THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 277, No. 40, Issue of October 4, pp. 37331–37338, 2002
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Insight into the Role of Low Molecular Weight Phosphotyrosine Phosphatase (LMW-PTP) on Platelet-derived Growth Factor Receptor (PDGF-r) Signaling

LMW-PTP CONTROLS PDGF-r KINASE ACTIVITY THROUGH TYR-857 DEPHOSPHORYLATION*

Received for publication, May 28, 2002, and in revised form, July 15, 2002
Published, JBC Papers in Press, July 30, 2002, DOI 10.1074/jbc.M205203200

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Low molecular weight phosphotyrosine phosphatase (LMW-PTP) is an enzyme involved in platelet-derived growth factor-induced mitogenesis and cytoskeleton rearrangement. Our previous results demonstrated that LMW-PTP is able to bind and dephosphorylate activated platelet-derived growth factor receptor (PDGF-r), thus inhibiting cell proliferation. Here we revisit the role of LMW-PTP on activated PDGF-r dephosphorylation. We demonstrate that LMW-PTP preferentially acts on cell surface PDGF-r, excluding the internalized activated receptor pool. Many phosphotyrosine phosphatases act by site-selective dephosphorylation on several sites of PDGF-r, but until now, there has been no evidence of a direct involvement of a specific phosphotyrosine phosphatase in the dephosphorylation of the 857 kinase domain activation tyrosine. Here we report that LMW-PTP affects the kinase activity of the receptor through the binding and dephosphorylation of Tyr-857 and influences many of the signal outputs from the receptor. In particular, we demonstrate a down-regulation of phosphatidylinositol 3-kinase, Src homology phosphatase-2, and phospholipase C-γ1 binding but not of MAPK activation. In addition, we report a slight action of LMW-PTP on Tyr-716, which directs MAPK activation through Grb2 binding. On the basis of these results, we propose a key role for LMW-PTP in PDGF-r down-regulation through the dephosphorylation of the activation loop Tyr-857, thus determining a general negative regulation of all downstream signals, with the exception of those elicited by internalized receptors.

Although PDGF[1] plays an important role in normal development, accumulating evidence suggests that its abnormal expression also contributes to a variety of diseases. This is emphasized by the fact that PDGF and its receptor are currently under investigation as targets in numerous proliferative disorders, including cancer and cardiovascular and fibrotic diseases (1). Binding of PDGF to the extracellular domain of the receptor is thought to induce receptor dimerization, allowing transphosphorylation of adjacent dimerized receptors on specific tyrosine residues within the intracellular region (2). The phosphorylated tyrosines supply docking sites for recruitment of cytosolic signaling proteins with appropriate binding motifs. The cellular function of receptor-associated proteins is believed to be modified as a consequence of association with and phosphorylation by the receptor itself. Thus, ligand binding initiates intracellular signaling events involving the synthesis of second messenger molecules, activation of small G proteins, protein phosphorylation cascades, and, finally, gene transcription. All these signaling pathways are thought to mediate biological responses to PDGF through cell cycle progression (3). The systems used to fine-tune receptor-induced signaling and the full variety of the signaling potential of the receptor remain uncertain. It has been demonstrated that PDGF-r expression is down-regulated for the duration of the second phase of the cell cycle and during cell differentiation. In particular, PDGF-r down-regulation appears to be regulated at two stages: a short time level, which is thought to be played essentially by an immediate endocytosis of ligand-receptor complexes, and a long-term down-regulation reply, played by receptor degradation by ubiquitin-dependent proteolysis (4) and by decline of PDGFβ-r mRNA expression.

It is well established that receptor tyrosine kinases are negatively controlled by phosphotyrosine phosphatases (PTPs) (5, 6). Down-regulation of tyrosine kinase receptors by PTPs could be a more rapid system with respect to the elimination of activatable PDGF-r from the membrane by clathrin-mediated internalization and the decrease of PDGF-r molecules by ubiquitin-mediated or lysosomal proteolysis or mRNA repression (7). There is little in the literature about the role of PDGF-r dephosphorylation by PTPs, although many PTPs have been found to interact with the receptor. In particular, SHP-2 (8), density-enhanced phosphatase-1 (9), LMW-PTP (10), and CD45 (11) have been depicted to dephosphorylate the activated PDGF-r, although the real significance of this dephosphorylation is largely unclear. In fact, PTP action on tyrosine kinase receptors could lead either to a broad reduction of receptor signaling, by targeting to all receptor phosphoty-

* This work was supported in part by Consiglio Nazionale delle Ricerche (target project on Biotechnology, Strategic project "Controlli post-trascrizionali dell'espressione genica"), the Italian Association for Cancer Research, the Ministero della Università e Ricerca Scientifica e Tecnologica (MIUR-PRIN 2000 and MIUR-Consiglio Nazionale delle Ricerche Biotechnology program L.95/95), and 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.

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[1] The abbreviations used are: PDGF, platelet-derived growth factor; PDGF-r, PDGF receptor; PI3K, phosphatidylinositol 3-kinase; PLC-γ1, phospholipase C-γ1; PTP, phosphotyrosine phosphatase; LMW-PTP, low molecular weight PTP; dnLMW-PTP, dominant negative LMW-PTP; SHP, Src homology phosphatase; MAPK, mitogen-activated protein kinase; SH2, Src homology 2.

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rosines, or to a modulation of signaling, through site-selective dephosphorylation.

LMW-PTPs are a group of 18-kDa enzymes that are widely expressed (12). Previous studies on the molecular biology of LMW-PTP in NIH3T3 cells demonstrated a well-defined role of this enzyme in PDGF-induced mitogenesis, showing that activated PDGF-r is a substrate for LMW-PTP (10, 13). The most relevant phenotypic effect of LMW-PTP overexpression is a strong reduction of the cell growth rate in response to PDGF stimulation. More recently, we have found that in NIH3T3 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 (13, 14). Cytoskeleton-associated LMW-PTP influences cell adhesion, spreading, and migration, controlling the phosphorylation level of p190Rho-GAP, a protein that is able to regulate Rho activity and, consequently, cytoskeleton rearrangement in response to PDGF stimulation. Hence, LMW-PTP is able to perform multiple functions in PDGF-induced mitogenesis: whereas cytosolic LMW-PTP binds and dephosphorylates PDGF-r, thus modulating part of its signaling cascade, cytoskeleton-associated LMW-PTP acts on phosphorylated p190Rho-GAP, consequently playing a role in PDGF-mediated cytoskeleton rearrangement (15). Recently, we demonstrated that during PDGF signaling, LMW-PTP is regulated by a redox mechanism involving the two cysteine residues of the catalytic site, which turn reversibly from the reduced to oxidized state after PDGF stimulation. The reversibility of *in vivo* LMW-PTP oxidation is glutathione-dependent (12). The additional catalytic pocket Cys-17 retains an intriguing and peculiar role in the formation of a S-S intramolecular bond, which protects the catalytic Cys-12 from further and irreversible oxidation. The presence of an additional cysteine near the catalytic one confers upon LMW-PTP the ability to rapidly recover its activity and finely regulate PDGF receptor activation.

In this study, we revisit the role of LMW-PTP in PDGF-r down-regulation, demonstrating that it acts as a real termination signal for receptor activation because it dephosphorylates the PDGF-r-regulatory tyrosine (Tyr-857), thus determining a general negative regulation of all downstream signals, with the exception of those elicited by internalized receptors.

EXPERIMENTAL PROCEDURES

Materials—Unless specified, all reagents were obtained from Sigma. NIH3T3 cells were purchased from American Type Culture Collection, and HepG2 cells expressing wild-type PDGF-r or Y857F mutant PDGF-r were a generous gift of Dr. A. Kazlauskas and have been described elsewhere (16). Human recombinant PDGF-BB was from Peprotech, and the Enhanced Chemiluminescence kit was from Amersham Biosciences. All antibodies were from Santa Cruz Biotechnology, with the exception of those against Tyr(P)-716 and p85-PI3K, which were from Upstate Biotechnology Inc. Antibodies against Tyr(P)-751 and Tyr(P)-857 were a generous gift of Dr. A. Kazlauskas and have been described elsewhere (17). BCA protein assay reagent was from Pierce. Dichlorodihydrofluorescein diacetate (DCFDA) was obtained from Molecular Probes.

Cell Culture and Transfections—NIH3T3 or HepG2 cells were routinely cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum in a 5% CO_2 humidified atmosphere. 10 μg of pRcCMV-C12S-LMW-PTP (expressing the dominant negative Cys-12 to Ser mutant of LMW-PTP) were transfected in NIH3T3 cells using the calcium phosphate method. Stable transfected clonal cell lines were isolated by selection with G418 (400 μg/ml). Mock-transfected cell lines were obtained by transfecting 2 μg of pRcCMVneo alone. The clonal cell 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.

Immunoprecipitation and Western Blot Analysis—1×10^6 cells were seeded in 10-cm plates in Dulbecco's modified Eagle's medium 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 radioimmune precipitation assay buffer lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, 2 mM EGTA, 1 mM sodium orthovanadate, 1 mM phenylmethanesulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). Lysates were clarified by centrifugation and immunoprecipitated for 4 h at 4 °C with 1–2 μg of the specific antibodies. Immunocomplexes were collected on protein A-Sepharose (Amersham Biosciences), separated by SDS-PAGE, 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 with specific antibodies and then with secondary antibodies conjugated with horseradish peroxidase, washed, and developed with the Enhanced Chemiluminescence kit.

PDGF-r Internalization by Trypsinization—PDGF-r internalization was determined by measuring the percentage of receptors that were resistant to trypsinization according to Ceresa *et al.* (18). Briefly, cells were serum-starved for 24 h and then stimulated with 30 ng/ml PDGF-BB. The cells were washed twice with phosphate-buffered saline and incubated on ice for 8 min with ice-cold, 20 mM sodium acetate, pH 3.7. The cells were washed with ice-cold phosphate-buffered saline and incubated with trypsin (1 mg/ml in phosphate-buffered saline) in ice for 30 min with occasional rocking. The reaction was stopped by the addition of soybean trypsin inhibitor (1 mg/ml). Cells were then washed and solubilized in lysis buffer for 10 min at 4 °C. The cell lysates were immunoprecipitated with anti-PDGF-r antibodies and subjected to SDS-PAGE. The trypsin-resistant 190-kDa PDGF-r is the internalized receptor. The total PDGF-r amount was evaluated in parallel in non-trypsinized cells.

PDGF-r Kinase Activity Assay—Cell lysates in lysis buffer were subjected to immunoprecipitation with anti-PDGF-r subunit antibodies (Santa Cruz Biotechnology). The immunoprecipitated proteins were washed twice in lysis buffer, washed twice in 50 mM Tris-HCl, pH 7.4, containing 1 mM sodium orthovanadate, and finally resuspended in 50 mM Hepes, pH 7.4, 10 mM $MnCl_2$. The reaction was started with the addition of [^{32}P]ATP (3000 Ci/mmol, 20 μCi) to all samples, which were incubated at 4 °C for 10 min. The beads were then washed once with 50 mM Tris-HCl, pH 7.4, and finally resuspended in 20 μl of Laemmli's sample buffer, boiled for 5 min, and separated by 7.5% SDS-PAGE (19). The autoradiogram was scanned using Chemidoc Quantity One software (Bio-Rad). Normalization was achieved by anti-PDGF-r immunoblot of a part of the analyzed samples. In addition, the PDGF-r kinase activity was assayed using an exogenous substrate, glutathione *S*-transferase-PLC-γ (20). Immunoprecipitates were incubated in the presence of 20 mM 1,4-piperazinediethanesulfonic acid, pH 7.0, 10 mM $MnCl_2$, 20 μg/ml aprotinin, and 10 μCi of [γ-^{32}-P]ATP (3000 Ci/mmol) for 10 min at 30 °C in the presence or absence of 0.5 μg of glutathione *S*-transferase-PLC-γ1. The fusion protein included amino acid residues 550–850 of rat PLC-γ1. The reaction was stopped by adding an equal volume of 2× sample buffer (10 mM EDTA, 4% SDS, 5.6 mM 2-mercaptoethanol, 20% glycerol, 200 mM Tris-HCl, pH 6.8, and 1% bromphenol blue). The samples were then incubated for 3 min at 95 °C, spun, and resolved on 7.5% SDS-polyacrylamide gel electrophoresis, and the radiolabeled proteins were detected by autoradiography. Normalization has been achieved by anti-PLC-γ1 immunoblot of a part of the analyzed samples.

Phosphatidylinositol 3-Kinase Assay—The PI3K assay was performed as described elsewhere (20). Briefly, serum-starved cells were incubated with 30 ng/ml PDGF for 10 min and then lysed in radioimmune precipitation assay buffer. Equal amounts of proteins were immunoprecipitated using PY20 anti-phosphotyrosine antibody (Santa Cruz Biotechnology). After washing, the immunobeads were resuspended in 50 μl of 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.5 mM EGTA. 0.5 μl of 20 mg/ml phosphatidylinositol was added, mixed, and incubated at 25 °C for 10 min. 1 μl of 1 mM $MgCl_2$ and 10 μCi of [^{32}P]ATP (3000 Ci/mmol) were then added simultaneously and incubated at 25 °C for an additional 10 min. The reaction was stopped by the addition of 150 μl of chloroform, methanol, and 37% HCl (10:20:0.2). The samples were extracted with chloroform and dried. Radioactive lipids were separated by thin-layer chromatography using chloroform, methanol, 30% ammonium hydroxide, and water (46:41:5:8). After drying, the plates were autoradiographed. The radioactive spots corresponding to phosphatidylinositol phosphate were scraped and counted in a liquid scintillation counter.

MAPK Activation—1.5×10^4 cells were plated on a 6-well dish in complete medium. Cells were serum-starved for 24 h before receiving 30 ng/ml PDGF-BB for the indicated times, lysed in radioimmune precip-

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itation assay buffer, and centrifuged to remove the insoluble debris. The total protein content was evaluated by the BCA protein assay, and 20 μg of lysates were resolved on a 12% SDS-PAGE. The resolved proteins were transferred to nitrocellulose membrane and probed overnight with anti-phospho-extracellular signal-regulated kinase 1/2 monoclonal antibodies.

LMW-PTP/PDGF-r in Vitro Binding Assay—The binding assay was performed as described elsewhere (21). Briefly, after PDGF-BB stimulation, HepG2 cells expressing wild-type or the Tyr to Phe mutant version of PDGF-r were lysed, and PDGF-r was immunoprecipitated. Immunocomplexes were collected as usual with protein A-Sepharose and washed several times with radioimmune precipitation assay buffer and once with binding buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM $MgCl_2$, 100 mM NaCl, and 0.1% Triton X-100). Agarose-bound receptors were resuspended in 0.1 ml of binding buffer and allowed to associate with 1 μg of dominant negative glutathione *S*-transferase-LMW-PTP at 4 °C for 1 h. The beads were washed twice in 1 ml of binding buffer and resuspended in SDS-PAGE sample buffer for anti-LMW-PTP immunoblot analysis.

RESULTS

LMW-PTP Controls the Tyrosine Phosphorylation Level of PDGF-r—Previous studies have shown that in NIH3T3 cells, overexpression of dnLMW-PTP causes an increased mitogenic response to PDGF, whereas overexpression of the wild-type LMW-PTP has the opposite effect (10, 23). The dominant negative form of LMW-PTP is a mutant of the catalytic site Cys-12 to Ser. As in all PTPs, the catalytic site cysteine is capable of transiently forming the cysteinyl-phosphate intermediate, and the mutation of this amino acid residue converts the enzyme in an inactive phosphatase. The preservation of the substrate binding site(s) in the C12S mutant (*i.e.* the Arg residue in the catalytic pocket) maintains the potential to bind intracellular natural substrates, leading to the increase in their tyrosine phosphorylation level. Here we perform a time course experiment (Fig. 1*A*) in which cells overexpressing dnLMW-PTP were serum-starved for 24 h and stimulated with PDGF for 10, 45, and 120 min. The tyrosine phosphorylation level of PDGF-r was evaluated by anti-phosphotyrosine immunoblot, in comparison with mock-transfected cells. The blot has been reprobed with anti-PDGF-r antibodies for normalization (Fig. 1*B*). The results, shown in Fig. 1*A,* demonstrated that overexpression of dnLMW-PTP leads to a great increase in the tyrosine phosphorylation level of the activated receptor. Notably, we observe an increase in both the peak of PDGF-r tyrosine phosphorylation (10 min) and the duration of activation of the receptor itself (Fig. 1*C*). Hence, overexpression of dnLMW-PTP produces a dramatic effect on the activation of PDGF-r, leading to a greater and longer activation with respect to mock-transfected cells.

LMW-PTP Does Not Affect the Tyrosine Phosphorylation Level of Internalized PDGF-r—The internalization and trafficking of ligand-activated cell surface receptor have long been reported as a possible down-regulation mechanism for many receptors (24). In addition, it has been reported recently that endosomes retain a role in either initiating or extending the signal elicited at the plasma membrane, namely, MAPK pathway activation (25–27). We analyzed the tyrosine phosphorylation level of internalized PDGF-r by trypsin treatment of agonist-stimulated dnLMW-PTP-overexpressing cells in comparison with mock-transfected ones. This method allows a direct analysis of receptor endocytosis by means of trypsin-directed proteolysis of the cell surface receptor. The internalized receptors are in fact the only ones retaining the original molecular weight, whereas the receptor molecules remaining at the cell surface are cleaved by trypsin. Our results (Fig. 2*A*) indicate that receptor internalization shows similar kinetics in both dnLMW-PTP-overexpressing cells and mock-transfected cells, with a maximum at 10 min after stimulation, followed by a slow decrease. The data have been normalized on the basis of

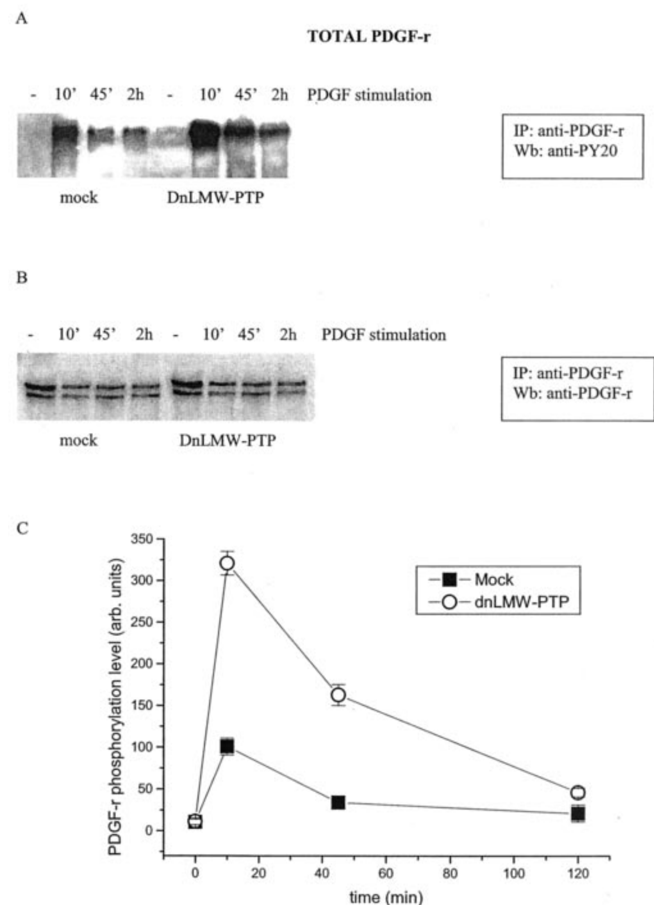

FIG. 1. **LMW-PTP controls PDGF-r tyrosine phosphorylation level.** 1×10^6 dnLMW-PTP or mock-transfected NIH3T3 cells were serum-starved for 24 h and then stimulated with 30 ng/ml PDGF-BB for the indicated periods. *A*, PDGF-r was immunoprecipitated from lysates, and the anti-phosphotyrosine immunoblot was performed. *B*, the blot was stripped and reprobed with anti-PDGF-r antibodies. Data have been analyzed by Chemidoc Quantity One software, and the normalized values obtained are plotted in *C* ($n = 5$).

anti-PDGF-r immunoblot by stripping and reprobing the same filter (Fig. 2*B*) and plotting the values in Fig. 2*C*. The results indicate that LMW-PTP does not affect the tyrosine phosphorylation level of the internalized receptor and that the tyrosine phosphorylation level of the internalized receptor does not decrease as rapidly as the cell surface receptor phosphorylation level (Fig. 1) but remains high for at least 2 h.

LMW-PTP Binds and Dephosphorylates the Tyr-857 of PDGF-r, Thus Influencing Receptor Kinase Activity—The tyrosine phosphorylation level of PDGF-r after stimulation is due to the regulation of the rate of phosphorylation or dephosphorylation. It has been reported that in PDGF-r, phosphorylation of the activation loop tyrosine in position 857 temporally precedes the complete autophosphorylation of the receptor in multiple SH2 domain binding sites and is required for the full activation of receptor kinase activity (16). To investigate the effect of LMW-PTP on Tyr-857, we analyzed the binding of LMW-PTP to Tyr-857 after PDGF-r activation. We have already reported that LMW-PTP binds only to agonist-activated PDGF-r through the phosphatase catalytic site, although we had no information about the specific tyrosine(s) involved in its binding (10, 13). HepG2 cells overexpressing the wild-type PDGFβ receptor or the Y857F mutant were stimulated with PDGF-BB, and the binding of dnLMW-PTP to agarose-bound activated PDGF-r was analyzed (Fig. 3*A*). Receptor activation in cells expressing either wild-type or mutant PDGF-r was

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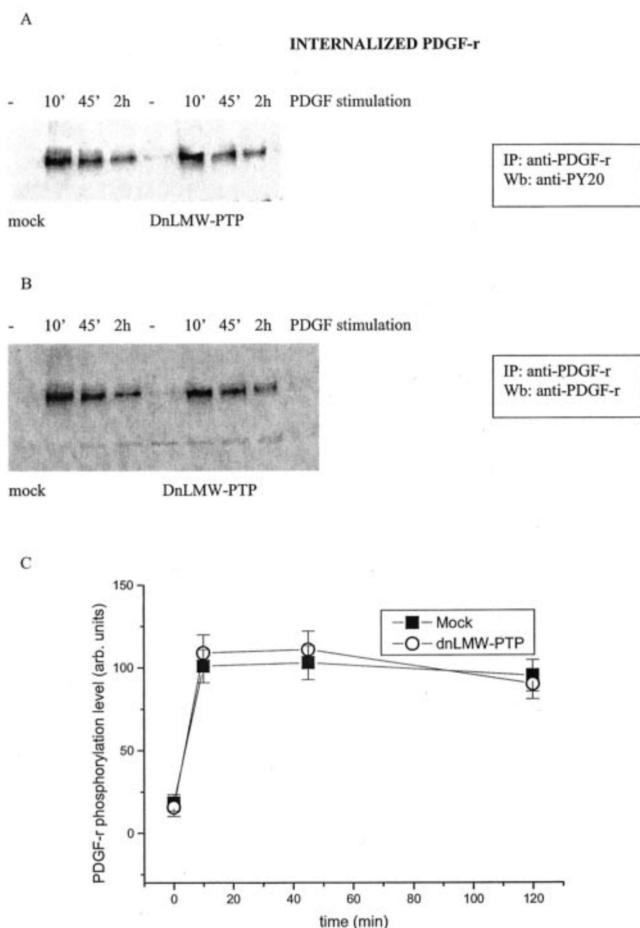

FIG. 2. **LMW-PTP does not affect the tyrosine phosphorylation of the internalized PDGF-r.** 1×10^6 dnLMW-PTP or mock-transfected NIH3T3 cells were serum-starved for 24 h and then stimulated with 30 ng/ml PDGF-BB for the indicated periods. PDGF-r internalization was evaluated by trypsin treatment as reported under "Experimental Procedures." *A*, PDGF-r was immunoprecipitated from lysates, and an anti-phosphotyrosine immunoblot was performed. *B*, the blot was stripped and reprobed with anti-PDGF-r antibodies. Data have been analyzed by Chemidoc Quantitation Analysis software, and the normalized data values obtained are plotted in *C* (n = 4).

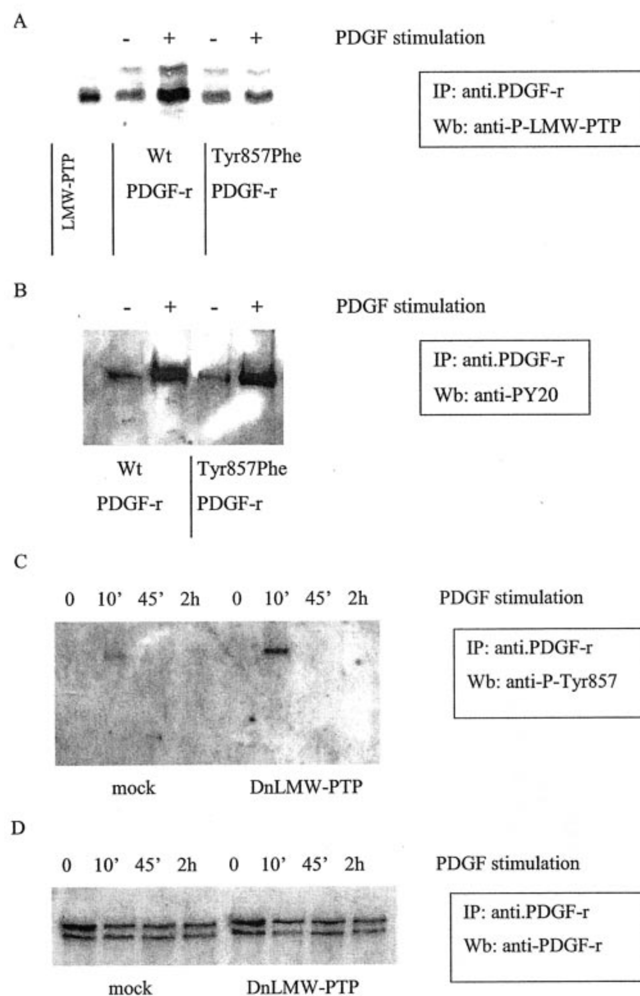

FIG. 3. **LMW-PTP binds and dephosphorylates Tyr-857 in the PDGF-r.** *A*, LMW-PTP *in vitro* binding assay. After PDGF-BB stimulation, HepG2 cells were lysed, and PDGF-r was immunoprecipitated. Immunocomplexes were collected, washed several times, and allowed to associate with 1 μg of glutathione *S*-transferase-LMW-PTP. The beads were washed and resuspended in SDS-PAGE sample buffer for anti-LMW-PTP immunoblot analysis. The activation of PDGF-r in the experiment was checked by anti-phosphotyrosine immunoblot of part of the same samples (*B*). The experiment is representative of three independent repetitions. *C*, tyrosine phosphorylation of Tyr-857. 1×10^6 dnLMW-PTP or mock-transfected NIH3T3 cells were stimulated with 30 ng/ml PDGF-BB for the indicated times. PDGF-r was immunoprecipitated from lysates, and an anti-phospho-Tyr-857 immunoblot was performed. Equalization was checked by stripping the blot and reprobing with anti-PDGF-r antibodies. *D*, the blot was stripped and reprobed with anti-PDGF-r antibodies. The results are the mean of at least three experiments.

checked by antiphosphotyrosine immunoblot of the agarose-bound receptors (Fig. 3*B*). The results show that the association between PDGF-r and LMW-PTP is dramatically reduced in cells expressing the Y857F mutant of PDGF-r compared with cells expressing the wild-type receptor, suggesting that Tyr-857 is a key binding site for LMW-PTP in the activated receptor. In addition, we analyzed the phosphorylation level of this residue by means of anti-phospho-Y857 antibodies (Fig. 3*C*). PDGF-r content has been checked by anti-PDGF-r immunoblot of the stripped blot (Fig. 3*D*). The results indicate that phosphorylation of tyrosine 857, although transient, is dramatically increased by dnLMW-PTP overexpression. LMW-PTP appears to influence only the phosphorylation level of Tyr-857 in the maximum peak (namely, 10 min after PDGF stimulation), and does not appear to influence the temporal extent of its phosphorylation.

The requirement of phosphorylation in Tyr-857 for receptor tyrosine kinase activity is well documented (29, 30). In this light, we assayed PDGF-r autophosphorylation activity in cells overexpressing dnLMW-PTP (Fig. 4*A*). PDGF-r kinase activity on an exogenous substrate such as PLC-γ1 was monitored under the same experimental conditions (Fig. 4*B*), obtaining results in agreement with the PDGF-r autophosphorylation assay. The results of the normalized data (Fig. 4, *A* and *B*) show that dnLMW-PTP overexpression affects receptor kinase activity only at 10 min, and not at longer times. It has been proposed that the phosphorylation of the different tyrosines in PDGF-r display temporal and spatial distribution, suggesting that receptor functions corresponding to each of the phosphorylated sites were regulated as a function of time. In particular, Bernard and Kazlauskas (17) propose that whereas the kinase activity of the receptor is regulated within the first 10 min after stimulation, other receptor functions persist for longer times. It is likely that LMW-PTP affects receptor kinase activity only at 10 min because the phosphorylation of tyrosine 857, which is required for activation of receptor kinase activity, is present only within that 10-min period.

Analysis of the Tyrosine Phosphorylation level of PDGF-r Sites—To investigate the hypothetical site selectivity of LMW-PTP in PDGF-r dephosphorylation, we analyzed the tyrosine

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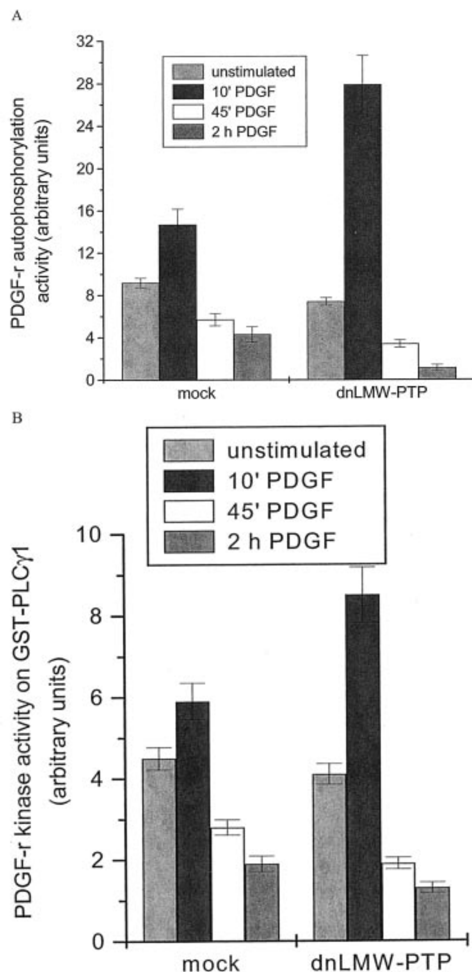

FIG. 4. **LMW-PTP affects the kinase activity of PDGF-r.** *A* and *B*, 1×10^6 dnLMW-PTP or mock-transfected NIH3T3 cells were serum-starved for 24 h and then stimulated with 30 ng/ml PDGF-BB. PDGF-r was immunoprecipitated from lysates, and an immunokinase assay was performed as reported under "Experimental Procedures." PDGF-r autophosphorylation kinase activity is reported in *A,* and PDGF-r kinase activity toward glutathione *S*-transferase-PLC-γ1 is reported in *B*. The ratio between the densitometric analyses of kinase assays and of normalization blots (anti-PDGF-r or anti-PLC-γ1 immunoblots) is shown in both *A* and *B* (n = 3).

phosphorylation level of different sites in a time course experiment with mock-transfected and dnLMW-PTP-overexpressing cells. We stimulated cells for the period necessary to commit cells to S-phase entry, namely, 8–9 h (28). We used phosphospecific antibodies directed toward Tyr(P)-857 (activation loop), Tyr(P)-751 (PI3K binding site), Tyr(P)-716 (Grb2 binding site), and Tyr(P)-1021 (PLC-γ1 binding site). The results in Fig. 5 show that dnLMW-PTP appears to greatly increase the tyrosine phosphorylation level of Tyr-857 (Fig. 5*A*) and Tyr-751 (Fig. 5*B*) and partially increase that of Tyr-1021 (Fig. 5*C*). On the contrary, the effect on Tyr-716 (Fig. 5*D*) is absent, in agreement with our previous observation about the lack of dephosphorylation of internalized activated receptors.

Taken together, these data suggest that LMW-PTP acts as a general PDGF-r signaling terminator by lowering the phosphorylation level of the regulatory Tyr-857 and/or many of the phosphotyrosines that mediated SH2-binding protein activation but not of the Grb2 binding site Tyr-716.

Analysis of the PDGF-r Downstream Pathways Affected by LMW-PTP—The tyrosine phosphorylation of PDGF-r leads to the recruitment of many SH2 domain-containing proteins. For many of them, different tyrosines have been found to be specific binding sites. In particular PI3K binds to Tyr-751, PLC-γ1 binds to Tyr-1021, and SHP-2 binds to Tyr-1009 (3, 31). Because the ability of these proteins to bind to the receptor is directly proportional to the phosphorylation of the binding site, we analyzed the recruitment of many SH2 domain-containing proteins to the receptor in mock-transfected and dnLMW-PTP-expressing cells. Fig. 6 shows the results obtained. dnLMW-PTP overexpression leads to increased binding of PI3K (Fig. 6*A*), PLC-γ1 (Fig. 6*B*), and SHP-2 (Fig. 6*C*). In addition, to confirm the general action of LMW-PTP on PDGF-r downstream signaling, we analyzed the activity of PI3K and MAPK in a time course experiment in dnLMW-PTP-expressing cells in comparison with mock-transfected cells. dnLMW-PTP overexpression leads to enhanced PI3K activity in both the immediate (10 min) and the tardive (5–8 h) waves of phosphoinositide production, suggesting a role for tyrosine phosphorylation in both PI3K activity waves (Fig. 6*D*). These data confirm a specific role of LMW-PTP in the long-lasting PDGF-r tyrosine phosphorylation regulation. On the contrary, we do not find any variation of MAPK activation in dnLMW-PTP-overexpressing cells in comparison with mock-transfected cells (Fig. 6*E*). This finding is in agreement with the lack of effect of dnLMW-PTP on Tyr-716 phosphorylation because this residue mediates the binding of Grb2 to the activated receptor and hence the activation of the Ras/Raf/MAPK pathway.

Taken together, these data indicate that LMW-PTP acts on PDGF-r signaling, regulating many signal transduction pathways. The role of LMW-PTP in down-regulation of the activation of PI3K, SHP-2, and PLC-γ1 recruitment may be due to a direct dephosphorylation of the phosphotyrosines that begin these signals or may be a simple consequence of a down-regulation of the kinase activity of the receptor through dephosphorylation of Tyr-857.

DISCUSSION

The notion that PTPs act as negative regulators of receptor tyrosine kinase signaling by direct dephosphorylation of receptor tyrosine kinases has received some experimental support. For example, overexpression of CD45 reduces PDGF receptor tyrosine phosphorylation (11), and antisense-mediated suppression of leukocyte common antigen-related (LAR) phosphatase potentiates signaling of various growth factor receptors, including insulin and epidermal growth factor receptors (32). In addition, data for negative regulation of epidermal growth factor receptor signaling by rPTPσ in A431 cells have been obtained by inducible overexpression of rPTPσ and by down-regulation of endogenous rPTPσ expression levels with an antisense approach (33). Receptor tyrosine kinase dephosphorylation by PTPs can lead to two outcomes: complete abrogation of the signal through dephosphorylation of the regulatory tyrosine and/or through general dephosphorylation or, alternatively, selective modulation of the signal output by dephosphorylation of a subset of SH2 domain or phosphotyrosine binding (PTB) domain binding phosphotyrosines. There are few reports concerning this feature. Kovalenko *et al.* (9) reported that a receptor-like PTP called density-enhanced phosphatase-1 displays site selectivity dephosphorylation of PDGF-r, preferring Tyr-763, Tyr-771, and Tyr-778 and not affecting the Tyr-857 regulatory site. Again, SHP-2 dephosphorylation of PDGF-r revealed preferential dephosphorylation of Tyr-771, Tyr-751, and Tyr-750 (34), namely, the PI3K and Ras-GAP binding sites. For both these phosphatases, the activation loop regulatory Tyr-857 was not a preferential substrate, suggesting that both SHP-2 and density-enhanced phosphatase-1 could be modulators of some signal transduction pathways, instead of general signal termination enzymes.

The aim of our work was to study the specific role of LMW-

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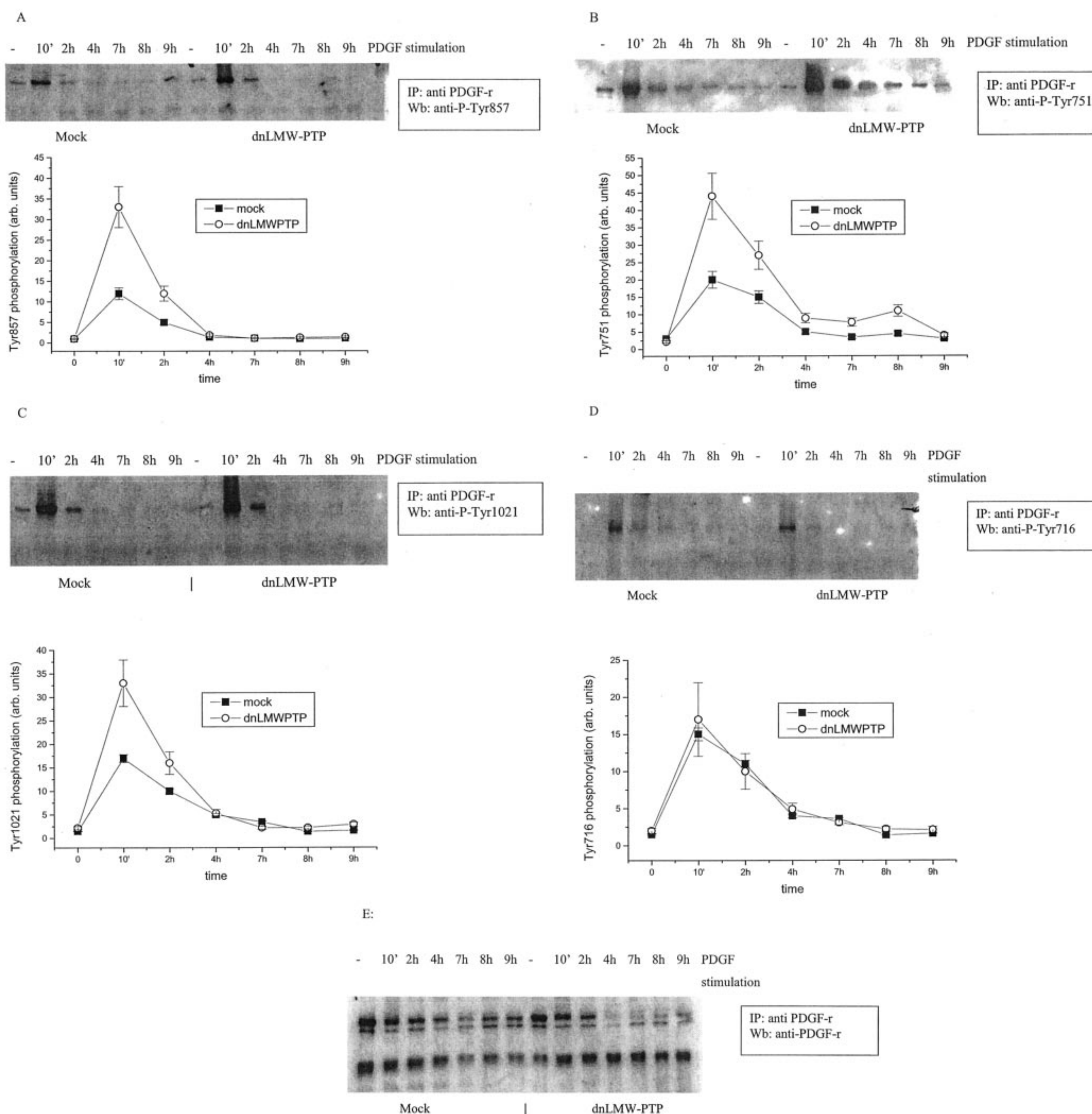

FIG. 5. **Analysis of the tyrosine phosphorylation level of several PDGF-r sites.** 1×10^6 dnLMW-PTP or mock-transfected NIH3T3 cells were serum-starved for 24 h and then stimulated with 30 ng/ml PDGF-BB for the indicated times. PDGF-r was immunoprecipitated from lysates, and sequential Western blots were performed. The ratios between the densitometric analyses of the anti-phosphospecific immunoblots and the anti-PDGF-r blots have been plotted and reported in each panel. *A*, anti-Tyr(P)-857 phosphorylation. *B*, anti-Tyr(P)-751 phosphorylation. *C*, anti-Tyr(P)-1021 phosphorylation. *D*, anti-Tyr(P)-716 phosphorylation. *E*, the blot has been stripped several times for each phosphospecific antibody and finally reprobed with anti-PDGF-r antibodies for normalization. These data are representative of at least three independent experiments with similar results.

PTP in the context of PDGF-r down-regulation by dephosphorylation. We state that LMW-PTP greatly influences the activation of PDGF-r. In fact, the overexpression of a dominant negative mutant of LMW-PTP induces a dramatic increase in both the phosphorylation peak (10 min) and the duration of phosphorylation. This effect explains the great increase we observed in cell proliferation and G_1 cell cycle phase length in dnLMW-PTP-expressing cells (10, 13).

First, our findings indicate that LMW-PTP does not act on internalized receptors because we show that this phosphatase does not influence the tyrosine phosphorylation level of the internalized activated receptor (Fig. 2). The internalization of the PDGF-r upon stimulation with the agonist is one of the earliest responses elicited by PDGF. The internalization is mediated by clathrin-coated pits in which the 100-kDa GTPase dynamin is responsible for the pinch-off step of the vesicle. Internalized receptors can recycle back to the cell surface or be sorted to lysosomal degradation (35). Many authors have reported that ligand-induced internalization retains a specific role in signal transduction, especially in MAPK activation. In fact, inhibition of insulin receptor internalization leads to a partial inhibition of Shc phosphorylation and MAPK down-

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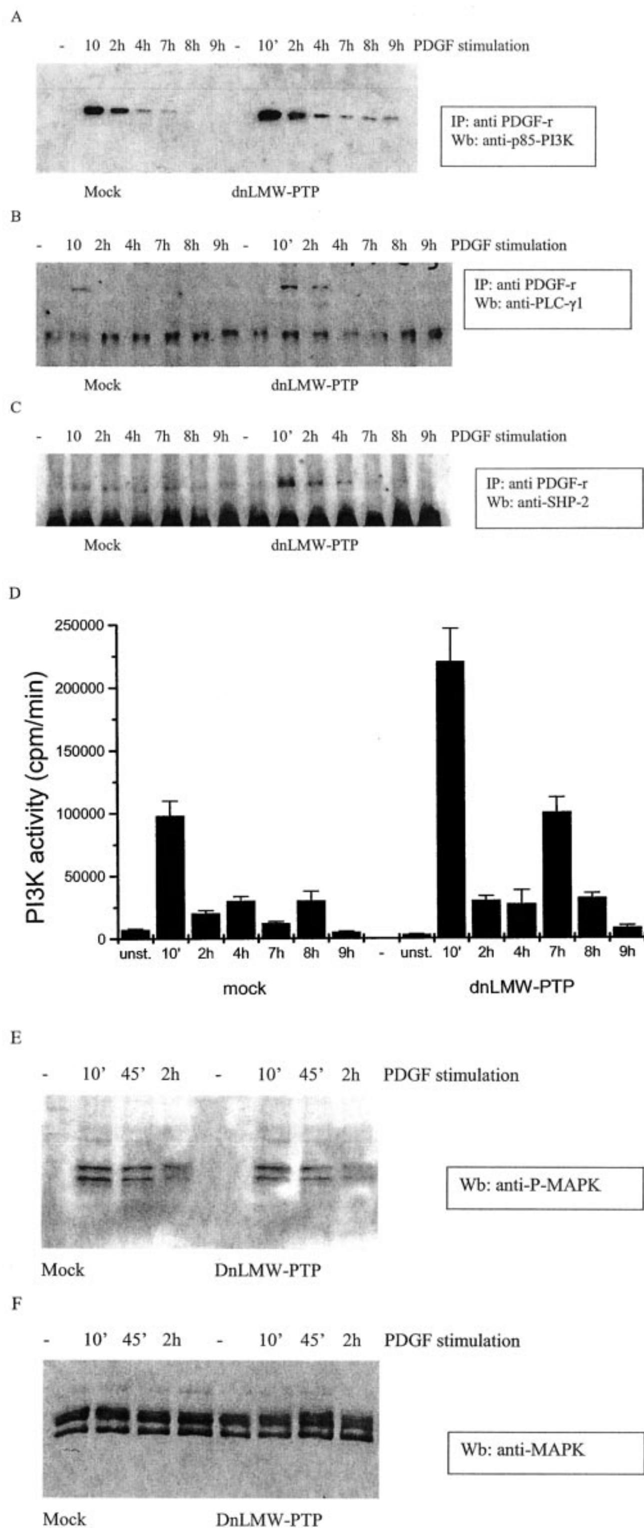

FIG. 6. **Analysis of PDGF-r downstream pathways affected by LMW-PTP.** 1×10^6 dnLMW-PTP or mock-transfected NIH3T3 cells were serum-starved for 24 h and then stimulated with 30 ng/ml PDGF-BB for the indicated times. PDGF-r was immunoprecipitated from lysates, and sequential Western blots were performed. *A*, anti-p85-PI3K immunoblot. *B*, anti-PLC-γ1 immunoblot. *C*, anti-SHP-2 immunoblot. These data are representative of at least three independent experiments with similar results. *D*, 400 μg of total proteins were used in a PI3K assay as reported under "Experimental Procedures" ($n = 5$). *E*, 40 μg of total proteins from lysates were used for an anti-phospho-MAPK immunoblot. *F*, equalization was confirmed by stripping the blot and reprobing with anti-MAPK antibodies. The results are representative of at least three independent experiments.

regulation (18, 36, 37). These findings indicate that the internalized insulin receptors have a positive signal transduction role, at least in Shc and MAPK pathways. In addition, Burke (25) has recently demonstrated that epidermal growth factor signaling is positively regulated by endocytosis and intracellular trafficking. It is likely that dephosphorylation of the internalized receptor is not a mechanism of down-regulation of the selective pathways activated by this subset of receptors. In fact, the internalized receptor remains highly phosphorylated for very long times, suggesting that this receptor is not a substrate for any PTPs, as already reported (38), and not only for LMW-PTP. It has been reported that down-regulation of MAPK activation is achieved by an alternative route: activated p42 and p44 MAPKs are specifically and directly dephosphorylated by MAPK phosphatases, a group of dual specificity PTPs transcriptionally induced upon agonist treatment (39). In this context, the direct dephosphorylation of the internalized receptors could be a marginal phenomenon.

Furthermore, we demonstrate that LMW-PTP specifically binds and dephosphorylates PDGF-r Tyr-857, affecting both the autophosphorylation activity of the receptor and its kinase activity toward exogenous substrates. Tyr-857 is one of the autophosphorylation sites of the PDGF-r, is located in the kinase domain, and regulates the catalytic activity of the receptor kinase (29, 40). We suggest that LMW-PTP affects the kinase activity of the receptor within 10 min after stimulation, dephosphorylating the kinase loop tyrosine. Later on, LMW-PTP does not influence receptor tyrosine kinase activity any more, in agreement with previous reports, suggesting that Tyr-857 is only briefly phosphorylated (17, 41). We emphasize that LMW-PTP is, to date, the only PTP able to dephosphorylate the activation loop regulatory tyrosine, thus influencing the kinase activity of the receptor both toward exogenous substrates and the receptor itself. In fact, only for SHP-2 and density-enhanced phosphatase-1 has site selectivity has been proven, although for both these phosphatases the Tyr-857 is not a preferential dephosphorylation site (9, 34). 11 of the 15 tyrosine residues outside the catalytic domain of PDGF-r (3) have been demonstrated to undergo autophosphorylation after ligand stimulation. We analyzed the role of LMW-PTP in the control of the tyrosine phosphorylation level of some of these phosphotyrosines, namely, Tyr(P)-857 (kinase activation loop), Tyr(P)-751 (PI3K binding site) Tyr(P)-716 (Grb2 binding site), and Tyr(P)-1021 (PLC-γ1 binding site). Although PDGF-r has many other tyrosines that can be phosphorylated upon agonist stimulation, in this way we spanned all functions of the activated receptor: (*a*) the relay of the kinase activity of the receptor itself by Tyr-857, (*b*) the activation of PI3K, the main mitogenic pathway starting from the receptor, by Tyr-751, (*c*) the activation of MAPK pathway by Tyr-716, through the Grb2/Sos/Ras/Raf/Mek route, which is mediated mainly by the internalized PDGF-r, and (*d*) the activation of PLC-γ1 by Tyr-1021. We show that LMW-PTP influences the tyrosine phosphorylation level of Tyr-857and Tyr-751 and partially influences that of Tyr-1021 (Fig. 5).

The tyrosine phosphorylation level of any Tyr(P) site in PDGF-r could be due to the regulation of the rate of phosphorylation or dephosphorylation. We speculate that the LMW-PTP-dependent dephosphorylation of Tyr-857, which acts as the regulatory site of the kinase activity of the receptor, could be responsible for all other downstream effects, including the decreased tyrosine phosphorylation level of signaling phosphotyrosines, although we could not exclude that LMW-PTP partially affects the phosphorylation level of other tyrosines. In fact, we observed a strongly reduced but not completely ablated binding of LMW-PTP to the Y857F mutant PDGF-r.

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In contrast, we report a very slight action of LMW-PTP on Tyr-716, lower with respect to that against Tyr-857, Tyr-751, and Tyr-1021. Tyr-716 directs the main route for MAPK activation through Grb2 binding (42), although this pathway could be activated by Grb2-independent ways (Fig. 5). Because the link between internalized receptor signaling and MAPK activation has been demonstrated, the observation that LMW-PTP does not act on Tyr(P)-716 supports our previous data about the inability of LMW-PTP to affect the tyrosine phosphorylation level of the internalized receptor (Fig. 2). In this light, it is likely that LMW-PTP has an only apparent preference for cell surface receptors, due to real receptor phosphotyrosine recognition. In agreement with these data, we demonstrated that LMW-PTP influences the activation of many signal transduction pathways such as PI3K, SHP-2, and PLC-γ1 but not of MAPK (Fig. 6), confirming the silencing of many, but not all, phosphotyrosines. Taken together, these data suggest that LMW-PTP acts as a general PDGF-r signaling terminator enzyme by dephosphorylating the regulatory Tyr-857 and many of the phosphotyrosines that mediated SH2-binding protein activation, such as PI3K, PLC-γ1, and SHP-2. The action of LMW-PTP on SH2 binding phosphotyrosines may be a direct dephosphorylation or a consequence of a down-regulation of the kinase activity of the receptor through dephosphorylation of Tyr-857. Our data on the selective binding of LMW-PTP of Tyr-857 appear to strengthen the second of these hypotheses.

LMW-PTP down-regulatory activity on PDGF-r might be controlled by several physiological conditions. In particular, we observed an increase of both the amount (by translational control) and activity (by redox regulation) of LMW-PTP by cell growth arrest stimuli, as cell-cell contact and cell differentiation. In both these situations, we demonstrated an increased down-regulation of PDGF-r (43). The regulated activity and expression of several PTPs might be a mechanism whereby receptors with various levels and/or patterns of tyrosine phosphorylation and concomitant variations in the pattern of associated SH2 domain-containing proteins and signaling output could be generated. Additional studies on PTP-dependent variations in the tyrosine phosphorylation level and/or pattern of the PDGF-r and other receptor tyrosine kinases in cells grown under various culture conditions, as well as in intact tissues, are therefore highly warranted.

Acknowledgment—We thank Dr. A. Kazlauskas for anti-Tyr(P)-857 and anti-Tyr(P)-751 antibodies and for HepG2 cells expressing wild-type and Y857F PDGF-r.

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J. Biol. Chem. 2002, 277:37331-37338.
doi: 10.1074/jbc.M205203200 originally published online July 30, 2002

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