Two Vicinal Cysteines Confer a Peculiar Redox Regulation to Low Molecular Weight Protein Tyrosine Phosphatase in Response to Platelet-derived Growth Factor Receptor Stimulation*

Low molecular weight protein tyrosine phosphatase (LMW-PTP) is an enzyme involved in platelet-derived growth factor (PDGF)-induced mitogenesis and cytoskeleton rearrangement because it is able to bind and dephosphorylate the activated receptor. LMW-PTP presents two cysteines in positions 12 and 17, both belonging to the catalytic pocket; this is a unique feature of LMW-PTP among all protein tyrosine phosphatases. Our previous results demonstrated that in vitro LMW-PTP is oxidized by either H$_2$O$_2$ or nitric oxide with the formation of a disulfide bond between Cys-12 and Cys-17. This oxidation leads to reversible enzyme inactivation because treatment with reductants permits catalytic activity rescue. In the present study we investigated the in vivo inactivation of LMW-PTP by either extracellularly or intracellularly generated H$_2$O$_2$, evaluating its action directly on its natural substrate, PDGF receptor. LMW-PTP is oxidized and inactivated by exogenous oxidative stress and recovers its activity after oxidant removal. LMW-PTP is oxidized also during PDGF signaling, very likely upon PDGF-induced H$_2$O$_2$ production, and recovers its activity within 40 min. Our results strongly suggest that reversibility of in vivo LMW-PTP oxidation is glutathione-dependent. In addition, we propose an intriguing and peculiar role of Cys-17 in the formation of a S-S intramolecular bond, which protects the catalytic Cys-12 from further and irreversible oxidation. On the basis of our results we propose that the presence of an additional cysteine near the catalytic cysteine could confer to LMW-PTP the ability to rapidly recover its activity and finely regulate PDGF receptor activation during both extracellularly and intracellularly generated oxidative stress.

Protein tyrosine phosphorylation plays a key role in the regulation of many cellular processes in eukaryotes such as cellular metabolism, proliferation, differentiation, and oncogenic transformation (1). Accumulating evidence indicates that the contribution of phosphotyrosine protein phosphatases (PTPs)\(^1\) to the control of the cell phosphorylation state is as relevant as that of phosphotyrosine protein kinases. The PTP superfamily is composed of over 70 enzymes that, despite very limited sequence similarity, share a common CX$_2$R active site motif and an identical catalytic mechanism. On the basis of their function, structure, and sequence, PTPs can be classified in four main families: 1) tyrosine-specific phosphatases, 2) VH1-like dual specificity PTPs, 3) the cdc25 phosphatases, and 4) the low molecular weight phosphatase (2).

The low molecular weight protein tyrosine phosphatase (LMW-PTP) is an 18-kDa enzyme that is expressed in many mammalian tissues (3). Our previous studies on the molecular biology of LMW-PTP in NIH3T3 cells demonstrated a well defined role of this enzyme in platelet-derived growth factor (PDGF)-induced mitogenesis. The most relevant phenotypic effect of LMW-PTP overexpression is a strong reduction of the cell growth rate in response to PDGF stimulation. We have previously shown that activated PDGF-R is a LMW-PTP substrate (4) and that LMW-PTP is involved in the control of specific pathways triggered by PDGF-R activation. In particular, LMW-PTP is able to modulate both myc expression, interfering with the Src pathway, and fos expression through a mitogen-activated protein kinase-independent pathway mediated by the signal transducers and activators of transcription (STAT) proteins (5). 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 (6). As a consequence of its phosphorylation, LMW-PTP shows an average 20-fold increase in its in vitro catalytic activity (7, 8, 9). 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 roles in PDGF-induced mitogenesis: while cytosolic LMW-PTP binds and dephosphorylates PDGF-R (4), thus modulating part of its signaling cascade, cytoskeleton-associated LMW-PTP acts on phosphorylated p190Rho-GAP, consequently play-
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Experimental Procedures

Materials—Unless specified all reagents were obtained from Sigma. NIH3T3 and C2C12 cells were purchased from ATCC, human recombinant PDGF-BB was from Peprotech, the Enhanced Chemi-Luminescence kit was from Amersham Pharmacia Biotech, all antibodies were from Santa Cruz Biochemicals, and BCA protein assay reagent was from Pierce. 5'-F-IAA and affinity-purified rabbit antibodies to fluorescein were obtained from Molecular Probes.

Site-specific Mutagenesis and Cloning of LMW-PTP Mutants in Eukaryotic Expression Vector—Oligonucleotide-directed mutagenesis was performed using the Unique Restriction Elimination Site kit from Amersham Pharmacia Biotech. The 26-base-long target mutagenesis primer contained an ACA codon (alanine) substituted for the original TGC codon (cysteine). The mutated LMW-PTP coding sequence was completely sequenced by the Sanger method and subcloned into the HindIII and Apal restriction sites of the pRCMV eukaryotic expression vector harboring the neomycin resistance gene.

Cell Culture and Transfections—NIH3T3 cells were routinely cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum in a 5% CO₂-humidified atmosphere. 10 μg of pRcCMV-wtLMW-PTP or pRcCMV-C17A-LMW-PTP were transfected in NIH3T3 cells using the calcium phosphate method. Stable transfected cell lines were isolated by selection with G418 (400 μg/ml). Control cell lines were obtained by transfecting 2 μg of pRcCMVneo alone. The clonal 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, which do not cross-react with murine endogenous LMW-PTP.

Immunoprecipitation 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. Cells were then lysed for 20 min on ice in 500 μl of RIPA lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, 1 mM sodium orthovanadate, 1 mM sodium vanadylsulfonate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin). Lysates were clarified by centrifugation and immunoprecipitated for 4 h at 4 °C with 0.1 μg of the specific antibodies. Immune complexes were collected on protein A-Sepharose (Amersham Pharmacia Biotech), separated by SDS-polyacrylamide gel electrophoresis, 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, then probed with secondary antibodies conjugated with horse-radish peroxidase, washed, and developed with the Enhanced Chemiluminescence kit.

Cell Lysate Fractions—Cell lysate fractions were obtained as already described (8). Briefly, PDGF-stimulated NIH3T3 cells were lysed in RIPA buffer, and the lysates were clarified by centrifugation for 30 min at 20,000 × g. Pellets were washed twice with 1 ml of RIPA and then resuspended in complete RIPA buffer (cRIPA), which is RIPA buffer plus 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate, by shaking for 1 h at room temperature and clarifying by centrifugation at 20,000 × g for 30 min. RIPA or cRIPA fractions were then used for immunoprecipitation analysis.

PTP Activity Assay—The PTP activity was measured as previously reported (7). Briefly, 1.5 × 10⁶ cells were collected in 300 μl of 0.1 mM sodium acetate, pH 5.5, 10 mM EDTA, 1 mM β-mercaptoethanol and sonicated for 10 s. The lysates were clarified by centrifugation, and 50 μl were used in the PTP activity assay with 50 μl of 10 mM para-nitrophenylphosphate, at 37 °C for 30 min. The production of p-nitrophenol was measured colorimetrically at 410 nm. The results were normalized to the basis of total protein content. Furthermore, PTP activity was measured in anti-LMW-PTP immunoprecipitates in 300 μl of 0.1 mM sodium acetate, pH 5.5, 10 mM EDTA, 1 mM β-mercaptoethanol at 37 °C for 15 and 45 min for wtLMW-PTP or C2C12 myoblasts, respectively.

In Vitro Labeling with 5'-F-IAA—Affinity-purified wtLMW-PTP was obtained using a procedure reported elsewhere (19). 1 μg of the purified enzyme was incubated in a final volume of 20 μl at 37 °C in 50 mM buffers of the indicated pH (sodium acetate for pH 5.5, MES for pH 6.5, and Tris-HCl for pH 7.5). H₂O₂ was added at the concentration of 0.2 or 2 mM, and the mixture was incubated for an additional 10 min at 37 °C. After the addition of 20 μM 5'-F-IAA from freshly prepared stock in dimethylformamide, the mixture was incubated for an additional 10 min. The labeling reaction was stopped by the addition of 2 × SDS sample buffer.

In Vivo 5'-F-IAA Labeling—Cells were lysed directly in RIPA buffer at pH 7.5, and 5'-F-IAA was added from freshly prepared stock to a final concentration of 5 μM. The lysates were maintained for 1 h at 4 °C for the labeling step and then were treated for immunoprecipitation with anti-fluorescein antibodies.

Measurement of Intracellular ROS—2 × 10⁶ cells were plated in 6-cm plates in standard culture medium, serum-starved for 24 h, and then stimulated with 30 ng/ml PDGF-BB. 200 μg/ml 2',7'-dichlorodihydrofluorescein diacetate, an oxidant-sensitive fluorescent dye, were added 5 min before analysis. Cells were then rapidly detached from the substrate by trypsinization and analyzed immediately by flow cytometry using a Becton Dickinson FACScan flow cytometer equipped with an argon laser lamp (FL-1, 488 nm; band pass filter, 530 nm).

RESULTS

Regulation of LMW-PTP Activity on PDGF Receptor during Extracellularly Generated Oxidative Stress—As already reported LMW-PTP is oxidized in vitro by H₂O₂ and NO (11, 12) in its catalytic site cysteines 12 and 17. To study if the generation of an oxidative stress in vivo could lead to LMW-PTP oxidation, thus influencing its catalytic activity, we have used the NIH3T3 fibroblast cell line overexpressing wtLMW-PTP. We have quantitated the LMW-PTP activity directly on activated PDGF-R because we previously reported that the tyrosine-phosphorylated receptor is an in vivo natural substrate for LMW-PTP (4). We immunoprecipitated the PDGF-R from cells and evaluated its tyrosine phosphorylation level by anti-phosphotyrosine immunoblotting as an indication of the LMW-PTP activity.
PTP in vivo activity. We exposed NIH3T3 cells, which were either overexpressing wtLMW-PTP or mock-transfected, to an exogenous oxidative stress generated by adding 100 milliunits/ml glucose oxidase (G/O) to the culture medium. It has been reported that after 10 min of treatment H\textsubscript{2}O\textsubscript{2} concentration reaches a steady state level of 30 \(\mu\text{M}\) (20). Fig. 1A shows the PDGF-R phosphorylation level in mock-transfected and wtLMW-PTP-expressing cells during G/O treatment. Dephosphorylation of agonist-activated PDGF-R by ectopically expressed wtLMW-PTP was almost completely blocked by the oxidative stress. The same results were obtained using 0.1 mM sodium pervanadate as the oxidant (Fig. 1B). It has been reported that although sodium orthovanadate is a competitive inhibitor for phosphotyrosine phosphatase, its oxidized form pervanadate is a strong oxidant of the PTPs active site cysteine (21).

We have previously demonstrated that LMW-PTP exists in two different cellular pools: a cytosolic pool, which is recruited to the membrane upon PDGF stimulation acting directly on PDGF-R, and a second pool anchored to the cytoskeleton that acts on a different substrate, p190Rho-GAP (10). LMW-PTP is thus able to influence cell growth through PDGF-R dephosphorylation and cytoskeleton rearrangement through Rho regulation. The results shown in Fig. 1C demonstrate that H\textsubscript{2}O\textsubscript{2} can down-regulate LMW-PTP activity on p190Rho-GAP as well as on PDGF-R, suggesting a general and strong action of oxidative stress on both cytosolic and cytoskeleton-associated LMW-PTP pools.

Furthermore we were interested in directly evaluating the influence of an exogenous oxidative stress on LMW-PTP activity, using a synthetic substrate, para-nitrophenylphosphate (PNPP), in an ex vivo assay. Fig. 1D shows the results obtained with phosphorylated PDGF-R and p190Rho-GAP. The PTP activity of wtLMW-PTP-expressing cells is strongly inhibited by H\textsubscript{2}O\textsubscript{2} treatment, confirming the results obtained with phosphorylated PDGF-R and p190Rho-GAP. Caselli et al. (11, 12) have demonstrated that LMW-PTP is oxidized in vitro by both H\textsubscript{2}O\textsubscript{2} and NO and is able to rescue its catalytic activity after treatment with reducing agents. It is interesting to note that further treatment with catalase for 10 min (to remove H\textsubscript{2}O\textsubscript{2}) led to a complete recovery of LMW-PTP activity (Fig. 1D), indicating that in the cell LMW-PTP is efficiently reduced again after removal of the oxidant.

To analyze whether the oxidized wtLMW-PTP is able to recover its catalytic activity in vivo, we evaluated the wtLMW-PTP activity on PDGF-R after the removal of the oxidative stress. After 10 min of G/O and PDGF treatment the produced H\textsubscript{2}O\textsubscript{2} was removed by adding 1 \(\mu\text{g/ml}\) catalase to the medium. The activity of wtLMW-PTP was quantitated by anti-phosphotyrosine immunoblotting of anti-PDGF-R immunoprecipitates as above. Band intensities were quantitated by analytical software, and the results are shown in Fig. 2A. Our data show that 20 min after H\textsubscript{2}O\textsubscript{2} removal the level of PDGF-R phosphorylation was comparable with that of the untreated control, suggesting that wtLMW-PTP is able to recover its catalytic activity on PDGF-R. Hence, in vivo the oxidation of LMW-PTP active site cysteines is followed by a reduction process, which permits the recovery of the activity of the phosphatase on PDGF-R. To
study the reactivation mechanism of oxidized LMW-PTP we analyzed the role of the redox cellular system based on reduced glutathione, which has been shown to be involved in the safety mechanism of various cell molecules (22). We reduced the availability of cellular reduced glutathione by using a strong depleting agent, diethyl maleate (DEM). NIH3T3 cells overexpressing wtLMW-PTP were incubated for various times with PDGF and then lysed in RIPA buffer, pH 7.0, containing 5 mM catalase was added for an additional 20 min. PDGF-R was immunoprecipitated, and anti-phosphotyrosine immunoblotting was performed. The plots show the data from densitometric analysis of the blots.

oxidation/inactivation of LMW-PTP during PDGF signaling is really due to ROS lead to the specific oxidation of Cys-12 and Cys-17 in the catalytic site with a low $pK_a$ (at pH 7.52 and 9.05 for iodoacetamide). Caselli et al. (11, 12) have demonstrated that H$_2$O$_2$ and NO lead to the specific oxidation of Cys-12 and Cys-17 in the catalytic pocket of LMW-PTP. In addition the active site cysteines of LMW-PTP, as in other PTPs, are specifically targeted by the sulfhydryl-modifying reagent iodoacetamide (25, 26). Recently an iodoacetamide-fluorescein (5'-F-IAA) labeling method of proteins containing low $pK_a$ cysteine residues has been reported (28), and we applied this method to study the oxidation state of LMW-PTP in vivo. We verified that in vitro the reaction of purified wtLMW-PTP with 5'-F-IAA was almost completely blocked by 0.2 mM H$_2$O$_2$ (Fig. 3A) at pH 6.5–7.5, suggesting a specificity of 5'-F-IAA for LMW-PTP active site reduced cysteines in these conditions.

To verify whether during PDGF signaling oxidized LMW-PTP becomes oxidized at the two catalytic cysteines, wtLMW-PTP-expressing cells were incubated for various times with PDGF and then lysed in RIPA buffer, pH 7.0, containing 5 mM catalase was added for an additional 20 min. PDGF-R was immunoprecipitated, and anti-phosphotyrosine immunoblotting was performed. The result is shown in Fig. 3B. LMW-PTP was strongly oxidized during PDGF signaling, reaching a maximum 10 min after stimulation with PDGF. The oxidation was followed by a new reduction, which reached the level of untreated cells 40 min after stimulation, suggesting that the oxidation/inactivation of LMW-PTP is transient and the enzyme is reactivated after the removal of the oxidants by endogenous mechanisms. The blot was reprobed with anti-LMW-PTP antibodies, and band intensities of both anti-fluorescein and anti-LMW-PTP immunoblots were quantitated. The ratio between these two values is reported in Fig. 3C as percentage of LMW-PTP reduction, allowing us to demonstrate that about 80% of LMW-PTP is already oxidized 2 min after the stimulation with PDGF and that oxidation becomes almost complete after 10 min. Moreover, within 40 min about 70% of LMW-PTP recovered the reduced state. Moreover, in a parallel experiment, we quantitated the LMW-PTP activity of immunoprecipitated LMW-PTP in PDGF-treated cells by using an enzymatic assay with PNPP as substrate. The results, reported in Fig. 3D, demonstrate that the oxidation of the phosphatase temporally coincided with enzyme inactivation while the re-reduction was concomitant with the rescue of catalytic activity. Interestingly, oxidation/inactivation of LMW-PTP reached the maximum 10 min after stimulation with PDGF, which was measured by 2',7'-dichlorodihydrofluorescein diacetate labeling (data not shown).

It has been reported that the H$_2$O$_2$ transient increase observed after growth factor stimulation is due to the activity of a nonphagocytic NADPH oxidase (29). To assess whether the oxidation of LMW-PTP during PDGF signaling is really due to...
endogenously produced H$_2$O$_2$, we used a strong inhibitor of the NADPH oxidase complex, diphenyl iodide (DPI). wtLMW-PTP-expressing cells were pretreated or not for 30 min with 10 $\mu$M DPI, and the oxidation of LMW-PTP was assayed by 5'-F-IAA labeling and anti-fluorescein immunoblotting as described above (Fig. 3E). In addition, DPI pretreatment caused a decrease of the PDGF receptor phosphorylation level in agreement with the absence of phosphatase inhibition (data not shown). The result demonstrates that the oxidation of LMW-PTP after PDGF treatment is impaired by the inhibition of NADPH oxidase by DPI, suggesting a central role of endogenously produced H$_2$O$_2$ in LMW-PTP oxidation.

To confirm the redox regulation of LMW-PTP during PDGF signaling, we assayed the redox state of endogenous phosphatase in NIH3T3 cells and in C2C12 myoblasts. Unfortunately the level of LMW-PTP in growing NIH3T3 cells is almost undetectable but rapidly increases when cells reach confluence (3).\(^2\) On the other hand, C2C12 mouse myoblasts naturally express a level of LMW-PTP higher than that in NIH3T3 cells. For these reasons, we have analyzed the redox state of endogenous LMW-PTP in PDGF-stimulated confluent NIH3T3 cells and in C2C12 cells. The oxidation of LMW-PTP was assayed by 5'-F-IAA labeling. LMW-PTP was then immunoprecipitated from lysates, and anti-fluorescein immunoblotting was performed. The result is shown in Fig. 4A. Endogenous LMW-PTP was strongly oxidized after PDGF treatment. Moreover, the oxidation was followed by a new reduction thereafter. These data confirm that the oxidation/inactivation of LMW-PTP (either endogenous or overexpressed) is transient, and the enzyme is reduced/reactivated after the removal of the oxidants.

In addition, in a parallel experiment we quantitated PTP activity in anti-LMW-PTP immunoprecipitates after PDGF treatment. Endogenous LMW-PTP from C2C12 cells was immunoprecipitated from PDGF-stimulated cells, and enzyme activity is shown in the plot of Fig. 4B. The data confirm that the oxidation of LMW-PTP during PDGF stimulation is followed by enzyme inactivation, while the re-reduction thereafter is accompanied by enzyme reactivation.

Furthermore, we studied the role of the reactivation of LMW-PTP during PDGF signaling. For this purpose, we blocked the

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\(^2\) P. Chiarugi, unpublished observations.
glutathione-dependent cellular system of oxidized protein reduction by using an inhibitor of the γ-glutamylcysteine synthetase, buthionine sulfoxide (BSO) (30). The cellular glutathione concentration was decreased to 90% by a 24-h pretreatment of NIH3T3 cells (data not shown). Mock-transfected and wtLMW-PTP-expressing NIH3T3 cells were pretreated or not for 16 h with 25 mM BSO, and the tyrosine phosphorylation level of PDGF-R was assayed by anti-phosphotyrosine immunoblotting. The results are shown in Fig. 5. In wtLMW-PTP-expressing cells the BSO treatment almost completely blocked the in vivo activity of the C17A mutant, whereas in mock-transfected cells the BSO treatment almost completely blocked the in vivo activity of the C17A mutant.

Role of Cys-17 in Rescue of LMW-PTP Activity on PDGF Receptor after Removal of Oxidative Stress—It has been reported both in vivo and in vitro that the oxidation of a sulfhydryl group to sulfenic acid (SOH) rapidly and spontaneously evolved into further oxidation products such as sulfenic acid and sulfonic (SO$_2$ and SO$_3$) (22). The milder oxidation to sulfenic acid of the catalytic site cysteine of a phosphatase is sufficient to inactivate the enzyme, although the reversibility of the reaction (i.e. the reduction to SH) is yet possible. The further oxidation states (SO$_2$ and SO$_3$) are probably terminal products that do not permit cellular reduction systems to operate on them, thus leading to inactive modified proteins (see Scheme 1, where cat. is catalytically). In this light, only a milder oxidation could be intended as a possible redox functional regulation.

| cat. active | cat. inactive | cat. inactive |
|------------|--------------|--------------|
| SH         | SOH          | SO$_2$       |
| reversible | irreversible | intramolecular disulfide |

It has been reported very recently that in vitro PTP1B is able to recover its catalytic activity after the removal of the oxidative stress by the thioredoxin system (14) or by the GSH system (15). The authors (14, 15) suggest that the cellular reduction system could play a central role in maintaining a low oxidation grade in PTP1B through the formation of a mixed disulfide between PTP1B and GSH. All members of the LMW-PTP subfamily have two vicinal cysteines positions 12 and 17, which are both in the catalytic pocket and totally conserved among the members of the subfamily. These two cysteines in vitro are able to form an intramolecular S–S bridge (12). We supposed that the redox functional regulation of this enzyme in vivo could be simply achieved by the formation of an intramolecular disulfide bond between Cys-12 and Cys-17. Mutations at the two cysteines had already been generated. Although the mutation in the Cys-12 totally inactivates the enzyme in vivo (4), the C17A mutant retains in vitro about 70% of its activity, suggesting a minor involvement of this residue in the catalytic mechanism (27, 28). To evaluate the in vitro activity of the C17A-LMW-PTP mutant, we transfected this mutant in NIH3T3 cells. First we demonstrated that C17A-LMW-PTP was active on phosphorylated PDGF-R. The results are reported in Fig. 6A. C17A-LMW-PTP retained almost 70% of the activity on the receptor with respect to wtLMW-PTP. This finding is in agreement with the activity of the C17A-LMW-PTP mutant measured in vitro on PNPP (32).
To study the role of the cysteine in position 17 on the oxidation/inactivation and reduction/reactivation processes of LMW-PTP, we treated NIH3T3 cells expressing wtLMW-PTP or C17A-LMW-PTP with PDGF and G/O for 10 min (10'), PDGF-R was immunoprecipitated. The anti-phosphotyrosine immunoblot is shown. Panel B, 1 × 10^6 NIH3T3 cells overexpressing wtLMW-PTP (wt) or C17A-LMW-PTP (C17A) were serum-starved for 24 h and then stimulated with 30 ng/ml PDGF-BB and 100 milliunits/ml G/O for 10 min. Panel C, cells were treated as in panel B, the medium was changed, and then 1 µg/ml of catalase was added for an additional 30 min. PDGF-R was immunoprecipitated, and anti-phosphotyrosine immunoblotting was performed. The histogram shows the data from the densitometric analysis of the blots. Panel D, cells were treated as in panels B and C, but fresh lysates were used for an in vitro PTP assay using PNPP as substrate. PTP-specific activity is shown in units/mg. C, control; G/OX, G/O; cat, catalase.

FIG. 6. Role of Cys-17 in the redox regulation of LMW-PTP during extracellularly generated oxidative stress. Panel A, 1 × 10^6 NIH3T3 cells overexpressing wtLMW-PTP or C17A-LMW-PTP were serum-starved for 24 h and then stimulated or not with 30 ng/ml PDGF-BB for 10 min (10'). PDGF-R was immunoprecipitated. The anti-phosphotyrosine immunoblot is shown. Panel B, 1 × 10^6 NIH3T3 cells overexpressing wtLMW-PTP (wt) or C17A-LMW-PTP (C17A) were serum-starved for 24 h and then stimulated with 30 ng/ml PDGF-BB and 100 milliunits/ml G/O for 10 min. Panel C, cells were treated as in panel B, the medium was changed, and then 1 µg/ml of catalase was added for an additional 30 min. PDGF-R was immunoprecipitated, and anti-phosphotyrosine immunoblotting was performed. The histogram shows the data from the densitometric analysis of the blots. Panel D, cells were treated as in panels B and C, but fresh lysates were used for an in vitro PTP assay using PNPP as substrate. PTP-specific activity is shown in units/mg. C, control; G/OX, G/O; cat, catalase.
by H₂O₂ production. To test this hypothesis, we analyzed the oxidation of inactivation/reactivation of LMW-PTP is achieved during stress.

C17A-LMW-PTP was inactivated by oxidation, as was wtLMW-PTP (although to a lower extent), but was completely unable to recover its enzymatic activity upon GSH-dependent reduction. This disulfide bond can be considered formed upon oxidation. This disulfide bond can be considered a protection system of LMW-PTP against further oxidation. On the basis of these data we propose that LMW-PTP is able to rescue its enzymatic activity upon GSH-dependent reduction with the extremely rapid diffusion of small oxidants such as H₂O₂ among cellular compartments, we observed a strong inhibition of LMW-PTP (via oxidation) on both activated PDGF-R and phosphorylated p190Rho-GAP.

The inactivation of LMW-PTP by oxidation is reversible in vitro upon reductant treatment with such agents as dithiothreitol (11, 12). Herein we show that LMW-PTP was able to rescue its catalytic activity in vivo on its substrate PDGF-R after the removal of the oxidative stress. The reversibility of oxidation and the consequent recovery of enzymatic activity are generally recognized as key points in the redox regulation of a protein. In fact, it has been widely discussed that protein oxidation should have two different meanings in cell behavior. Oxidation of the protein backbone or the simple oxidation of methionine and/or cysteine residues could lead to direct protein fragmentation or to irreversibly oxidized “dead” products (37). On the other hand, regulated oxidation of amino acid side chains and in particular of cysteine residues should be considered a reversible functional regulation of proteins because the oxidation might be reversed by the redox cellular systems (thioredoxin and GSH/glutaredoxin).

We proposed that the same Cys-17-mediated redox regulation of inactivation/reactivation of LMW-PTP is achieved during the PDGF signaling-dependent oxidative stress mediated by H₂O₂ production. To test this hypothesis, we analyzed the redox state of LMW-PTP by in vivo 5′-F-IAA labeling and anti-fluorescein immunoblotting after PDGF treatment of wt- and C17A-LMW-PTP-expressing cells (Fig. 7). The results show again that the position 17 was irrelevant in the oxidation of LMW-PTP after PDGF stimulation. On the contrary, Cys-17 was essential in the reduction process of LMW-PTP, which leads to enzyme reactivation.

On the basis of these data we propose that LMW-PTP is able to rescue its enzymatic activity upon GSH-dependent reduction of the disulfide bridge between Cys-12 and Cys-17 that is formed upon oxidation. This disulfide bond can be considered as a protection system of LMW-PTP against further oxidation. In fact, protection of the catalytic Cys-12 from oxidation states higher than sulfenic acid of the catalytic Cys-12 could be achieved either by GSH mixed disulfide or by an intramolecular Cys-12–Cys-17 disulfide. The impairment of oxidized C17A-LMW-PTP reduction (which still possess a functional Cys-12) excludes the possibility of a Cys-12–GSH mixed disulfide as a protection mechanism and supports our hypothesis of the formation of an intramolecular Cys-12–Cys-17 bridge.

**DISCUSSION**

On the basis of the growing evidence of the requirement of endogenously produced oxidants during growth factor signal transduction (13, 27, 28), we investigated in vivo the possible redox regulation of LMW-PTP by H₂O₂ during PDGF stimulation in NIH3T3 cells. It has been reported that the H₂O₂ produced upon growth factor stimulation is really a cellular second messenger directly influencing many signal transducers such as the STAT proteins, p70 S6k, protein kinase D, and PTPs (33–36). We have already demonstrated that LMW-PTP is a key regulator of PDGF-R phosphorylation upon recruitment to the membrane when cells are stimulated with the growth factor (5, 6, 10).

In this article we first show that LMW-PTP was oxidized in vivo by an exogenous oxidative stress such as H₂O₂ produced by G/O or sodium pervanadate. We have already demonstrated that in NIH3T3 cells LMW-PTP is active on phosphorylated PDGF-R and on p190Rho-GAP, thus influencing both mitosis rate and cytoskeleton rearrangement. Herein, we quantitated the in vivo activity of LMW-PTP directly on its natural phosphorylated substrates and demonstrate that the oxidation of LMW-PTP led to the inactivation of the enzyme preventing dephosphorylation of both PDGF-R (Fig. 2, A and B) and p190Rho-GAP (Fig. 2B). We have previously demonstrated that LMW-PTP is distributed in the cell in two different pools, a cytosolic pool, which acts on PDGF-R, and a cytoskeleton-associated pool, which is active on p190Rho-GAP (10). In agreement with the extremely rapid diffusion of small oxidants such as H₂O₂ among cellular compartments, we observed a strong inhibition of LMW-PTP (via oxidation) on both activated PDGF-R and phosphorylated p190Rho-GAP.

The inactivation of LMW-PTP by oxidation is reversible in vitro upon reductant treatment with such agents as dithiothreitol (11, 12). Herein we show that LMW-PTP was able to rescue its catalytic activity in vivo on its substrate PDGF-R after the removal of the oxidative stress. The reversibility of oxidation and the consequent recovery of enzymatic activity are generally recognized as key points in the redox regulation of a protein. In fact, it has been widely discussed that protein oxidation should have two different meanings in cell behavior. Oxidation of the protein backbone or the simple oxidation of methionine and/or cysteine residues could lead to direct protein fragmentation or to irreversibly oxidized “dead” products (37). On the other hand, regulated oxidation of amino acid side chains and in particular of cysteine residues should be considered a reversible functional regulation of proteins because the oxidation might be reversed by the redox cellular systems (thioredoxin and GSH/glutaredoxin). Herein, we show that the reduction process that permits the recovery of LMW-PTP activity was dependent on the cellular reduced glutathione content as indicated by the data obtained using BSO or DEM to deplete glutathione (Figs. 2 and 5). Thioredoxin/thioredoxin reductase/NADPH and glutaredoxin/glutathione/glutathione reductase/NADPH are the two major redox cellular systems (38). Our findings indicate that in vivo the LMW-PTP reactivation is under the control of the glutaredoxin/glutathione/glutathione reductase/NADPH system.

Moreover, we have shown that the redox regulation of LMW-PTP in vivo activity i) was active upon PDGF stimulation, ii) was performed by the endogenously produced H₂O₂, and iii) was required for LMW-PTP dephosphorylation of PDGF-R itself. In fact, we demonstrate that upon PDGF treatment almost 80% of LMW-PTP was oxidized and inhibited after 10 min and that after 45 min almost 70% of the phosphatase was reduced and had recovered its catalytic activity. Also in these conditions the reduction process was dependent on the availability of reduced glutathione because the reduction process was impaired by BSO treatment (Fig. 7). In addition, we have shown that this redox regulation was active also on the endogenous...
envelope and hence that was not a particular feature of overexpressed LMW-PTP.

It has been proposed that the transient increase of ROS observed in response to growth factor administration is due to the induced activation of a membrane NADPH oxidase complex (39). This event is dependent on the administration of PDGF and is probably under the control of the signal transduction pathway of phosphatidylinositol 3-kinase (40). We have shown that the oxidation of LMW-PTP was performed by an endogenously produced oxidative stress as indicated by the use of DPI, an inhibitor of the NADPH oxidase. This treatment clearly prevented the oxidation of LMW-PTP in response to PDGF (Fig. 3D).

Furthermore, our data suggest that the redox regulation of LMW-PTP during PDGF stimulation was required for PDGF-R dephosphorylation. In fact, the inhibition of the recovery of the LMW-PTP catalytic activity by reduction with glutathione-depleting agents such as BSO severely impaired the action of LMW-PTP on phosphorylated receptor. These data, together with the time-dependent oxidation/inactivation and reduction/reactivation of LMW-PTP upon PDGF stimulation, indicate a specific role of the inhibition of LMW-PTP during PDGF-R signal transduction. In fact, the phosphatase was oxidized/inactivated (up to 80%) at the very beginning of agonist stimulation and reduced/reactivated later when switching off of the receptor signal is required. Lee et al. 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35. Sun, X., Majumbder, P., Shyoia, H., Wu, F., Kumar, S., Weichselbaum, R., Kharbanda, S., and Kufe, D. (2000) J. Biol. Chem. 275, 17237–17240
36. Waldron, R. T., and Rozengurt, E. (2000) J. Biol. Chem. 275, 17114–17121
37. Berlett, B. S., and Stadtman, E. R. (1997) J. Biol. Chem. 272, 20313–20316
38. Mustacich, D., and Powis, G. (2000) Biochem. J. 346, 1–8
39. Pagano, P. J., Clark, J. K., Cifuentes-Pagano, J. K., Clark, S. M., Callis, J. M., and Quinn, M. T. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 14483–14488
40. Bae, Y. S., Sung, J., Kim, O., Kim, Y., Hur, K. C., Kazlauskas, A., and Rhee, S. G. (2000) J. Biol. Chem. 275, 10527–10531
41. Gross, S., Knebel, A., Tenev, T., Neininger, A., Gaestel, M., Herrlich, P., and Bohmer, F. (1999) J. Biol. Chem. 274, 26378–26386
Two Vicinal Cysteines Confer a Peculiar Redox Regulation to Low Molecular Weight Protein Tyrosine Phosphatase in Response to Platelet-derived Growth Factor Receptor Stimulation

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