The low M_r phosphotyrosine protein phosphatase behaves differently when phosphorylated at Tyr[131] or Tyr[132] by Src kinase

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Abstract The low molecular weight phosphotyrosine protein phosphatase (LMW-PTP) is phosphorylated by Src and Src-related kinases both in vitro and in vivo; in Jurkat cells, and in NIH-3T3 cells, it becomes tyrosine-phosphorylated upon stimulation by PDGF. In this study we show that pp60[Src] phosphorylates in vitro the enzyme at two tyrosine residues, Tyr[131] and Tyr[132], previously indicated as the main phosphorylation sites of the enzyme, whereas phosphorylation by the PDGF-R kinase is much less effective and not specific. The effects of LMW-PTP phosphorylation at each tyrosine residue were investigated by using Tyr[131] and Tyr[132] mutants. We found that the phosphorylation at either residue has differing effects on the enzyme behaviour: Tyr[131] phosphorylation is followed by a strong (about 25-fold) increase of the enzyme specific activity, whereas phosphorylation at Tyr[132] leads to Grb2 recruitment. These differing effects are discussed on the light of the enzyme structure.

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Key words: Phosphotyrosine protein phosphatase; Low molecular weight PTP; PTP phosphorylation; PTP activation; Docking protein; Grb2; Src kinase

1. Introduction

Protein tyrosine phosphorylation is a crucial event in the regulation of cell proliferation, differentiation and chemotaxis [1–3]. Protein tyrosine phosphatases (PTPs) contribute to the protein phosphorylation balance by counteracting the role of protein tyrosine kinases. On the basis of structural and kinetic features, PTPs are currently grouped into four classes: tyrosine specific PTPs (receptor-like and soluble), VH1-like dual specificity PTPs, cdc25, and low molecular weight PTPs (LMW-PTP) displaying very limited sequence similarity, though sharing a common active site motif (CX_5R) and catalytic mechanism [4].

The LMW-PTPs are 18 kDa enzymes, widely expressed in animal tissues as two isoforms (IF1 and IF2) originating through alternative splicing, as well as in yeast and in prokaryotes [4]. We have previously demonstrated that the LMW-PTP is involved in mitogenic signal cascades triggered by several growth factors, such as insulin and PDGF [5,6]. In particular, the enzyme interacts with the activated PDGF receptor (PDGF-R) directly through its catalytic site, negatively affecting both Src and the STAT pathways and leading to down-regulation of immediately early genes such as c-*myc* and c-*fos* [6]. Recently, we have shown that the LMW-PTP resides constitutively both in the cytosolic and in the cytoskeleton-associated fractions; however, it is tyrosine-phosphorylated directly by Src only in the cytoskeletal fraction, while only the enzyme present in the cytosolic fraction is able to interact with the phosphorylated PDGF-R [7].

Previous results from our and other laboratories demonstrated that the LMW-PTP is phosphorylated in v-Src transformed NIH-3T3 cells and in resting Jurkatt cells at Tyr[131] and, to a lesser extent, at Tyr[132], thus becoming activated [8,9]. However, these data are not conclusive for what the effective specific activity of the phosphorylated enzyme is concerned. In addition, the possibility of differential effects following the phosphorylation of either tyrosine residue was not investigated. Therefore, we used Y131A and Y132A LMW-PTP mutants to study the effects of phosphorylation of each residue on enzyme behaviour. Furthermore, the finding that the residues following Tyr[132] (Y[132]-G[133]-N[134]-D[135]) conforms to the consensus motif recognised by the Grb2 SH2 domain [10] led us to investigate whether Grb2 was able to bind in vitro the phosphorylated recombinant LMW-PTP. The reported results are discussed in the light of the LMW-PTP three-dimensional structure.

2. Materials and methods

Unless otherwise specified, all reagents were obtained from Sigma; NIH-3T3 cells were purchased from ATCC; human recombinant PDGF-BB was from Peprotech; AG1296 was from ICN; Enhanced Chemi-Luminescence Kit was from Amersham; anti-pp60[Src] antibodies were from Upstate Biotechnology Inc.; anti-PDGF-R antibodies and PY20 anti-phosphotyrosine antibodies were from Santa Cruz; anti-Grb2 monoclonal antibodies (clone 81) were from Transduction Laboratories; Peptide synthesis reagents were from NovaBiochem (Switzerland); HATU was from PerSeptive Biosystems.

2.1. Site-directed mutagenesis
The recombinant Y131A, Y132A and Y131A-Y132A LMW-PTP (isoform 2) mutants were prepared as fusion proteins with GST by

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oligonucleotide-directed mutagenesis using an USE mutagenesis kit (Pharmacia) based upon the unique site elimination method [11]. Each mutation was confirmed by DNA sequencing.

2.2. Expression, purification and cleavage of GST fusion proteins

The nucleotide sequences of the wild-type and mutant bovine liver LMW-PTP were cloned in frame with the sequence of the glutathione *S*-transferase in the pGEX-2T bacterial expression vector. Enzyme expression and purification were achieved in the *Escherichia coli* TB1 strain as previously described [12].

2.3. Src and PDGF-R kinase assays

Phosphorylation of wild-type and mutant LMW-PTP was carried out as previously described [8]. Briefly, 5 μg of enzyme was dissolved in 40 μl of 25 mM HEPES, pH 7.5, containing 100 mM NaCl, 10 mM MgCl₂, 10 mM MnCl₂, 0.1% Nonidet P-40, 10% glycerol, and 50 μM ATP. The mixture was incubated with 5 units of Src either purchased from UBI or immunoprecipitated from c-Src overexpressing NIH-3T3 cells stimulated with 30 ng/ml PDGF-BB. Alternatively, the mixture was incubated with v-Src immunoprecipitated from v-Src overexpressing NIH-3T3 cells (kindly provided by Prof. V. Chiarugi) or in the presence of the activated PDGF-R immunoprecipitated from PDGF-stimulated NIH-3T3 cells. [γ-S]ATP was used in the non-radioactive phosphorylation assays to determine the wild-type or mutant LMW-PTP activity. The phosphorylation stoichiometry of the wild-type and mutant LMW-PTPs was calculated by a kinase assay carried out using [γ-^{32}P]ATP (10 μCi per sample) in the presence of 1 mM vanadate to prevent enzyme autodephosphorylation. The samples were incubated for 3 h at 30°C, resuspended in 4×SDS sample buffer and run in SDS-PAGE; finally, the dried gels were autoradiographed.

2.4. PTP assay

LMW-PTP activity was determined as previously reported using PNPP as a substrate [8]. Kinetic parameters were determined by using a non-linear fitting of the Michaelis equation (Fig. P program, Biosoft).

2.5. Immunoprecipitation and Western blot

1×10⁶ cells were seeded in 10 cm plates in DMEM supplemented with 10% FCS and serum starved for 24 h before receiving 30 ng/ml PDGF-BB. A freshly made 0.1 mM pervanadate solution (20 μl of a solution containing 50 mM sodium vanadate [NaVO₄] and 50 mM H₂O₂ in 10 ml of medium) was added to the cells 30 min before stimulation with PDGF. Then, cells were lysed for 20 min in 500 μl of ice-cold RIPA lysis buffer (50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 1% Nonidet P-40, 2 mM EGTA, 1 mM sodium orthovanadate, 1 mM PMFS, 10 μg/ml aprotinin, 10 μg/ml leupeptin), centrifuged and immunoprecipitated for 4 h at 4°C with 1 μg of the specific antibodies. Immune complexes were collected on protein A-Sepharose (Pharmacia), run in SDS-PAGE, and transferred onto nitrocellulose (Sartorius). Immunoblots were incubated for 1 h at room temperature in 10 mM Tris-HCl buffer, pH 7.5, containing 3.0% bovine serum albumin, 1 mM EDTA and 0.1% Tween-20, probed with specific antibodies followed by horseradish peroxidase-conjugated secondary antibodies, washed and developed with the Enhanced Chemi-Luminescence Kit (Amersham).

2.6. Peptide synthesis and immobilisation

Two 14-mer phosphopeptides (I-I-E-D-P-*PY*-Y-G-N-D-S-D-F-E and I-I-E-D-P-Y-*PY*-G-N-D-S-D-F-E) (*PY*^{131} and *PY*^{132}, respec-

tively) encompassing residues 126–139 in the LMW-PTP sequence were synthesised in solid phase by the 9-fluorenylmethoxycarbonyl (Fmoc) chemistry using a MilliGen 9050 PepSynthesizer according to the manufacturer's protocol. Phosphotyrosine was incorporated as Fmoc-Tyr(PO(OBzl)OH)-OH and coupled by HATU coupling reagent, all other residues were incorporated as pentafluorophenyl ester derivatives. Peptide deprotection and cleavage from the resin were performed by incubating each resin for 2 h at room temperature with a TFA/TIS/H₂O (90:5:5) mixture. The deprotected peptides were purified by reverse-phase HPLC on a C18 Vydac (20×250 mm) column and their purity assayed by amino acid analysis and mass spectrometry. The peptides, dissolved in coupling buffer (0.1 M NaHCO₃, pH 8.3, containing 0.5 M NaCl), were covalently coupled to NHS-activated Sepharose 4 Fast Flow beads (Pharmacia) by overnight incubation at 4°C. Then the free groups on the resin were blocked by 2 h incubation in 1.0 M Tris (pH 8.0). The coupling yield was monitored by reverse-phase HPLC; typically, 4.0 mg (about 2.3 μmol) of peptide/ml of beads was coupled.

2.7. Binding assay of Grb2 to the Tyr-phosphorylated peptides and LMW-PTP

1×10⁶ NIH-3T3 cells were seeded in 10 cm plates in DMEM supplemented with 10% FCS. Whole cells were lysed by incubation for 30 min in 1.0 ml of ice-cold RIPA buffer supplemented with protease and phosphatase inhibitors. The lysates were centrifuged and 0.3 ml of supernatant was incubated overnight at 4°C with about 50 μl of peptide-bound beads. Then the beads were collected by centrifugation, washed four times with the lysis buffer at room temperature and eluted with 100 μl of SDS-PAGE sample buffer. The eluted proteins were analysed by SDS-PAGE and transferred onto nitrocellulose (Sartorius). Anti-Grb2 Western blot was performed as described above.

Binding experiments between Grb2 and GST-LMW-PTP, either wild-type and Y131A or Y132A mutant, were carried out as follows: equal amounts (50 μg) of purified GST-LMW-PTP, either wild-type and mutant at Y131A or Y132A, were phosphorylated in vitro by Src (see above). After phosphorylation, complexes were recovered with glutathione-coupled agarose beads and incubated overnight with an NIH-3T3 cell lysate (see above) in the presence and in the absence of *PY*^{132} or *PY*^{131} (10 μM); each resin was washed with lysis buffer and the proteins, eluted with SDS sample buffer, were run in 12% SDS-PAGE and blotted onto nitrocellulose. Anti-Grb2 Western blot was performed as described above.

2.8. Computer graphics modeling

A model of the complex of LMW-PTP and GRB2 was constructed using the program Quanta. The structure of Grb2 was docked on to the LMW-PTP structure (PDB entry 1PHR) using the peptide bound to the Grb2 as a guide. The three different PDB files 1TZE, 1BM2, 1BMB, all containing a Grb2 SH2 domain with bound peptides, were used for the modeling. All three peptides contain a P-Tyr-X-Asn sequence. The peptides of the Grb2 structures were positioned so that phosphotyrosine and Asn side chains were superimposed with the side chains of Tyr^{132} and Asn^{134} of the LMW-PTP.

3. Results

Recent reports from our and other groups showed that Src is able to phosphorylate in vitro the LMW-PTP, which be-

Table 1
Kinetic parameters and activation upon tyrosine phosphorylation of the wild-type and mutant LMW-PTP

	Wild-type	Y131A	Y132A	Y131A-Y132A
k_{cat} (s^{-1})	30.0±0.81	12.6±0.42	20.7±0.53	11.6±0.52
K_m (mM)	0.19±0.01	8.45±0.21	0.42±0.01	8.2±0.81
k_{cat}/K_m (s^{-1} M^{-1}) 10³	157.89	1.49	49.32	1.41
Phosphorylation (%)	4.44	1.40	2.96	0
Actual activation upon phosphorylation (fold)[a]	18.00 (1.78)	1.00 (1.00)	25.00 (1.71)	–

The assays were performed in triplicate and repeated three times using PNPP as a substrate. The amount of tyrosine phosphorylation was evaluated using [γ-^{32}P]ATP in the kinase reaction; enzyme activation was assayed after 120 min incubation in the presence of c-Src and [γ-thio]ATP (see Section 2).

[a]Actual activation is the effective activation of each phosphorylated derivative with respect to the unphosphorylated form. In parentheses are reported the values of the apparent enzyme activation in the kinase reaction mixture with respect to the unphosphorylated counterpart.
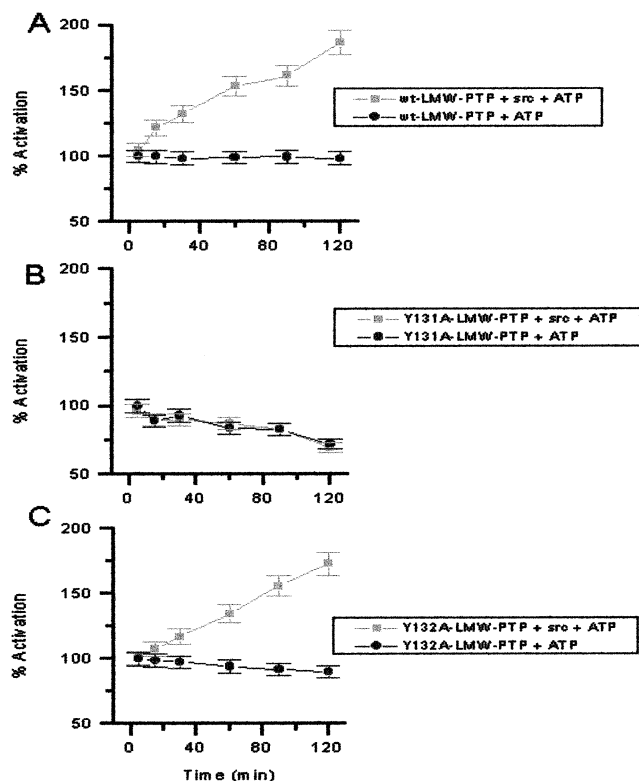
Fig. 1. Phosphorylation at Tyr[131] activates the LMW-PTP. In vitro phosphatase assays of the wild-type and mutant LMW-PTP phosphorylated by c-Src. Five μg of enzyme was incubated at 30°C with 5 I.U. of c-Src in the presence of [γ-thio]ATP. Four μl aliquots were assayed for enzyme activity at the indicated times using PNPP as a substrate. Percent of activation of the wild-type LMW-PTP and of the Y131A and Y132A mutants following phosphorylation by c-Src is reported in panels A, B, and C, respectively. Experiments were carried out in triplicate (S.D.s are indicated).

comes activated about two-fold [8,9]. To further investigate the molecular features of such phosphorylation, we used the Y131A, Y132A and Y131A-Y132A enzyme mutants. These mutants were phosphorylated in vitro by the activated PDGF receptor immunoprecipitated from stimulated NIH-3T3 cells, by v-Src or c-Src (see Section 2). The immunoprecipitated PDGF-R is able to phosphorylate the LMW-PTP, and this phosphorylation is strongly reduced in the presence of AG1296, a specific PDGF-R inhibitor belonging to the tyrphostin family [13] (not shown). The site(s) of LMW-PTP phosphorylation by the PDGF-R were investigated by using either the Y131A, the Y132A or the Y131A-Y132A mutant as a substrate; we found that the phosphorylation levels of all mutants were comparable to that of the wild-type enzyme, indicating that the phosphorylation by PDGF-R can be considered rather aspecific.

The LMW-PTP tyrosine phosphorylation by the viral and cellular Src tyrosine kinase was also investigated. We immunoprecipitated v-Src from subconfluent v-Src transformed NIH-3T3 and c-Src from NIH-3T3 cells serum starved for 24 h and stimulated for 5 min with PDGF-BB. Both v-Src and c-Src were able to phosphorylate in vitro the enzyme (not shown). In particular, on the basis of the specific [γ-^{32}P]ATP radioactivity and of the amount of protein used in the tests, we calculated a 4% and a 5% extent of tyrosine phosphorylation in the c-Src and v-Src kinase experiments, respectively;

these values are higher than in the presence of the PDGF-R kinase. The same in vitro kinase assay was carried out in the presence of each LMW-PTP mutant (Y131A, Y132A and Y131A-Y132A). The extent of Y131A and Y132A mutant phosphorylation by c-Src (very similar results were obtained in the presence of v-Src) was reduced by 3.2- and 1.5-fold respectively as compared to the wild-type enzyme, whereas the Y131A-Y132A mutant phosphorylation was almost undetectable (see Table 1). Therefore, the LMW-PTP phosphorylation by Src in vitro appears more effective (about 20-fold) and specific (restricted to only Tyr[131] and, to a lesser extent, Tyr[132]) with respect to the phosphorylation by the activated PDGF-R.

To check the effects of phosphorylation on the LMW-PTP activity, we previously determined the main kinetic parameters of the wild-type and mutant LMW-PTP (Table 1). An increase (about 45-fold) of the K_m values of the Y131A and Y131A-Y132A mutants and a decrease of the catalytic efficiency of Y131A mutant (about 100-fold) can be noted, indicating that Tyr[131] is important for the enzyme catalytic activity. This finding is not surprising, considering that this
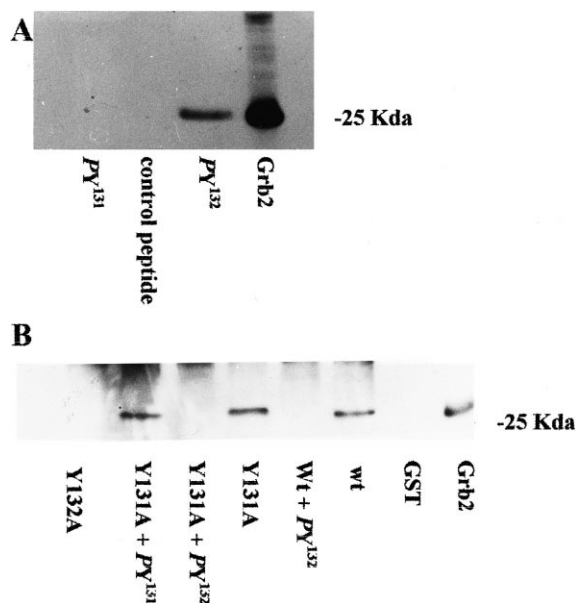

Fig. 2. Grb2 binds to a Tyr-phosphorylated peptide derived from the LMW-PTP sequence and to the wild-type and Y131A mutant enzymes phosphorylated by c-Src. Panel A: 10^6 NIH-3T3 cells were lysed and the supernatants were incubated with resin-bound synthetic peptides phosphorylated at Tyr[131] (lane 1), Tyr[132] (lane 3) or unphosphorylated (lane 2), as indicated in Section 2. Proteins bound to each resin peptide were eluted, run in SDS-PAGE and blotted onto nitrocellulose. The nitrocellulose was incubated with anti-Grb2 monoclonal antibodies (see Section 2). A PC12 cell lysate was used as a positive control (lane 4). Panel B: Y132A (lane 1), Y131A (lanes 2, 3 and 4) and wild-type (lanes 5 and 6) LMW-PTP-GST fusion proteins were phosphorylated in vitro by c-Src and recovered with glutathione-agarose beads (see Section 2). Then each resin protein was incubated with an NIH-3T3 cell lysate alone (lanes 1, 4 and 6), in the presence of PY[132] (lanes 3 and 5) or in the presence of PY[131] (lane 2); each resin was extensively washed and resin-bound proteins were eluted, run in 12% SDS-PAGE and transferred to a nitrocellulose sheet. The nitrocellulose was washed, saturated and incubated with anti-Grb2 antibodies. Immunopositive bands were revealed by incubation with the horseradish-conjugated secondary antibody (see Section 2). Lane 7, control of a cell lysate incubated with GST-agarose; lane 8, positive Grb2 control. Similar results were obtained in three separate experiments.
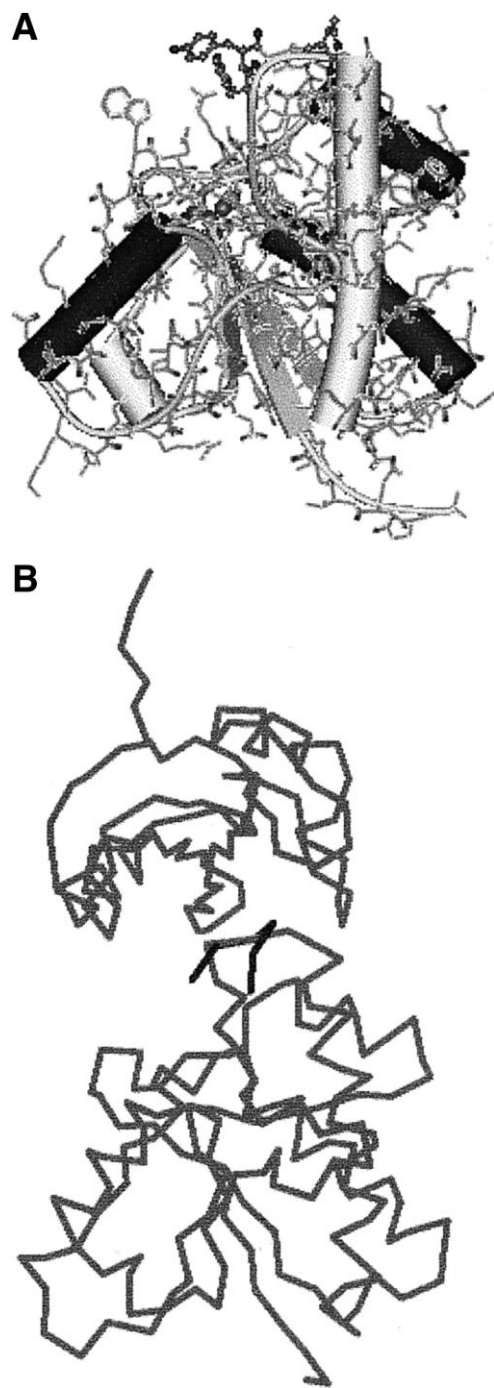
Fig. 3. Outline of the LMW-PTP IF2 structure. A: The positions and the relative orientations of the Tyr[131] and Tyr[132] and N134 side chains (ball and stick) are depicted (drawn by WebLab Wiever program). B: Molecular model of the complex between LMW-PTP and the Grb2 SH2 domain. The LMW-PTP (PDB entry 1PHR) (bottom) and Grb2 SH2 domain (PDB entry 1BM2) (top) are depicted together with the Grb2 binding peptide (in dark lines) (drawn by Molscript, Kraulis). The LMW-PTP orientation is the same in A and B.

residue is structurally close to the general acid/base catalyst of the reaction (Asp[129]). In contrast, the kinetic parameters of the Y132A mutant are very similar to those shown by the wild-type enzyme, suggesting a minor role of Tyr[132] in the catalytic machinery. To evaluate the overall specific activity of the enzyme phosphorylated at either Tyr[131] or Tyr[132], a

kinase assay in the presence of [γ-S]ATP was performed. Fig. 1A shows that, after 120 min incubation, the apparent activity of the wild-type enzyme rises to about 200%. Enzyme labelling with [γ-^{32}P]ATP allowed to estimate the amount of LMW-PTP that is phosphorylated within the same time interval (about 4%). On the basis of these data, we calculated an average 18-fold activation of the mixture of the wild-type LMW-PTP molecules phosphorylated at either Tyr[131] or Tyr[132] or unphosphorylated with respect to the unmodified enzyme (Table 1, left column). To analyse the relative contribution of both Tyr[131] and Tyr[132] to enzyme activation, the same phosphorylation experiments were performed using the Y131A and Y132A mutants. Tyr[132] phosphorylation has no effect on the enzyme activity (Fig. 1B), whereas only Tyr[131] phosphorylation is followed by enzyme activation (Fig. 1C). The phosphorylation stoichiometry and the extent of the mutant LMW-PTP activation upon phosphorylation are depicted in Table 1. While the Y131A mutant does not show any detectable activation, a sharp increase in enzymatic activity of the Y132A mutant (about 25-fold) can be noted. The higher activation of the Y132A mutant with respect to that calculated for the wild-type enzyme (about 18-fold) can be explained considering that the latter is phosphorylated also at Tyr[132], that does not contribute to enzyme activation.

Since the sequence C-terminal to Tyr[132] conforms to the motif specifically recognised by the Grb2 SH2 domain (Y-X-N) [10], the possibility that *P*Tyr[132] could be a recruitment site for such a docking protein was investigated by performing two binding experiments in vitro. The first experiment was carried out using three synthetic peptides encompassing the sequence around Tyr[131]-Tyr[132] differing from each other in the phosphate group position. The control peptide was not phosphorylated, whereas peptides *P*Y[131] and *P*Y[132] contained a phosphotyrosine residue corresponding, in the LMW-PTP sequence, to Tyr[131] or Tyr[132], respectively. The peptides were bound to NHS-activated Sepharose 4 Fast Flow beads (see Section 2). The resins containing the control *P*Y[131] and *P*Y[132] peptides were separately incubated with an NIH-3T3 cell lysate, washed and eluted; the eluted proteins were run in SDS-PAGE in duplicate. One slab was silver stained and the other slab was blotted onto nitrocellulose. The stained slab (not reported) showed a sharp 25 kDa band that was absent when the NIH-3T3 cell lysate was run through the resins containing the control peptide or the *P*Y[131] peptide. The Western blot analysis of the second slab showed that the 25 kDa protein was immunoreactive with anti-Grb2 antibodies (Fig. 2A), thus indicating the possibility of a specific interaction of this docking protein with the LMW-PTP phosphorylated at Tyr[132].

To assess this possibility, we performed a second binding experiment using GST-LMW-PTP, either wild-type and mutant at Tyr[131] or Tyr[132], phosphorylated by c-Src. The phosphorylated enzymes were captured with glutathione beads and incubated with NIH-3T3 lysates in the presence and in the absence of the *P*Y[132] or *P*Y[131] (see Section 2). Fig. 2B shows the results of the Western blot with anti-Grb2 antibodies. A 25 kDa protein immunoreactive with anti-Grb2 antibodies associated with the phosphorylated GST-LMW-PTP, either wild-type and mutant at Tyr[131] (lanes 6 and 4, respectively); the addition of *P*Y[132] strongly inhibited such a binding (lanes 5 and 3, respectively), whereas the Grb2/Y131A complex was not affected by the presence of *P*Y[131] (lane 2). Both the

Y132A mutant and the GST alone were not able to associate to Grb2 (lanes 5 and 7, respectively); the unphosphorylated enzyme did not bind Grb2 (data not shown). These results indicate that the LMW-PTP/Grb2 interaction is specifically relieved only by PY^{132}, thus confirming the data about the interaction between Grb2 and the phosphorylated peptides. They also demonstrate that the Grb2/phosphorylated LMW-PTP interaction is specific, depending on the presence, in the enzyme, of the typical Grb2 binding sequence (PY-G-N-D) including $PTyr^{132}$.

The molecular features of the interaction between Grb2 and the Tyr^{132}-phosphorylated LMW-PTP were further assessed by molecular modeling (see Section 2). Computer graphic models of possible complexes between LMW-PTP and Grb2 could be assembled in slightly different ways depending on which of the two available Grb2 peptide complexes was used (PDB file 1TZE and 1BM2, 1BMB). However, none of the models led to steric clashes between Grb2 and LMW-PTP, which supports the feasibility of an interaction of the Grb2 SH2 domain with the Tyr^{132}-phosphorylated enzyme (Fig. 3B).

4. Discussion

The aim of the present paper was to address the effects of phosphorylation at either Tyr^{131} or Tyr^{132} on the LMW-PTP behaviour by using mutants at these residues. All mutants, particularly the Y131A mutant, displayed significant differences in the main kinetic parameters with respect to the wild-type enzyme. Previously reported data [8,9,14] showed a phosphorylation of the enzyme by Src kinases and by the Eph receptor kinase and that the former is specifically targeted to Tyr^{131} and Tyr^{132} and is followed by enzyme activation. However, the possibility of a differential contribution of Tyr^{131} and Tyr^{132} phosphorylation in enzyme activation was not investigated, nor the effective activation of the phosphorylated enzyme. By using Y131A and Y132A mutants, we have shown that the specific activity of the LMW-PTP phosphorylated at Tyr^{131} increases by roughly 25-fold, whereas phosphorylation at Tyr^{132} leaves enzyme activity unchanged. These results strengthen the previously reported findings on the LMW-PTP phosphorylation in vivo by Src kinases [9], further indicating that such a modification does have some biological and functional significance.

In the mammalian LMW-PTPs (but not in the corresponding enzymes from yeasts and prokaryotes), Tyr^{132} is followed by the conserved sequence G-N-D, which conforms to the consensus motif for phosphotyrosine recognition by the Grb2 SH2 domain (PY-X-N-X) [10]. We have shown that Grb2 binds in vitro synthetic phosphopeptides derived from the LMW-PTP sequence around Tyr^{131}-Tyr^{132} and to the enzyme phosphorylated in vitro by Src at Tyr^{132}, indicating that this enzyme derivative is specifically recognised by Grb2 (most likely at the level of the SH2 domain). The physiological significance of the LMW-PTP/Grb2 interaction needs to be further addressed. For example, it could target the Tyr^{132}-phosphorylated enzyme to specific locations into the cell; in fact, the LMW-PTP phosphorylated by c-Src has recently been found in the cytoskeletal fraction whereas only the cytosolic fraction of the enzyme is able to interact with the activated PDGF-R [7].

The differing effects following LMW-PTP phosphorylation can tentatively be explained on the basis of the X-ray structural data of the LMW-PTP isoforms 1 and 2 [15,16], showing that both isoforms display very similar structures, particularly with regard to the position of the Tyr^{131}-Tyr^{132} cluster. However, it is worth noting that these structures refer to enzymes complexed with substrate analogs and hence could differ in some features with respect to that of the substrate-free enzyme (the Src kinase substrate in our experiments), by analogy with previously reported structural data on PTP1B and Yersinia PTPase. In fact, upon substrate binding, these enzymes undergo a conformational reorganisation involving the WpD loop containing the catalytic aspartic acid, which moves to its catalytically competent position into the active site [17–19]. Fig. 3A shows that the Tyr^{131} side chain lies on the border of the crevice harbouring the catalytic site, while the Tyr^{132} side chain stretches out from protein surface. It can also be seen that the Tyr^{132}-Asn^{134} sequence in the 114–136 loop is fully accessible. Therefore, it is conceivable that Tyr^{132} has a minor role in the enzyme kinetic behaviour, whereas its phosphorylation can generate a solvent exposed, fully accessible docking site suitable to specifically recruit SH2 containing proteins. Instead, phosphorylation at Tyr^{131} might affect the P-loop conformation leading to enzyme activation possibly by increasing the k_{cat} value for phosphoenzyme hydrolysis (the rate-limiting step of the catalytic mechanism) by analogy with the previously reported activation mechanism by cGMP [20]. It appears intriguing that the alternative phosphorylation of adjacent residues by the same kinase sharply modulates differing LMW-PTP functions such as enzyme activity and recruitment of SH2 proteins. Overall, from these and other previously reported data on the effect of cGMP, NO, and H_2O_2 on the LMW-PTP activity [20–22], a picture does emerge indicating the existence of a complex pattern of regulation of this enzyme.

Considering the close proximity of Tyr^{132} to the active site crevice, we can expect that the interaction of Grb2 with the phosphorylated LMW-PTP affects the access of the ligand binding surface and active site entrance which could lead to enzyme inactivation. In the model of the LMW-PTP/Grb2 complex (Fig. 3B), the enzyme active site appears not completely blocked and is still accessible for low molecular weight substrates such as phosphopeptides. However, it is clear that complex formation would block the access to the active site of high molecular weight substrates, such as phosphorylated proteins. These considerations lead to hypothesise that the phosphorylation state of either tyrosine residue can be considered as a molecular switch controlling the level of enzyme activity and/or the substrate specificity of the enzyme. It is known that SH2 domain PTPs such as SHP2 and PTPα are inhibited intramolecularly through a steric block of the catalytic cleft by the SH2 domain from the protein itself and that such a steric hindrance is relieved by the interaction with a peptide substrate [23]. A similar mechanism of intramolecular inhibition is shown by SH2 kinases such as the Src family enzymes. Therefore, when demonstrated, the modulation of the Tyr^{132}-phosphorylated enzyme bound to Grb2 could be considered as a variation of a most general strategy, leading to control the catalytic activity of such a single-domain PTP by an intermolecular (rather than intramolecular) SH2 domain interaction.

Recently, an accurate study on the phosphorylation of PTP1B on tyrosine has been reported [24]. PTP1B becomes

phosphorylated at Tyr[66] upon binding to the EGF receptor, and the specific activity of the enzyme mixture containing the phosphorylated derivative rises to about 300%. A 12% phosphorylation stoichiometry was found, leading to roughly calculate a 20-fold increase of the specific activity of the Tyr[66]-phosphorylated PTP1B, a value very similar to that calculated for the Tyr[131]-phosphorylated LMW-PTP (about 25-fold); in addition, the phosphorylated PTP1B recruits Grb2. However, in this case, catalytic activity enhancement and Grb2 binding are triggered by the phosphorylation of the same Tyr residue. Other PTPs, such as PTP1C and SHP-2 [25,26] are activated upon phosphorylation at specific tyrosine residues; in addition, a number of PTPs have been shown to interact with SH2 containing targets following tyrosine phosphorylation. Phosphorylation at Tyr[542] or Tyr[584] by the PDGF-R makes SHP-2 able to recruit the adapter protein Grb2 thus modulating downstream events such as the Ras pathway [27,28]; RPTPα phosphorylated at Tyr[789] recruits Grb2 [29]. Therefore, our data add to a number of previously reported findings, supporting the conclusion that phosphorylation at specific tyrosine residues is one of the main mechanisms by which many PTPs finely tune their catalytic activity as well as their ability to interact with other proteins.

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