

Low Molecular Weight Protein-tyrosine Phosphatase Tyrosine Phosphorylation by c-Src during Platelet-derived Growth Factor-induced Mitogenesis Correlates with Its Subcellular Targeting*

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The low molecular weight phosphotyrosine phosphatase (LMW-PTP) is an enzyme that is involved in the early events of platelet-derived growth factor (PDGF) receptor signal transduction. Our previous results have shown that 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. In this study we have established that, in nontransformed NIH3T3 cells, LMW-PTP exists constitutively in cytosolic and cytoskeleton-associated localization and that, after PDGF stimulation, c-Src is able to bind and to phosphorylate LMW-PTP only in the cytoskeleton-associated fraction. As a consequence of its tyrosine phosphorylation, LMW-PTP significantly increases its catalytic activity. After PDGF stimulation these two LMW-PTP pools act on distinct substrates, contributing in different manners to the PDGF receptor signaling. The cytoplasmic LMW-PTP fraction exerts its well known action on activated PDGF receptor. On the other hand we have now demonstrated that the cytoskeleton-associated LMW-PTP acts specifically on a few not yet identified proteins that become tyrosine-phosphorylated in response to the PDGF receptor activation. Finally, these two LMW-PTP pools markedly differ in the timing of the processes in which they are involved. The cytoplasmic LMW-PTP pool exerts its action within a few minutes from PDGF receptor activation (short term action), while tyrosine phosphorylation of cytoskeleton-associated LMW-PTP lasts for more than 40 min (long term action). In conclusion LMW-PTP is a striking example of an enzyme that exerts different functions and undergoes different regulation in consequence of its subcellular localization.

Signal transduction cascades driven by tyrosine phosphorylation regulate many cellular processes in eukaryotes such as cell proliferation, differentiation, and migration (1, 2). The

extent of protein tyrosine phosphorylation is determined by the concerted activity of protein-tyrosine kinases and protein-tyrosine phosphatases (PTPs).¹ In recent years the importance of PTPs in the regulation of intracellular tyrosine phosphorylation level has been widely recognized. The PTPs superfamily consists of over 70 enzymes that, despite very limited sequence similarity, have a common active site motif CX₅R and an identical catalytic mechanism. On the basis of their function, structure, and sequence, PTPs are often 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 (3).

The low molecular weight protein-tyrosine phosphatase (LMW-PTP) is an 18-kDa enzyme that is expressed in a wide variety of mammalian tissues (4). In our previous studies on the physiologic functions of LMW-PTP, we have expressed in NIH3T3 cells a catalytically inactive Cys¹² to Ser LMW-PTP mutant, which has maintained its capacity of substrate binding (5, 6). Overexpression of this LMW-PTP mutant form caused enhanced cell proliferation and serum-induced mitogenesis, indicating that the C12S mutant is a dominant negative LMW-PTP (dnLMW-PTP) (7). Analysis of dnLMW-PTP overexpressing clones led to the identification of PDGF-R as a specific *in vivo* substrate of LMW-PTP and to the demonstration that this phosphatase is most probably involved in the control of one or more signaling pathways triggered by PDGF-R activation (8). The action of LMW-PTP is essentially restricted to G₁ phase of the cell cycle and influences *myc* and *fos* gene induction driven by PDGF-R activation. In particular, LMW-PTP appears to be involved in the regulation of *myc* expression interfering with Src pathway and in the regulation of *fos* activation through an extracellular signal-regulated kinase-independent pathway mediated by the STAT proteins (8).

Previous results (9) have shown that LMW-PTP is tyrosine-phosphorylated in unstimulated v-Src overexpressing cells, although no direct association was detected between v-Src and LMW-PTP. More recently, it has been reported that in resting Jurkat cells LMW-PTP is tyrosine-phosphorylated, apparently by Lck kinase, and rapidly dephosphorylated upon T cell receptor stimulation (10). The same paper reported that LMW-PTP phosphorylation occurs in Tyr¹³¹ and partially in Tyr¹³², leading to a 2-fold enhancement of LMW-PTP enzymatic activity.

Src tyrosine kinase associates with activated PDGF receptor,

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¹ The abbreviations used are: PTP, protein-tyrosine phosphatase; LMW-PTP, low molecular weight protein-tyrosine phosphatase; dnLMW-PTP, dominant negative LMW-PTP; PDGF, platelet-derived growth factor; PDGF-R, PDGF receptor; wtLMW-PTP, wild type LMW-PTP; STAT, signal transducer and activator of transcription.

becomes tyrosine-phosphorylated, slightly increases its catalytic activity, and translocates to the cell periphery by an actin-dependent process (11). Here Src becomes associated with pp125^{FAK}, and this fact represents an early and critical event in the assembly of focal adhesion complex (12). It has been demonstrated that Src/FAK association enhances Src activity, and this is a prerequisite for tyrosine phosphorylation of other focal adhesion or cytoskeleton-associated proteins (13). These considerations led us to examine the role of the LMW-PTP tyrosine phosphorylation in relation to its subcellular targeting upon PDGF-induced mitogenesis in NIH3T3 cells.

We find that in NIH3T3 cells LMW-PTP is localized in both cytoplasmic and cytoskeleton-associated fractions also in the absence of PDGF treatment. 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. As a consequence of this phosphorylation, LMW-PTP greatly increases its catalytic activity probably toward cytoskeleton-associated Tyr-phosphorylated substrate(s). Furthermore we show that these two intracellular LMW-PTP pools exert their actions at different times starting from PDGF-R stimulation. Altogether these data are consistent with the hypothesis that LMW-PTP is subjected to different regulatory mechanisms and has different substrate specificity in consequence of its subcellular localization.

EXPERIMENTAL PROCEDURES

Materials—Unless specified all reagents were obtained from Sigma. NIH3T3 cells were purchased from ATCC; human recombinant platelet-derived growth factor BB (PDGF-BB) was from Peprotech; enhanced chemiluminescence kit was from Amersham Pharmacia Biotech; Src kinase assay kit and anti-Src antibodies were from Upstate Biotechnology Inc.; anti-PDGF receptor antibodies and PY20 and PY99 anti-phosphotyrosine antibodies were from Santa Cruz.

Cell Culture and Transfections—NIH3T3 cells were routinely cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum in 5% CO₂ humidified atmosphere. 10 μg of pSVT7PTPC12S (7) or pSVT7PTP (14) and 0.5 μg of pSV2neo, conferring neomycin resistance, were cotransfected in NIH3T3 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 pSV2neo alone. The clonal lines were screened for expression of the transfected genes by (a) Northern blot analysis and (b) enzyme-linked immunosorbent assay polyclonal anti-LMW-PTP rabbit antibodies, which do not cross-react with murine endogenous LMW-PTP.

10 μg of pSGT vector-base constructs overexpressing dominant negative c-Src (SrcK⁻) (kindly provided by Dr. S. Courtneidge) and 0.5 μg of pBABEpuro, conferring puromycin resistance were cotransfected in NIH3T3 cells or in wtLMW-PTP overexpressing cells, using the calcium phosphate method. Stable transfected clonal cell lines were isolated by selection with puromycin (1 mg/liter). The clonal lines were screened for expression of the transfected genes by anti-p60 Src monoclonal antibodies (Santa Cruz).

Immunoprecipitations and Western Blot Analysis—1 × 10⁶ 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. Freshly made pervanadate solution (50 mM sodium vanadate and 50 mM H₂O₂) was added to the cells at a final concentration of 0.1 mM 30 min before the cells were lysed. 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). Complete RIPA lysis buffer (cRIPA) is RIPA plus 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate. Lysates were clarified by centrifugation and immunoprecipitated for 4 h at 4 °C with 1 μg of the specific antibodies. Immune complexes were collected on protein A-Sepharose (Amersham Pharmacia Biotech), 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 first with specific antibodies and then with secondary antibodies conjugated with

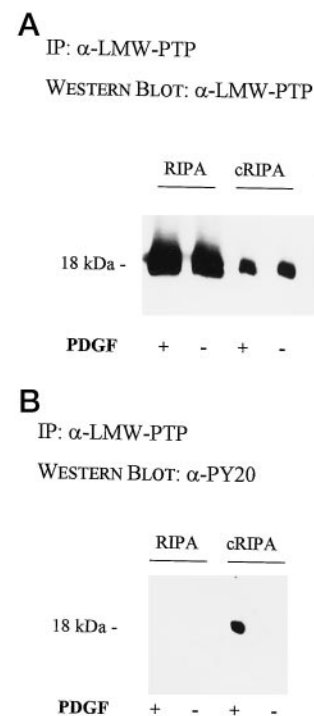


FIG. 1. *In vivo* LMW-PTP phosphorylation during PDGF stimulation is restricted to the cRIPA fraction. Cells, stimulated with 30 ng/ml of PDGF-BB for 5 min, were fractionated using RIPA and cRIPA buffers as reported under "Experimental Procedures." The samples were immunoprecipitated with anti-LMW-PTP polyclonal antibodies. *A*, anti-LMW-PTP immunoblot. *B*, the blot was stripped and reprobed with anti-phosphotyrosine (PY20) antibodies. The result is representative of three independent experiments.

horseradish peroxidase, washed, and developed with the enhanced chemiluminescence kit (Amersham Pharmacia Biotech).

Cell Lysates Fractionation—PDGF-stimulated NIH3T3 were lysed in RIPA buffer, and the lysates were clarified by centrifugation at 20,000 × g for 30 min. Pellets were washed twice with 1 ml of RIPA and then resuspended in cRIPA buffer by shaking for 1 h at room temperature and newly clarified by centrifugation at 20,000 × g for 30 min. RIPA or cRIPA fractions were then used for immunoprecipitation analysis.

RESULTS

Tyrosine-phosphorylated LMW-PTP Preferentially Associates with cRIPA-soluble Fraction—Subcellular localization of many enzymes, such as protein kinase C, Raf, Src, etc., is influenced by extracellular stimuli, and detergent phase partitioning is a commonly used separation method (15, 16). RIPA buffer is a commonly used lysis buffer containing non-ionic detergents such as Triton X-100 or Nonidet P-40. It is currently accepted that the RIPA-soluble fraction contains cytosolic and many of the plasma membrane structures. Caveolae, focal adhesions, and cytoskeleton-associated structures need additional detergents to be lysed. Caveolae are solubilized by octyl glucoside treatment, while cytoskeleton-associated structures are solubilized using stronger ionic detergent such as deoxycholate or SDS (16, 17). We have demonstrated previously that LMW-PTP is an enzyme that upon PDGF stimulation interacts with the activated PDGF receptor (7). To assess if LMW-PTP action is restricted to cytosolic soluble fraction or not, we have evaluated the relative amount LMW-PTP in RIPA and cRIPA fractions after PDGF stimulation. Serum-starved NIH3T3 cells overexpressing LMW-PTP were stimulated with 30 ng/ml of PDGF for 5 min, and the lysates were fractionated as described under "Experimental Procedures." Both RIPA and cRIPA-soluble fractions were used in immunoprecipitation assays with anti-LMW-PTP antibodies. Anti-LMW-PTP Western blot (Fig.

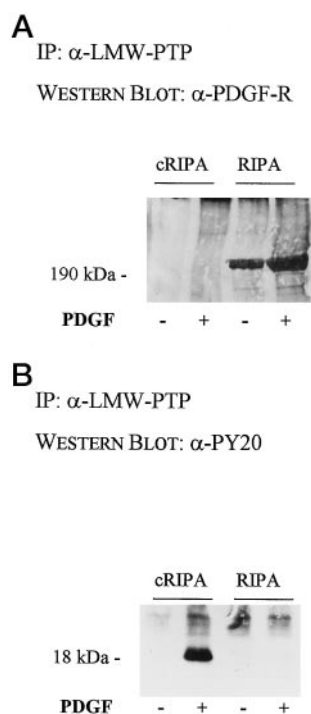


FIG. 2. LMW-PTP association with the activated PDGF receptor is restricted to the RIPA fraction. Cells stimulated with 30 ng/ml of PDGF-BB for 5 min were fractionated using RIPA and cRIPA buffers as reported under "Experimental Procedures." The samples were immunoprecipitated with anti-LMW-PTP polyclonal antibodies. *A*, anti-PDGF-R immunoblot of the samples loaded on a 7.5% SDS-PAGE. *B*, anti-phosphotyrosine immunoblot (PY20) of the same sample loaded on a 15% SDS-PAGE. The result is representative of three independent experiments.

1A) reveals that LMW-PTP resides in both fractions and that its relative amount is not dependent on growth factor administration. We reported previously that PDGF-R-associated LMW-PTP is not tyrosine-phosphorylated, neither in the resting state nor during activation of the receptor transduction pathway (7). Accordingly, the LMW-PTP in the RIPA-soluble fraction did not become tyrosine-phosphorylated in response to PDGF stimulation (Fig. 1*B*, left). Surprisingly, we found that in the same conditions the LMW-PTP associated to the cRIPA fraction becomes tyrosine-phosphorylated upon PDGF stimulation as indicated by reprobing the same Western blot with anti-phosphotyrosine antibodies (Fig. 1*B*, right).

The Association between LMW-PTP and the Activated PDGF Receptor Is Restricted to the RIPA-soluble Fraction—We demonstrated previously that LMW-PTP associates with the activated PDGF-R. This interaction leads to PDGF-R dephosphorylation and modulates specifically the Src and the STAT pathways of the mitogenic signaling (8). In Fig. 2*A* we report the analysis of the association between PDGF-R and LMW-PTP after subcellular fractionation with RIPA and cRIPA. In this experiment we have used NIH3T3 overexpressing dnLMW-PTP to maximize the interaction between the PDGF-R and the phosphatase, which occurs via the LMW-PTP catalytic site (7). Cells were PDGF-stimulated for 5 min. Lysates were then fractionated as indicated above and used in anti-LMW-PTP immunoprecipitation. The anti-PDGF-R immunoblot (Fig. 2*A*) reveals that PDGF-R coimmunoprecipitates with LMW-PTP only in the RIPA fraction, while it does not in the cRIPA fraction. The same samples were then checked for LMW-PTP tyrosine phosphorylation (Fig. 2*B*). Results clearly indicate that the interaction of LMW-PTP with the activated PDGF-R is not accompanied by LMW-PTP tyrosine phosphorylation, since

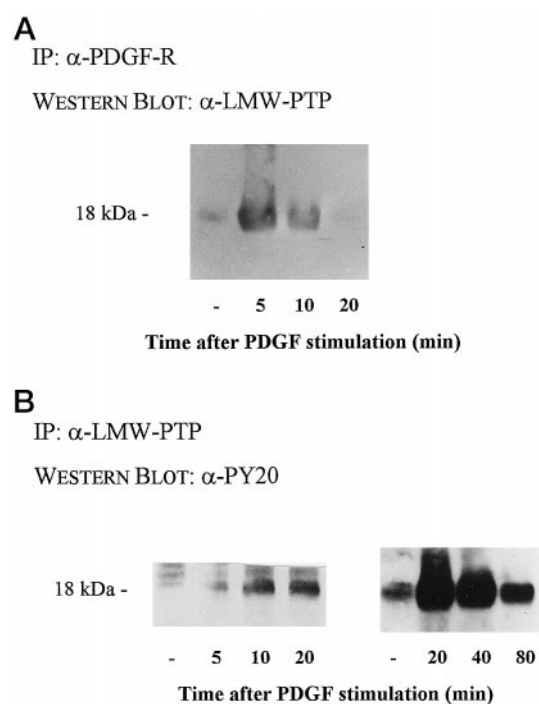


FIG. 3. Time courses of LMW-PTP association with PDGF-R and of LMW-PTP phosphorylation. dnLMW-PTP overexpressing cells, stimulated with 30 ng/ml of PDGF-BB for the indicated times, were fractionated using RIPA and cRIPA buffers as reported under "Experimental Procedures." The samples were immunoprecipitated with anti-LMW-PTP or anti-PDGF-R polyclonal antibodies. *A*, RIPA fractions were used for anti-PDGF-R immunoprecipitation, and the samples were analyzed by means of an anti-LMW-PTP Western blot. *B*, cRIPA fractions were used for anti-LMW-PTP immunoprecipitation. The samples were then analyzed by means of anti-phosphotyrosine immunoblot. The result is representative of three independent experiments.

this phenomenon is restricted to the cRIPA cytoskeleton-associated fraction.

Time Course Analysis of PDGF-R/LMW-PTP Association and LMW-PTP Tyrosine Phosphorylation—According to the results of the previous sections, the RIPA and cRIPA-soluble pools of LMW-PTP are spatially separated within the cell and hence could exert different functions in cell physiology. We have examined the time courses of both PDGF-R/LMW-PTP association and the LMW-PTP tyrosine phosphorylation. NIH3T3 cells overexpressing dnLMW-PTP were serum-starved for 24 h and then stimulated with 30 ng/ml of PDGF for 5, 10, or 20 min. For the association analysis cells were lysed using RIPA buffer and immunoprecipitated with anti-PDGF-R antibodies. The anti-LMW-PTP immunoblot (Fig. 3*A*) shows that the association between the two molecules is transient, reaching a maximum 5 min after PDGF treatment and then rapidly decreasing. In the phosphorylation analysis cells were lysed in cRIPA buffer, and the lysates were immunoprecipitated using anti-LMW-PTP antibodies. The anti-phosphotyrosine immunoblot, presented in Fig. 3*B*, shows that LMW-PTP tyrosine phosphorylation is a long lasting phenomenon, since it reaches a maximum about 10 min after PDGF stimulation and then slowly decreases.

In Vivo LMW-PTP Phosphorylation during PDGF Signaling Is Performed by c-Src—Previous reports showed that LMW-PTP is phosphorylated on tyrosine in cells overexpressing *v*-Src and in resting Jurkat T-cells (9, 10). To study the role of *c*-Src tyrosine kinase in LMW-PTP phosphorylation during PDGF signal transduction, we decided to use cells overexpressing both the dominant negative form of *c*-Src

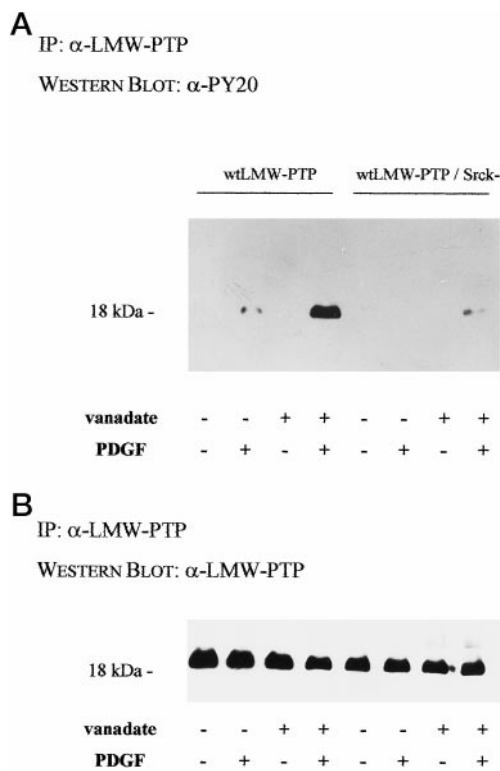


FIG. 4. LMW-PTP is tyrosine-phosphorylated by *c-Src* tyrosine kinase. NIH3T3 cells overexpressing both SrcK⁻ and wtLMW-PTP or LMW-PTP alone were treated with 0.1 mM pervanadate for 30 min and then with 30 ng/ml of PDGF-BB for 5 min. 0.5 mg of total proteins from cRIPA lysates were used for anti-LMW-PTP immunoprecipitation. *A*, anti-phosphotyrosine (PY) immunoblot. *B*, the same filter was stripped and reprobed with anti-LMW-PTP antibodies for normalization. The result is representative of three independent experiments.

(SrcK⁻) and the wtLMW-PTP. After PDGF stimulation, we have evaluated the LMW-PTP tyrosine phosphorylation level in SrcK⁻/wtLMW-PTP overexpressing cells compared with control cells overexpressing wtLMW-PTP alone. To increase the tyrosine phosphorylation level of LMW-PTP, both cell lines were pretreated with 1 mM pervanadate for 30 min before PDGF stimulation to inhibit phosphotyrosine phosphatases activity. Pervanadate is a specific and strong inhibitor of PTPs whose action leads to general increase of tyrosine phosphorylation level of many intracellular proteins (18). Cells were lysed with cRIPA and immunoprecipitated with anti-LMW-PTP antibodies. Results of the anti-phosphotyrosine immunoblot are shown in Fig. 4*A*. After PDGF stimulation, in SrcK⁻/wtLMW-PTP overexpressing cells we observe a lower LMW-PTP phosphorylation level with respect to cells overexpressing wtLMW-PTP alone, both in pervanadate-treated and untreated cells. In Fig. 4*B* we report, as control, the LMW-PTP content in the same sample. In a recent work we reported that SrcK⁻ expression does not affect PDGF-R functionality as indicated by the fact that, in these cells, the level of phosphorylation of PDGF-R is equal with respect to not transfected cells (19). SrcK⁻/wtLMW-PTP overexpressing cells contain a fully active PDGF-R while possessing a defective Src kinase activity. Hence, these data indicate a specific role of Src tyrosine kinase and not of PDGF-R in LMW-PTP tyrosine phosphorylation during PDGF stimulation.

LMW-PTP Associates Directly with *c-Src* in the cRIPA-soluble Fraction during PDGF Stimulation—To assess whether LMW-PTP phosphorylation is performed by *c-Src* itself or by a Src-dependent tyrosine kinase, we decided to investigate the

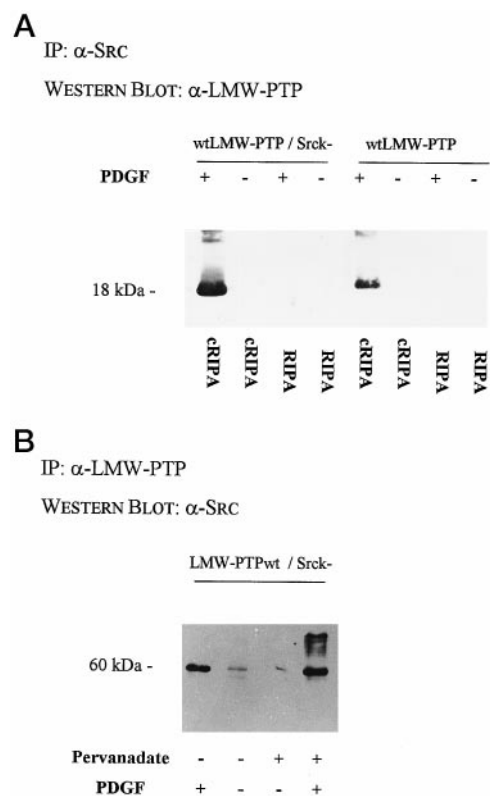


FIG. 5. LMW-PTP directly associates with *c-Src* tyrosine kinase in the cRIPA fraction. *A*, NIH3T3 cells overexpressing both SrcK⁻ and wtLMW-PTP were treated with 0.1 mM pervanadate for 30 min and then with 30 ng/ml of PDGF-BB for 5 min. Equal amounts of RIPA and cRIPA fractions were used for anti-*c-Src* immunoprecipitation. The anti-LMW-PTP immunoblot is presented. *B*, NIH3T3 cells were treated as above. cRIPA fractions were used for anti-LMW-PTP immunoprecipitation. The anti-Src immunoblot of the samples is presented. The result is representative of three independent experiments.

possible association between LMW-PTP and Src by means of coimmunoprecipitation analysis both in RIPA and cRIPA fractions. wtLMW-PTP and SrcK⁻/wtLMW-PTP overexpressing cells were serum-starved for 24 h and stimulated with PDGF for 5 min. Fig. 5*A* shows the anti-LMW-PTP immunoblot of the anti-Src immunoprecipitation of the fractionated lysates. Our results indicate that there is an association between LMW-PTP and *c-Src* both in SrcK⁻/wtLMW-PTP and wtLMW-PTP overexpressing cells, after PDGF stimulation. The LMW-PTP/*c-Src* association is restricted to the cRIPA fraction in agreement with our previous observation about the lack of interaction between LMW-PTP and *c-Src* in the cytosolic fraction during PDGF stimulation (8).

A similar experiment was performed pretreating SrcK⁻/wtLMW-PTP cells with pervanadate. The results shown in Fig. 5*B* indicate that LMW-PTP/Src interaction is independent from pervanadate administration, thus suggesting that the association between the two molecules is not mediated by LMW-PTP catalytic site.

LMW-PTP Acts Preferentially on a p200 Tyrosine-phosphorylated Protein in the cRIPA Fraction—Activated PDGF-R is a substrate of LMW-PTP only in the RIPA fraction, since there is not association between these two proteins in the cRIPA fraction (Fig. 2). To find possible substrate(s) of LMW-PTP in the cRIPA fraction, we analyzed the tyrosine phosphorylation level of the cytoskeleton-associated proteins upon PDGF stimulation. Analysis of cRIPA lysates was performed by means of anti-phosphotyrosine (PY20) immunoprecipitation and anti-phosphotyrosine (PY99) immunoblot in cells overexpressing

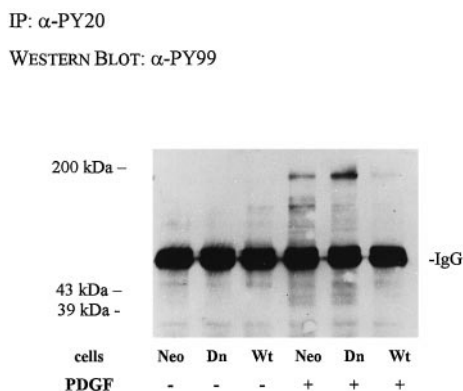


FIG. 6. LMW-PTP acts on a tyrosine-phosphorylated protein of about 200 kDa in the cytoskeleton-associated fraction NIH3T3 cells overexpressing wtLMW-PTP (*wt*) or dnLMW-PTP (*dn*), and mock-transfected cells (*neo*) were stimulated with PDGF-BB for 5 min. The cRIPA fractions were obtained as reported under "Experimental Procedures." Equalized amounts of proteins of the fractionated lysates were used for anti-phosphotyrosine immunoprecipitations using PY20 antibodies. The samples were divided in two aliquots and separately analyzed by means of anti-phosphotyrosine (PY99) and anti-PDGF-R immunoblot. The anti-phosphotyrosine immunoblot of the samples is presented. The approximate molecular mass of the bands of interest (see text) is indicated. The result is representative of three independent experiments.

wtLMW-PTP, dnLMW-PTP, and mock-transfected cells. This experiment (shown in Fig. 6) led us to the identification of a major differentially tyrosine-phosphorylated band with an apparent molecular mass of about 200 kDa and two minor bands of about 43 and 39 kDa. These proteins could represent possible LMW-PTP substrates in the cRIPA fraction. Since in PDGF-stimulated cells the principal tyrosine-phosphorylated protein in the 180–220 kDa range is PDGF-R itself, we reprobated the filter with anti-PDGF-R antibodies with negative results. Nevertheless these data suggest that LMW-PTP may have different substrates in distinct subcellular localization.

DISCUSSION

Our previous studies pointed out the role of LMW-PTP in the PDGF-induced mitogenesis. We have shown that LMW-PTP is a key intermediate in the early steps of PDGF-R signal transduction, because it binds directly to activated receptor and consequently modulates the activation of Src tyrosine kinase and the phosphorylation state of STAT proteins. We have also underlined that LMW-PTP action on PDGF signal transduction is specific, since this enzyme influences only these two signaling pathways. In fact, recent data from our laboratory have shown that in PDGF-stimulated NIH3T3 cells, *c*-Src overexpression leads to an up-regulation of both STAT1 and STAT3, hence the effect of LMW-PTP on STAT pathway could be a consequence of the concomitant action of LMW-PTP on Src activity (19). However, the interaction of LMW-PTP with the Src and the STATs pathways modulates *myc* and *fos* expression, two protooncogenes crucial for G_1 progression (8).

In this study we have identified, by means of subcellular fractionation, two different and constitutive LMW-PTP pools. The first one, that we called RIPA soluble, contains the cytoplasm and many of the plasma membrane structures. The second, the cRIPA-soluble fraction, contains cytoskeleton- and plasma membrane-associated structures such as caveolae, focal adhesion plaques, etc. We have established that the LMW-PTP associated with cRIPA fraction is tyrosine-phosphorylated upon PDGF stimulation, while cytosolic LMW-PTP is not (Fig. 1). The RIPA-soluble LMW-PTP pool is the only one that interacts with the activated PDGF-R, as indicated by our data in coimmunoprecipitation assay in RIPA and cRIPA fractions

(Fig. 2). In addition, no tyrosine phosphorylation is observed in PDGF-R-bound LMW-PTP, thus suggesting that LMW-PTP phosphorylation is not performed by PDGF-R. In agreement with our previously reported results (7), these data suggest that another tyrosine kinase must be implicated in this process. We have now shown that LMW-PTP tyrosine phosphorylation is performed by Src tyrosine kinase on the basis of two evidences. First, in cells overexpressing SrcK⁻, the dominant negative form of *c*-Src, which is catalytically inactive but still able to bind its substrates, we observed a dramatic reduction of LMW-PTP phosphorylation in response to PDGF with respect to control cells (Fig. 4). Second, *c*-Src directly interacts with LMW-PTP, as indicated by LMW-PTP/*c*-Src coimmunoprecipitation assays. Interestingly, this association is restricted to the cytoskeleton-associated LMW-PTP, and it is not present in the RIPA fraction, according to the observation that LMW-PTP is phosphorylated only in the cytoskeleton-associated fraction (Fig. 5). On the other hand, we demonstrated previously that the functionality of PDGF-R is not impaired in SrcK⁻ overexpressing cells, as indicated by tyrosine phosphorylation level of PDGF-R in SrcK⁻ cells in comparison with NIH3T3 control cells (19). Taken together these observations suggest that the difference in LMW-PTP tyrosine phosphorylation be mainly due to *c*-Src kinase activity. In addition, the association between these two molecules is independent of pervanadate, a potent competitive inhibitor of PTPs. These data support the hypothesis that the interaction between LMW-PTP and *c*-Src is not mediated by LMW-PTP catalytic site. On the contrary, we reported previously that in the case of the activated PDGF-R/LMW-PTP interaction in the RIPA fraction, the presence of the competitive inhibitor orthovanadate completely inhibits this association (20).

The *c*-Src protooncoprotein has been recognized to reside in both cellular adhesion plaques and within the plasma membrane during growth factor stimulation (21), although its cognate oncogenic counterpart *v*-Src has been found almost exclusively in adhesion plaques in transformed cells (11, 22). This fact accounts for the previously reported *v*-Src-directed LMW-PTP phosphorylation (9). In this report we show that the LMW-PTP tyrosine phosphorylation is a phenomenon not restricted to transformed cells, but occurs also in normal cells in response to PDGF stimulation.

Src tyrosine kinase has many different targets; one of them, the FAK tyrosine kinase, is active in focal adhesions. Activation of cell surface integrins and growth factor receptors lead to a rapid increase in tyrosine phosphorylation of FAK and of focal adhesion-associated proteins such as paxillin and tensin (23). The formation of a bipartite kinase between FAK and *c*-Src is an early and critical event in the formation of focal adhesion complexes during the activation of growth factors and integrins signaling pathways. Recent evidence indicated that the association of Src and FAK enhances Src activity, and this is a prerequisite for cytoskeleton-associated Src activation leading to the phosphorylation of proteins in focal adhesion plaques (13). Most likely, LMW-PTP is among these newly phosphorylated proteins. We have evidence that the phosphorylation of LMW-PTP performed *in vitro* by *c*-Src leads to an increase of about 25-fold in the LMW-PTP enzymatic activity.² Hence, after PDGF stimulation the two constitutive LMW-PTP pools possess very marked differences in enzymatic activity that could reflect distinct roles in signal transmission. Furthermore, we analyzed the duration of the two phenomena in which LMW-PTP is involved. LMW-PTP interaction with PDGF-R,

² P. Cirri, P. Chiarugi, L. Taddei, G. Raugeri, G. Camici, G. Manao, and G. Ramponi, submitted for publication.

restricted to the RIPA fraction, is transient and reaches a maximum 5 min after PDGF stimulation, while LMW-PTP phosphorylation in cytoskeleton-associated fraction is a long lasting phenomenon and decreases only after 40 min (Fig. 3). These temporal and spatial differences between the two LMW-PTP pools may reflect distinct substrate specificity and different roles of the two LMW-PTP pools in different subcellular localization. Finally, we have looked for tyrosine-phosphorylated proteins that could represent new LMW-PTP substrate in the cytoskeleton-associated fraction. Our results with wtLMW-PTP or dnLMW-PTP overexpressing cells in cRIPA fraction show mainly a protein of about 200 kDa, which is differentially phosphorylated in these cell lines (Fig. 6). We have found that this p200 is not PDGF-R, but we were not able to identify this protein that should be a cytoskeleton-associated substrate of LMW-PTP. Among proteins that: 1) have a molecular mass of about 200 kDa, 2) reside in the cytoskeleton fraction, and 3) become tyrosine-phosphorylated in response to PDGF, tensin, talin, p190GAP, and a yet unidentified p200 (24) are possible candidates to be a LMW-PTP substrate in the cRIPA fraction.

In conclusion, the role of LMW-PTP during the transduction of the PDGF mitogenic signaling appears more complex than the bare regulation of PDGF-R phosphorylation. LMW-PTP is constitutively distributed in distinct intracellular localization, most probably with different physiological implications. The cytosolic LMW-PTP fraction is recruited to the activated PDGF-R, which is a LMW-PTP-specific substrate. This is a short lasting event that does not lead to LMW-PTP tyrosine phosphorylation. On the other side, again in response to PDGF stimulation, the cytoskeleton-associated LMW-PTP pool becomes tyrosine-phosphorylated by c-Src. This event is instead a long lasting phenomenon, which leads to an increase in LMW-PTP catalytic activity and could be responsible for the dephosphorylation of cytoskeleton-associated proteins such as p200.

In conclusion, the two LMW-PTP pools, having distinct subcellular localization and showing different responses to PDGF stimulation, may play diverse roles in cell physiology.

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