A-LMW-PTP mutant on phosphorylated p190Rho-GAP with respect to wtLMW-PTP (Fig. 4). These data indicate that the role of LMW-PTP exerted in the regulation of PDGF-induced mitogenesis and Rho-dependent cytoskeletal rearrangement, such as chemotaxis toward growth factors and cell adhesion rate on ECM, is mainly mediated by the phosphorylation of LMW-PTP on tyrosine 131.

On the contrary, Y131A-LMW-PTP mutant fails to dephosphorylate both PDGF receptor and p190Rho-GAP upon PDGF stimulation (Figs. 2B and 4). It is very likely that the phosphorylation of LMW-PTP on Tyr 132 is not involved in neither PDGF-induced cell growth nor in the phenotypic effects of Rho-mediated cytoskeleton rearrangement, such as chemotaxis toward growth factors and cell adhesion rate on ECM, is mainly mediated by the phosphorylation of LMW-PTP on tyrosine 131.

Nevertheless, the Y131A-LMW-PTP mutant is indeed a functional protein that is correctly targeted to the cytoskeleton structure (Fig. 1A) and tyrosine phosphorylated (Fig. 1B) in response to PDGF. In addition, the in vivo catalytic activity of the Y131A-LMW-PTP mutant on p-nitrophenylphosphate is almost 40% with respect to the wild type enzyme.

Furthermore, our data indicate an intriguing inhibitory role of the phosphorylation in position 132 on LMW-PTP function in PDGF-induced mitogenesis, chemotaxis, and cell adhesion. It should be noted that in every analyzed phenomenon the wtLMW-PTP shows a softened behavior with respect to Y132A-LMW-PTP-expressing cells. In fact, the wtLMW-PTP, which can be phosphorylated in both 131 and 132 sites, is less active on activated PDGF-R and phosphorylated p190RhoGAP with respect to Y132A-LMW-PTP, which can be phosphorylated only in position 131. As a consequence, wt-LMW-PTP is a less effective enzyme than Y132A-LMW-PTP in PDGF signaling, suggesting that the phosphorylation of Tyr132 in LMW-PTP could represent a negative regulation of enzyme activity during PDGF signaling.
The behavior of the Y131A-LMW-PTP mutant overexpressing cells has suggested the involvement of the phosphorylation on Tyr^{132} in the matrix remodelling process. We analysed the resistance to cell detachment from the substratum as an indication of the strength of cell adhesion. The phosphorylation of LMW-PTP on Tyr^{132} only leads to a strong decrease of the production of matrix metalloproteinases, as indicated by a zymography analysis (Fig. 7). Studies on the regulation of the MMPs and serine-protease urokinase (17, 18) expression have revealed that the MMPs and urokinase genes are regulated at a transcriptional level (19, 20). The promoter regions of the genes for these proteases show conserved motifs for AP1 and NF-κB transcription factors (31, 32), which confer regulability by growth factors and cytokines (33, 34). The signal transduction pathway that leads to protease transactivation control, involves the Grb2/Sos1/Ras/Raf/MEK1/MAPK route (27, 28). In this light, we investigated the p44/p42 MAPK activation level in exponentially growing NIH-3T3 cells expressing wt-LMW-PTP and in cells expressing the single tyrosine mutants (Fig. 8). The phosphorylation levels of p44 and p42 correlate with the activity of metalloproteinases because the Y131A-LMW-PTP mutant overexpressing cells showed a decrease in MAPK stimulation together with a decrease in secreted MMPs level.

We already reported that the sequence C-terminal to Tyr^{132} conforms to the motifs specifically recognized by the Grb2 SH2 domain (Y^X) (7) and that in vitro only the phosphorylation in Tyr^{132} leads to Grb2 adapter binding by GST-LMW-PTP. Grb2 is a phosphotyrosine-binding protein that transduces the signal starting from activated tyrosine kinase receptors to the Ras/Raf/MAPK pathway. It has been reported that other PTPs, such as SHP1, SHP2, and RPTPs, possess the Grb2 consensus binding site (35, 36). We demonstrated herein that the phosphorylation in Tyr^{132} mediates in vivo the binding between LMW-PTP and Grb2 after PDGF stimulation (Fig. 6). In fact, the Y131A-LMW-PTP mutant is able to bind Grb2 even more efficiently than the wild type phosphatase, whereas the Y132A-LMW-PTP mutant lacks any detectable association, suggesting a specificity for Tyr^{132} and not for Tyr^{131} in the interaction between phospho-LMW-PTP and Grb2.

On the basis of structural data and computer modelling (7), we suggest that the interaction of LMW-PTP with Grb2 is most likely at the level of its SH2 domain, which is engaged with Grb2.

REFERENCES

1. Van de Geer, P., Hunter, T., and Lindberg, R. (1994) Annu. Rev. Cell Biol. 10, 251–337
2. Fauman, E. B., and Saper, M. A. (1996) Trends Biochem. Sci. 21, 413–417
3. Ramponi, G. (1994) Adv. Prot. Phosphatases 8, 1–25
4. Chiarugi, P., Cirri, P., Raugei, G., Camici, G., Dolfi, P., Berti, A., and Ramponi, G. (1995) FEBS Lett. 372, 49–53
5. Chiarugi, P., Cirri, P., Marra, F., Raugei, F., Fiaschi, T., Camici, G., Manao, G., Romanelli, R. G., and Ramponi, G. (1998) J. Biol. Chem. 273, 6767–6785
6. Cirri, P., Chiarugi, P., Taddei, L., Raugei, G., Camici, G., Manao, G., and Ramponi, G. (1998) J. Biol. Chem. 273, 35252–35257
7. Bucciantini, M., Chiarugi, P., Cirri, P., Taddei, L., Stefani, M., Raugei, G., Nordlund, P., and Ramponi, G. (1999) FEBS Lett. 456, 73–78
8. Rigacci, S., DeIlNocenti, D., Bucciantini, M., Cirri, P., Berti, A., and Ramponi, G. (1996) J. Biol. Chem. 271, 1287–1291
9. Tailor, P., Gilman, J., Williams, S., Couture, C., and Mustelin T. (1997) J. Biol. Chem. 272, 5371–5374
10. Chiarugi, P., Cirri, P., Taddei, L., Giannoni, E., Camici, G., Manao, G., Raugei, G., and Ramponi, G. (2000) FEBS Lett. 484, 460–464
11. Yu, D. H., Qu, C. K. Henegariu, O., Lu, X., and Feng G. S. (1998) J. Biol. Chem. 273, 21125–21131
12. Inagaki, K., Noguchi, T., Matsuzaki, H., Horikawa, T., Fukunaga, K., Teuda, M., Ichihashi, M., and Kusagawa, M. (2000) Oncogene 19, 75–84
13. Mott, J. D., Thomas, C. L., Rosenbach, M. T., Takahara, K., Greenspan, D., and Bando, M. J. (2000) J. Biol. Chem. 275, 1384–1390
14. Chang, J. H., Gill, S., Settleman, J., and Parsons, S. J. (1995) J. Cell Biol. 130, 335–368
15. Roof, R. W., Haskell, M. D., Dukes, B. D., Sherman, N., Kinter, M., and Parsons, S. J. (1998) Mol. Cell. Biol. 18, 7052–7063
16. Liu, Y., Bishop, A., Witucki, L., Krayahl, B., Shimizu, E., Tsien, J., Ubersax, J., Bietlrow, J., Morgan, D. O., and Shokat, K. M. (1999) J. Biol. Chem. 9, 671–678
17. Westermann, A., and Kallfelz, V. M. (1999) FEBS Lett. 13, 781–792
18. Johnsen, M., Lund, L. R., Romer, J., Almholt, K., and Dano, K. (1998) Curr. Opin. Cell Biol. 10, 667–671
19. Vincenti, M. P., White, L. A., Schroen, D. J., Benbow, U., and Brinckerhoff, C. E. (1996) Curr. Op. Cell. Biol. 6, 391–411
20. Benbow, U., and Brinckerhoff, C. E. (1997) Matrix Biol. 15, 519–526
21. Singer, C. F., Marbaix, E., Lemoine, P., Courtoy, P. J., and Reckott, Y. (1999) J. Biol. Chem. 274, 259, 435–446
22. Bond, M., Fabunmi, R. P., Baker, A. H., and Newby, A. C. (1998) FEBS Lett. 435, 29–34
23. Swifts, A. M., Klijn, J. G., Heenzen-Lobmans, S. C., and Foeckens, J. A. (1999) Breast Cancer Res. Treat. 55, 9–20
24. Fribi, G., Pucci, M., Grappone, C., Pellegrini, G., Salzano, R., Casini, A., Milani, S., and Del Rosso, M. (1999) Hepatology 29, 688–678
25. Lao, H. K. (1999) Cardiovasc. Res. 43, 1049–1059
26. Zeigler, M. E., Chi, Y., Schmidt, T., and Varani, J. (1999) J. Cell. Physiol. 180, 271–284
27. Reddy, R. B., Krueger, J. S., Kondapaka, S. B., and Diglio, C. A. (1999) Int. J.
28. Besser, D., Bardelli, A., Didicenko, S., Thelen, M., Comoglio, P. M., Ponzetto, C., and Nagamine, Y. (1997) Oncogene 14, 705–711
29. Ried, S., Jager, Jeffers, M., Vande Woude, G. F., Graeff, H., Schmitt, M., and Lengyel, E. (1999) J. Biol. Chem. 274, 16377–16386
30. Keyse, S. M. (2000) Curr. Opin. Cell Biol. 12, 186–192
31. Janulis, M., Silberman, S., Amwegaskar, A., Gutkind, J. S., and Shultz, R. M. (1999) J. Biol. Chem. 274, 801–813
32. Bond, M., Baker, A. H., and Newby, A. C. (1999) Biochem. Cell Biol. 264, 561–567
33. Lee, J. S., See, R. H., Deng, T., and Shi, Y. (1996) Mol. Cell. Biol. 16, 4312–4326
34. Westermarck, J., Seth, A., and Kahari, V. M. (1997) Oncogene 14, 2651–2660
35. Blanchetot, C., and Den Hertog, J. (2000) J. Biol. Chem. 275, 12446–12452
36. Ganjiu, R. K., Bruhaker, S. A., Chernok, R. D., Avraham, S., and Groopman, J. E. (2000) J. Biol. Chem. 275, 17263–17268
Low Molecular Weight Protein-tyrosine Phosphatase Controls the Rate and the Strength of NIH-3T3 Cells Adhesion through Its Phosphorylation on Tyrosine 131 or 132

Paola Chiarugi, Maria Letizia Taddei, Paolo Cirri, Doriana Talini, Francesca Buricchi, Guido Camici, Giampaolo Manao, Giovanni Raugei and Giampietro Ramponi

J. Biol. Chem. 2000, 275:37619-37627.
doi: 10.1074/jbc.M006375200 originally published online September 8, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M006375200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 36 references, 13 of which can be accessed free at http://www.jbc.org/content/275/48/37619.full.html#ref-list-1