PDGF receptor as a specific in vivo target for low M_r phosphotyrosine protein phosphatase

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Abstract **Low M_r phosphotyrosine protein phosphatase (LMW-PTP) is a 18 kDa cytosolic enzyme widely distributed in eukaryotic cells. LMW-PTP catalyses the hydrolysis of phosphotyrosine residues and overexpression of the enzyme in normal and transformed cells inhibits cell proliferation. Site directed mutagenesis, together with crystallographic studies, have contributed to clarify the catalytic mechanism, which involves the active site signature sequence $C_{12}XXXXXR_{18}$, a main feature of all PTPase family members. In order to identify the LMW-PTP substrate/s we have expressed in NIH-3T3 cells a catalytically inert Cys^{12} to Ser phosphatase mutant which has preserved its capacity for substrate binding. Overexpression of the mutant phosphatase leads to enhanced cell proliferation and serum induced mitogenesis, indicating that the mutation results in the production of a dominant negative protein. Analysis of mutant LMW-PTP expressing cells has enabled us to demonstrate an association between LMW-PTP and platelet derived growth factor receptor that appears to be highly specific. Our data suggest a catalytic action of LMW-PTP on the phosphorylated platelet derived growth factor receptor.**

Key words: Phosphotyrosine tyrosine phosphatase; PDGF-R; Negative dominant

1. Introduction

The phosphorylation that occurs on protein tyrosine residues plays a critical role in the regulation of various fundamental biological processes such as cell division, differentiation, and transformation [1]. Until recently, it was generally thought that protein tyrosine kinases (PTKs) play the main role in the regulation of protein phosphorylation levels, while phosphotyrosine protein phosphatases (PTPases) were considered to play 'housekeeping' roles, since they cause the dephosphorylation of tyrosine-phosphorylated proteins returning the kinase target substrates back to the basal levels. However, this point of view is now debated, since there are evidences that PTKs and PTPases cooperate in the control of cellular phosphorylation levels [2]. The main family of PTPases includes more than 40 different enzymes that have been cloned and characterized [3]. They share a common evolutionary conserved polypeptide stretch of about 250 residues that includes a strictly conserved CXXXXXR active site motif [3]. Both cysteine and arginine included in the motif are essential for catalysis. Dixon et al. [2,3] demonstrated that the active site cysteine forms a thiol phosphate transient intermediate during the catalytic process, whereas the arginine plays a role both in the substrate binding and in the stabilisation of the transition state. The active site motif folds in a three-dimensional phosphate binding site as demonstrated by X-ray crystal structure determined by Barford et al. [4] for human placenta PTP1B. On the other hand, the CXXXXXR motif is contained also in two additional phosphatase subfamilies, i.e. the dual specificity protein phosphatases and the low M_r protein phosphatase. The crystal structure of the LMW-PTP has been recently determined [5]. Although this enzyme shows no general homology with respect to the main PTPase family members, it forms a three-dimensionally folded phosphate binding loop that is structurally identical to that contained in the human placenta PTP1B and Yersinia PTPases, both included in the main PTPase family [4,6].

LMW-PTP is able to dephosphorylate phosphotyrosine containing protein in vitro [7]. Some interesting phenotype effects, due to the enzyme overexpression in normal and oncogene-transformed animal cells in culture, have been previously observed. In particular, we demonstrated that overexpression of a synthetic gene coding for the LMW-PTP causes a general decrease in PDGF stimulated thymidine incorporation and growth rate in both normal and oncogene-transformed cells, as well as the decrease in the ability of transformed cells to growth in soft-agar [8,9]. However, we think that the overexpression of an active enzyme in the cells can cause several perturbations of the cellular environment, such as possible unusual enzyme localisation and above all lack of substrate specificity, thus leading to 'pharmacological' effects. To overcome this point, it was decided to use, in similar experiment, a LMW-PTP mutant where Cys^{12} is replaced by Ser: we demonstrated that the mutation of this residue, included in the phosphate binding loop, causes the complete loss of catalytic activity, whereas the protein is still able to bind substrates [10,11,12].

Overexpression of this mutant in cultured animal cells could produce a dominant negative effect, since the overexpressed catalytically inactive enzyme could compete with the natural one for substrate binding, causing accumulation of unhydrolyzed tyrosine-phosphorylated LMW-PTP substrate(s). This could enable us to observe phenotype effects and to immunoprecipitate the catalytically inactive enzyme-substrate complexes.

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Abbreviations: LMW-PTP, low M_r phosphotyrosine protein phosphatase; PTPase, protein tyrosine phosphatase; PTK, protein tyrosine kinase; PDGF, platelet derived growth factor; EGF, epidermal growth factor; PDGF-R, platelet derived growth factor receptor; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum.

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On the other hand, we think that the limited overexpression of a catalytically inactive enzyme gives a lower perturbation of the cell, enabling us to identify LMW-PTP target protein substrate(s).

2. Materials and methods

2.1. Materials

Unless otherwise specified all reagents were obtained from Sigma. pSVT7 and pSV2neo eukaryotic expression vectors were gifts from G. Bensi (Biocine, Siena, Italy); restriction and modification enzymes were from Promega; Sequenase 2.0 DNA sequencing kit was from USBC. NIH-3T3 cells were purchased from ATCC; human recombinant platelet derived growth factor BB (PDGF-BB), human recombinant epidermal growth factor (EGF) and bovine insulin were from Peprotech; RC20 antiphosphotyrosine antibodies were from Affiniti; Enhanced Chemio-Luminescence Kit was from Amersham. [^{3}H]Thymidine was from NEN. Anti platelet derived growth factor receptor (PDGF-R) antibodies were from Santa Cruz.

2.2. Plasmid construction

The *Sph*I/*Sac*I fragment of pGEMPTPC12S [10] was subcloned in the *Eco*RI/*Sac*I sites of pSVT7 eukaryotic expression vector. The presence of C12S mutation was confirmed by DNA sequencing according to Sanger.

2.3. Antibodies generation

Antiserum to LMW-PTP was obtained by immunising rabbits with LMW-PTP, expressed as glutathione *S*-transferase fusion protein in *E. coli* with the use of pGEX-KT vector (Pharmacia). The product was purified by means of affinity chromatography and cleaved with bovine thrombin (Sigma) according to Taddei et al. [13].

2.4. Cell culture and transfections

NIH-3T3 cells were routinely cultured in DMEM additioned with 10% fetal calf serum (FCS) in 5% CO_2 humidified atmosphere. 10 μg pSVT7PTPC12S or pSVT7PTP and 0.5 μg pSV2neo (conferring neomycin resistance) were co-transfected in NIH-3T3 cells using the calcium phosphate method. Stable transfected clonal cell lines were isolated by selection in G418 (400 μg/ml). Control cell lines were obtained by transfection of 2 μg pSV2neo alone. The clonal lines were screened for expression of the transfected genes by: (a) Northern blot analysis; and (b) ELISA using polyclonal anti-LMW-PTP rabbit antibodies, which do not crossreact with murine endogenous NIH-3T3 LMW-PTP.

2.5. Growth kinetics experiments

Two independent LMW-C12SPTP overexpressing clones were chosen for growth-determination experiments; 2×10^4 cells of each clonal line were seeded in 24-multiwell plates, serum starved for 24 h and then 20% FCS was added to stimulate mitogenesis: 1 h pulses with 1 μCi/ml of [^{3}H]thymidine (NEN) were performed during a period of 24 h. Incorporation rate in serum-stimulated cells were normalised on the basis of cell number per well. Growth kinetics experiments were performed seeding 2×10^4 cells in 24-multiwell plates and counting them in triplicate at 24 h intervals for a total period of 72 h.

2.6. Immunoprecipitations and Western blot analysis

1×10^6 cells were seeded in 10 cm plates in DMEM supplemented with 10% FCS. Cells were serum starved for 24 h before receiving the appropriate mitogenesis stimulating agent (20% FCS, PDGF-BB 50 ng/ml, bovine insulin 300 nM or EGF 100 ng/ml) for 5 min at 37°C.

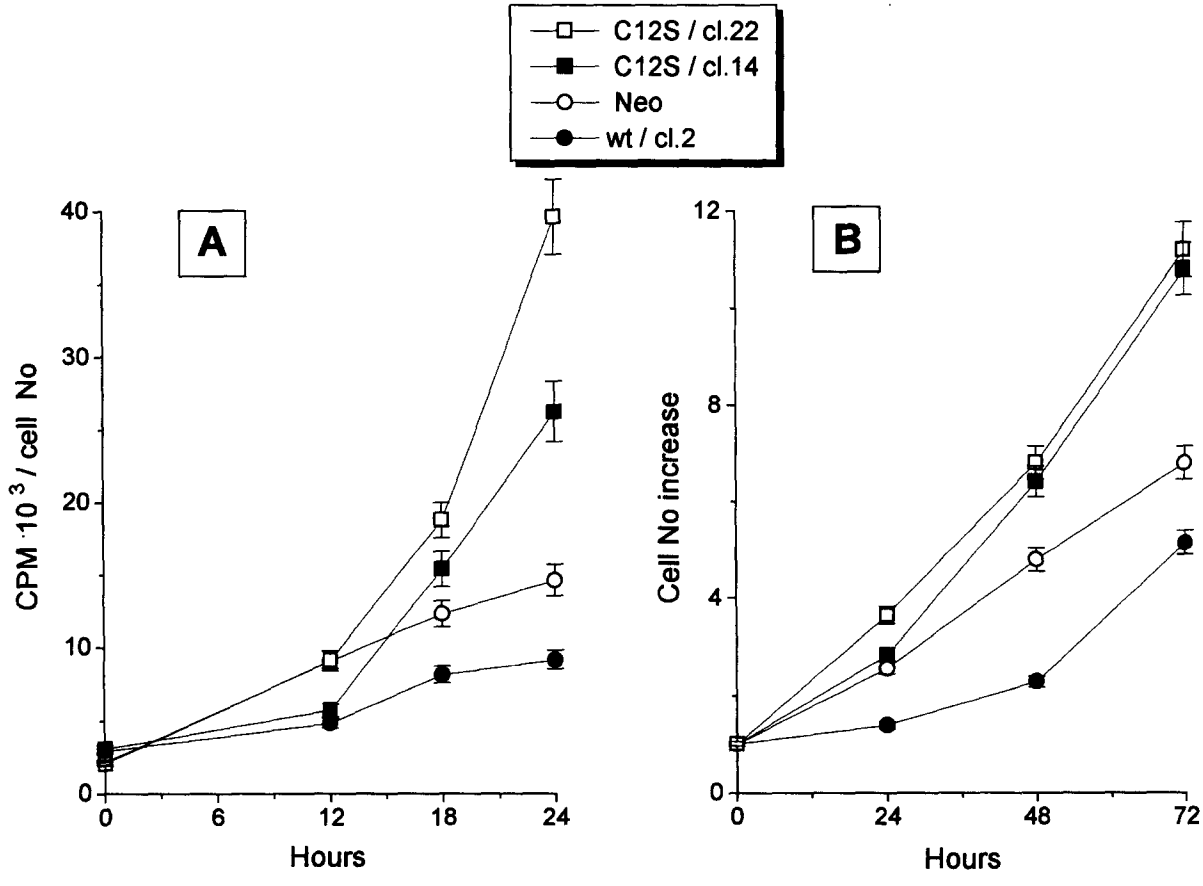

Fig. 1. Phenotypic analysis of NIH-3T3 transfected clones. (A) [^{3}H]Thymidine incorporation in NIH-3T3-C12SPTP, NIH-3T3-wtPTP and NIH-3T3neo transfected clones. 1 μCi/ml of [^{3}H]thymidine was added for 1 h to culture medium after 20% FCS refeeding of serum starved cells. Data obtained measuring [^{3}H]thymidine incorporation were normalised to the number of cells per well. (B) Growth kinetic analysis of NIH-3T3-C12SPTP, NIH-3T3-wtPTP and NIH-3T3neo transfected clones. Cells were seeded in DMEM supplemented with 10% FCS; exponentially growing populations were counted in triplicate at 24 h intervals. Data shown are relative to one experiment representative of three that gave qualitatively identical results; S.D. is indicated ($n = 4$).

Cells were then lysed for 20 min in ice in 700 μl of lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM EDTA, 10 mM sodium pyrophosphate, 0.1 mM sodium orthovanadate, 100 mM NaF, 1 mM phenylmethanesulphonylfluoride, 1 mg/ml bovine serum albumin, 10 μg/ml, aprotinin, 10 μg/ml leupeptin). Lysates were clarified by centrifugation and immunoprecipitated for 4 h at 4°C with 10 μg of polyclonal anti-LMW-PTP antibodies or 1 μg anti-PDGF receptor antibodies. Immune complexes were collected on protein G Sepharose (Pharmacia), separated by SDS/PAGE, and transferred onto nitrocellulose (Sartorius). Immunoblot was incubated in 1% bovine serum albumin in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1 mM sodium orthovanadate and 0.1% Tween-20, for 20 min at 37°C and then probed with 0.1 μg/ml of monoclonal RC20 anti-phosphotyrosine antibody (Affiniti) for 20 min at 37°C, washed three times in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA and 0.5% Tween-20 and developed with Enhanced Chemio-Luminescence kit (Amersham).

3. Results and discussion

3.1. Analysis of dominant negative phenotypic effects

LMW-PTP appears to be involved in the regulation of growth factor dependent mitogenesis showing a decrease of fetal calf serum and PDGF [14] stimulated DNA synthesis in wild-type LMW-PTP transfected cells (NIH-3T3-wtPTP). In order to assess whether the inactive phosphatase mutant (LMW-C12SPTP) was able to behave as a dominant negative enzyme, we have evaluated the ability of serum to stimulate DNA synthesis in transfected cells overexpressing the mutated LMW-PTP (NIH-3T3-C12SPTP). For this purpose we have selected several transfected clones and among them, by mean of ELISA, we have chosen two independent LMW-C12SPTP overexpressing clones, C12S-cl.14 and cl.22, together with a clone (wt-cl.2) overexpressing the wt-LMW-PTP. All these clones showed high levels of recombinant protein expression (data not shown), about 15-fold with respect to basal levels [14]. The two LMW-C12SPTP overexpressing clones showed a clear increase in DNA synthesis rate with respect to the control line (NIH-3T3neo) and in contrast to an evident decrease in the NIH-3T3-wtPTP clones, as demonstrated by [^{3}H]thymidine incorporation in serum-stimulated 24 h serum-starved cells (Fig. 1A). Furthermore, analysis of growth kinetics in asynchronous cells was performed in transfected clones during a period of 72 h, showing a net increase of cell number in NIH-3T3-C12SPTP-cl14 and cl.22 in comparison to control cell lines NIH-3T3neo and NIH-3T3-wtPTP (Fig. 1B). We emphasize that while overexpression of the active form of the enzyme within the cell might lead to pharmacological effects, due to the possible loss of substrate specificity and/or to a shift in intracellular location, the dominant negative approach engenders far less perturbation of the cell system, limiting itself to a variation of the level of specific substrate(s) of the enzyme. These considerations allow us to confirm that there is a stringent relationship between the observed phenotypic modification of NIH-3T3-C12SPTP cells and the interaction of LMW-C12SPTP with specific substrates.

3.2. Immunologic evidence for a LMW-PTP/PDGF receptor interaction

In order to find phosphorylated proteins, possibly associated to LMW-PTP, immunoprecipitation was performed on NIH-3T3-C12SPTP, NIH-3T3-wtPTP and NIH-3T3-neo cells using anti-LMW-PTP antibodies: after FCS stimulation, a 190 kDa protein was recognised by anti-phosphotyrosine antibodies only in the first two cell lines, but its amount was found to be several time higher in NIH-3T3-C12SPTP cells as compared to NIH-3T3-wtPTP cells (Fig. 2A). Because of the important role of tyrosine phosphorylation in the regulation of growth factor-dependent mitogenesis, we have tested the hypothesis that p190 could be a phosphorylated growth factor receptor. Serum starved NIH-3T3-C12SPTP cells were stimulated with three known NIH-3T3 mitogenic growth factors [15] such as EGF, PDGF and insulin. Fig. 2B shows that p190 is present in im-

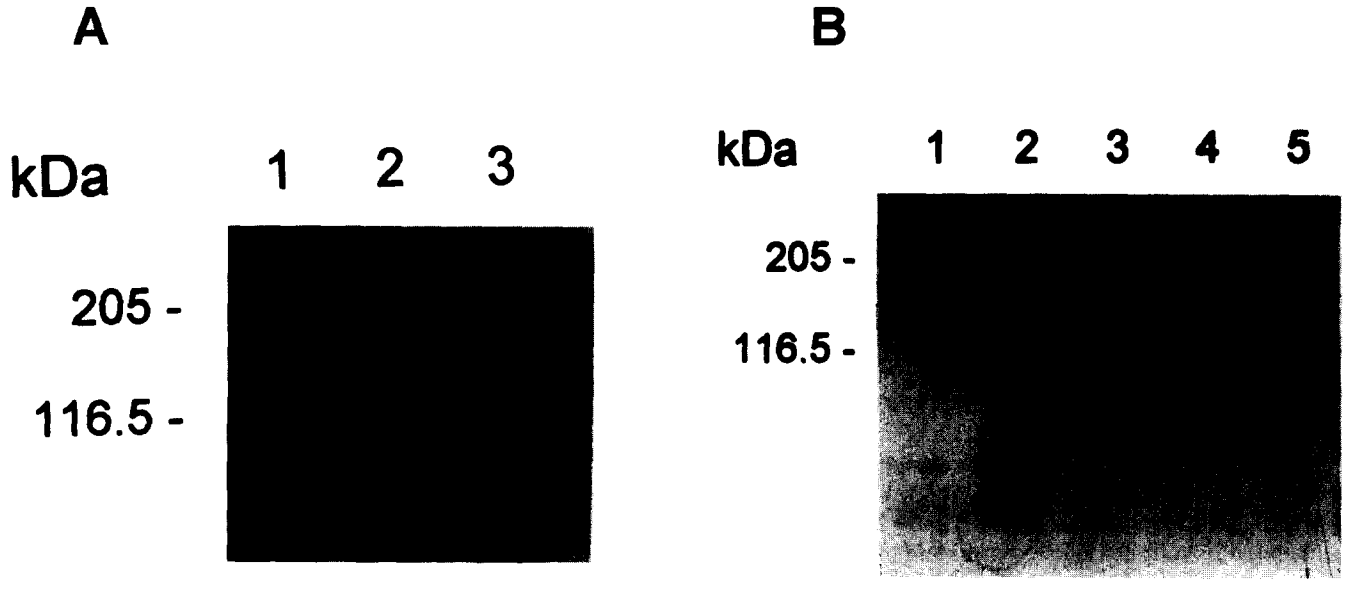

Fig. 2. Association of LMW-PTP with phosphotyrosine phosphorylated proteins: Lysates from 1×10^6 cells were immunoprecipitated with 10 μg of anti-LMW-PTP polyclonal antibodies. Immunoprecipitates were run on SDS-PAGE, transferred to nitrocellulose membrane and probed with antiphosphotyrosine antibodies. (A) Phosphotyrosine immunoblotting of 20% FCS stimulated NIH-3T3-C12SPTP (lane 1), NIH-3T3-wtPTP (lane 2) and NIH-3T3-neo cells (lane 3). (B) Phosphotyrosine immunoblotting of NIH-3T3-C12SPTP cells stimulated with different growth factors. Lane 1 = non-stimulated cells; lane 2 = PDGF-BB; lane 3 = EGF; lane 4 = insulin; lane 5 = A431, 170 kDa EGF-receptor overexpressing cells as a positive phosphotyrosine control.

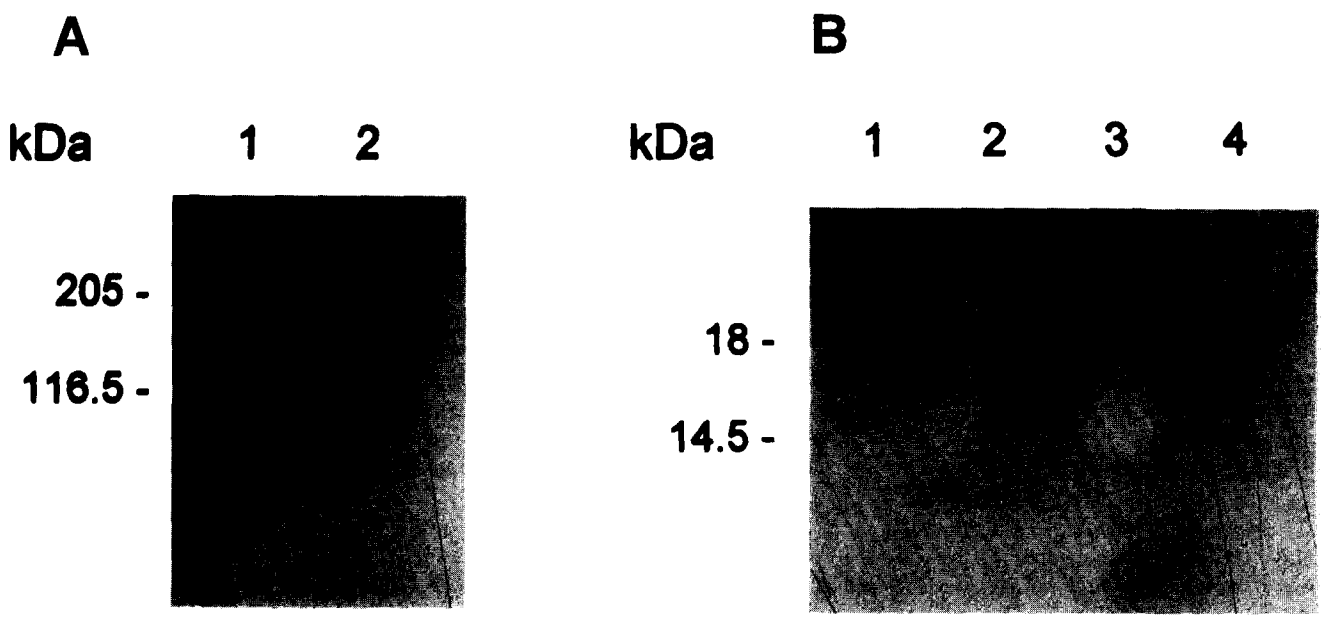

Fig. 3. Identification of p190 as PDGF receptor. (A) Lysates from 1×10^6 cells were immunoprecipitated with 10 μg of anti-LMW-PTP polyclonal antibodies. Immunoprecipitates were run on SDS-PAGE, transferred to nitrocellulose membrane and probed with anti PDGF-R polyclonal antibodies; lane 1 = NIH-3T3-C12SPTP; lane 2 = NIH-3T3-wtPTP. (B) In an experiment similar to that of panel A, cells were immunoprecipitated with 1 μg of anti-PDGF receptor antibodies and immunoprecipitates were probed with anti-LMW-PTP polyclonal antibodies; lanes 1 and 2 = unstimulated NIH-3T3-wtPTP and NIH-3T3-C12SPTP; lanes 3 and 4 = NIH-3T3-wtPTP and NIH-3T3-C12SPTP stimulated with 50 ng/ml of PDGF-BB.

munoprecipitate only after PDGF stimulation but after EGF or insulin treatments, as well as in non stimulated cells. These data led us to the hypothesis that p190 and PDGF receptor are identical.

Immunoprecipitates of NIH-3T3-C12SPTP and NIH-3T3-wtPTP cellular lysates with anti-PTPase antibodies were immunoblotted with anti-PDGR-R antibodies: the result (Fig. 3A) confirmed that p190 is actually PDGF-R. Furthermore, we immunoprecipitated lysates of PDGF stimulated/unstimulated NIH-3T3-C12 SPTP and NIH-3T3-wtPTP cells with anti-PDGF-R antibodies. Fig. 3B shows a Western blot of the immunoprecipitates revealed by anti- PTPase antibodies. The formation of LMW-PTP/PDGF-R complex was observed only in stimulated cells, in both NIH-3T3-C12SPTP and NIH-3T3-wtPTP, although in the former case the phenomenon was quantitatively higher. This difference in the amount of immunoprecipitate (lane 3 and 4) is not a consequence of different levels of overexpression, which resulted to be comparable in both NIH-3T3-C12SPTP and NIH-3T3-wtPTP cells as indicated by anti-LMW-PTP ELISA quantification (see previous section). This fact indicates that this phosphatase binds only to the activated receptor form. In NIH-3T3-C12SPTP cells the overexpressed inactive enzyme and the cellular wild-type enzyme compete for the natural substrate, resulting in a reduction of the hydrolysis rate of phosphorylated substrate, with its consequent concentration increase. Both Fig. 3A and B show that using NIH-3T3-C12SPTP cells the amount of immunoprecipitated complex is several times higher than in NIH-3T3-wtPTP cells, suggesting that the interaction only occurs via the catalytic site as also suggested by the lack of SH2 domains in the LMW-PTP. Hence, these experimental data indicate that PDGF-R is a specific in vivo LMW-PTP substrate.

The interaction of SH_2-domain-containing PTPase subfamily, such as PTP1C [16] and PTP1D [17,18] with activated PDGF-R can result in either an increase of their catalytic activity [19] or in a change of intracellular location mediated in both cases by PTPases tyrosine phosphorylation. Interestingly, phosphorylated PTP1D remains unable to dephosphorylate the activated receptor and, conversely, phosphorylated PTP1C displays a promiscuous activity on autophosphorylated PDGF, EGF, insulin and insulin-like receptors, showing no substrate specificity [19]. For this reason we investigated the possible LMW-PTP modification due to PDGF-R interaction. Immunoprecipitation with anti-LMW-PTP antibodies on cellular lysates from NIH-3T3-wtPTP cells and NIH-3T3-C12SPTP cells were analysed by antiphosphotyrosine immunoblotting with negative results in both samples: we can conclude that in the case of LMW-PTP, we found that association of LMW-PTP with PDGF-R was not correlated with LMW-PTP tyrosine phosphorylation (data not shown).

PDGF-R activation leads to tyrosine phosphorylation of several receptor substrates, such as: PLC-γ, PI-3kinase, PTP1D, Src, GAP, etc., [20]. Furthermore, protein interaction with PDGF-R can result in the positioning of an adaptor molecule (such as Grb2) leading to the activation of side signal pathways [21,22]. Finally, it is also possible that the generation of the mitogenic signal by PDGF-R is regulated via interactions modifying its kinase activity. While very little is known about PDGF-R direct inactivation, it recently appeared that ligand mediated endocytosis could be triggered by autophosphorylation of PDGF-R Tyr^{579} [23]. On the other hand, autophosphorylated receptor tyrosine kinases could be directly down-regulated by a simple dephosphorylation of specific phosphotyrosine residues, operated by a phosphotyrosine protein phosphatase, which is, in turn, recruited by the receptor itself [1]. Our data suggest that LMW-PTP tightly interacts with

activated PDGF-R. This interaction is apparantly performed via its catalytic site, acting on phosphotyrosine residues of the receptor cytoplasmic domain. This leads to receptor inactivation, thus reducing the mitogenic signal, which could be consistent with the phenotypic effects observed in wild-type and LMW-C12SPTP transfected cells, as well as in PDGF stimulated cells overexpressing the active enzyme [14]. Hence, LMW-PTP appears to be involved in the negative control of PDGF-R mediated signals, rather than in their amplification such as in the case of PTP1D [24,25]. Furthermore, specific dephosphorylation of one of the nine known phosphotyrosine residues in the activated receptor could result in the specific interruption of only one of the side signal pathways dependent on recruitment of SH_2-proteins following receptor activation.

These experiments demonstrate PDGF-R to be the first identified in vivo substrate of a member of the PTPase family. They also provide the first evidence of an alternative way to turn off the mitogenic signal via a Tyr-phosphorylated activated kinase cascade, promoted by a specific phosphatase directed dephosphorylation.

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