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LOW MOLECULAR WEIGHT PROTEIN TYROSINE PHOSPHATASE (LMW-PTP) AND ITS POSSIBLE PHYSIOLOGICAL FUNCTIONS OF REDOX SIGNALING IN THE EYE LENS*

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Abstract

Low molecular weight protein tyrosine phosphatase (LMW-PTP) was cloned from human lens epithelial B3 cells (HLE B3) and the recombinant enzyme was purified to homogeneity. The pure enzyme reacted positively with anti-LMW-PTP antibody, displayed tyrosine-specific phosphatase activity and was extremely sensitive to H_2O_2. The inactivated LMW-PTP could be regenerated by thioltransferase (TTase)/GSH system as demonstrated by both activity assay and by mass spectrometry (MS). The MS study also showed that an intramolecular disulfide bond was formed between C13 and C18 at the active site, and was reduced by the TTase/GSH system. The putative role of LMW-PTP in regulating platelet derived growth factor (PDGF)-stimulated cell signaling was demonstrated in wild type mouse lens epithelial cells (LEC) in which LMW-PTP was transiently inactivated, corroborated with the transient phosphorylation of Tyr857 at the active site of PDGF receptor and the downstream signaling components of Akt and ERK1/2. In contrast, LMW-PTP activity in PDGF-stimulated LEC from TTase −/− mice was progressively lost, concomitant with the high basal and sustained high phosphorylation levels at Tyr857, Akt and ERK1/2. We conclude that the reversible LMW-PTP activity regulated by ROS-mediated oxidation and TTase/GSH reduction is the likely mechanism of redox signaling in lens epithelial cells.

Keywords

Low Molecular Weight Protein Tyrosine Phosphatase; signal transduction; protein S-thiolation; reactive oxygen species; lens epithelial cells

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Introduction

It is well-known that redox balance is essential for proper cellular function. The cell uses antioxidants such as glutathione (GSH), and oxidation defense enzymes such as catalase and glutathione peroxidase to keep the level of reactive oxygen species (ROS) low. There are also effective oxidation repair enzyme systems that can keep the protein thiols in the reduced state. One is the GSH-dependent thioltransferase (TTase, also known as glutaredoxin, or GRx) system that includes TTase, GSH and glutathione reductase [1] while the other is the NADPH-dependent thioredoxin (TRx) system that includes TRx, NADPH and thioredoxin reductase (TR) [2]. Although high ROS can cause various cellular damages, overwhelming evidence accumulated in recent years indicate that low level of ROS has a physiological function for controlling cell signaling, known as redox signaling. ROS are produced during growth factor or cytokine stimulation, allowing cell signaling to proceed for proper gene expression leading to cell proliferation, differentiation and other cellular functions [3]. The mechanism of redox signaling is not fully understood and the targets for ROS are also unclear.

Phosphorylation of tyrosine residues of intracellular proteins is one of the most important regulatory cell signaling mechanisms involved in cytokine or growth factor-stimulated cellular functions. Cellular protein phosphotyrosine levels are regulated by the antagonistic activities of two classes of enzymes, protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). Much work has been done on PTKs in the past but recently, more attention has been shifted to the understanding of PTP function.

PTP superfamily consists of classical tyrosine-specific phosphatases, dual specificity phosphatases, cdc25 phosphatases, and low molecular weight protein tyrosine phosphatase (LMW-PTP) [4]. PTPs share little homolog among each other except that they have the conserved catalytic sequences, including the absolutely conserved cysteine at the active site. The catalytic cysteine moieties have a low pKa value and under physiological conditions they are present as thiolate anions that are highly susceptible to oxidation. It has been shown that PTPs, including PTP1B, PTEN, cdc25B and LMW-PTP, can all be oxidized and inactivated by oxidants [5]. Oxidation of the catalytic cysteines blocks the capacity of these enzymes to dephosphorylate their targets, because catalysis is mediated by transferring of the phosphate moiety from the substrate to the catalytic cysteine followed by hydrolysis of that phosphate. Extensive evidences have suggested that PTPs are the direct targets of intracellular ROS and redox signaling generated during various growth factor stimulations for cell proliferation [6, 7].

In recent years, attention has been focused on LMW-PTP for its potential involvement in cell proliferation and migration. Studies [8] have shown that LMW-PTP could dephosphorylate the receptor protein tyrosine kinase (RPTK), focal adhesion kinase (FAK) and GTPase-activated protein Rho, all essential elements in the processes of cell proliferation and migration. LMW-PTP is an 18-kDa enzyme widely distributed in eukaryotic cells. Two isoforms were first discovered in human red blood cells originating through an alternative splicing mechanism [9], in which two exons (exon 3 or 4) are alternatively incorporated in the mRNA sequence. A third form with deletion in both 3 and 4 exons was later discovered [10].

Little is known about the nature of the protein tyrosine phosphatase in the lens. Blanquet and Croquet first detected PTPase activity in bovine lens [11] and observed an increased PTPase activity with aging of the lens [12]. Umeda et al. [13] purified an 18-kDa PTPase from the lenses of chick embryos and characterized it to be LMW-PTP. Recently, Rao, et al. [14] confirmed the expression of LMW-PTP in human lens and human lens epithelial cells. Here we report the cloning of LMW-PTP from human lens epithelial cells as three different splicing forms: LMW-PTP1, LMW-PTP2 and LMW-PTP3. Purified recombinant LMW-PTP2 was

characterized to be highly susceptible to oxidation, and the inactive enzyme could be partially restored by reduction with the TTase system in vitro. We further proved the putative role of LMW-PTP in mediating PDGF-signaling in mouse lens epithelial cells. We also provided evidence on the importance of TTase in regulating the function of LMW-PTP during PDGF signaling using primary epithelial cell culture from the lenses of TTase −/− mice and found that LMW-PTP function was compromised in comparison with that of the cells from TTase +/+ wild type mice.

Materials and Methods

NADPH, GSH, GSSG, iodoacetamide, dithiothreitol (DTT), p-nitrophenyl phosphate (pNPP), hydrogen peroxide, Dulbecco's phosphate buffered saline (PBS), cell culture medium (MEM, DMEM), fetal bovine serum, gentamicin were all from Sigma Chemical Co. (St. Louis, MO). Bicinchoninic acid (BCA) protein assay reagent and SuperSignal west chemiluminescent substrate were from Pierce (Rockford, IL). Agarose, *Taq* DNA polymerase, *Eco*RI, *Nde*I, and isopropyl-β-D-thiolgalactoside (IPTG) were all purchased from Gibco-BRL (Grand Island, NY). Eukaryotic TA cloning kit and cDNA cycle kit were from Invitrogen (Carlsbad, CA). QIAfilter plasmid mini kit and QIAquick gel extraction kit were obtained from Qiagen, Inc. (Valencia, CA). Bugbuster protein extraction reagent and pET system were from Novagen, Inc. (Madison, WI). Platelet-derived growth factor (PDGF) BB homodimer (human recombinant) were purchased from Calbiochem. (San Deigo, CA). Anti-human LMW-PTP antibody and IP ready protein A/G agarose suspension were from Exalpha Biologicals, Inc. (Watertown, MA). The PDGFR-β antibody was purchased from Upstate (Lake Placid, NY). The phospho-PDGFR-β (P-Tyr 857) polyclonal antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The phospho-Akt (P-Akt) and phospho-ERK1/2 (P-ERK1/2) monoclonal antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA). Western blotting luminal reagent and horseradish peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). His-Bind column was from Clontech Laboratories, Inc. (Palo Alto, CA). All other chemicals and reagents were of analytical grade.

Human Lens Cell Culture

Human lens epithelial cell lines, HLE B3 and SRA 01/04, were kindly provided by Dr. Uhsa Andley of Washington University and Dr. Venkat Reddy of University of Michigan, respectively. The B3 cells were grown and maintained in medium consisting of MEM supplemented with 20% FBS and 50 g/ml gentamicin in a humidified CO_2 incubator, while SRA 01/04 cells were cultured in DMEM supplemented with 15% FBS and 50 g/ml gentamicin. Medium was changed every four days. For H_2O_2-exposed experiments, cells were gradually deprived of serum in the medium by first incubating in 2% FBS overnight and then in serum-free medium for 30 minutes.

Mouse Lens Epithelial Primary Cell Culture

Primary lens epithelial cell (LEC) cultures were prepared from both wild-type (129SV) and $TTase^{-/-}$ mice. The $TTase^{-/-}$ mouse line has been developed following previous methods [15,16,17] and is available in our laboratory [18] Mouse lens capsules with attached epithelial layers were obtained under sterile conditions and placed in a 96-well plate containing 75 l of MEM medium (with 10% FBS and 50 g/ml gentamicin) per well. These tissues were incubated for 2–3 weeks in a humid atmosphere with 5% CO_2 at 37°C. Medium was changed every week, and the cell plate was observed under an inverted light microscope to detect cell growth. The primary LECs outgrew from the lens capsules were subdivided and plated at 5×10^5 cells in 60 mm plates.

PDGF-induced cell signaling in mouse lens cells

Cells were incubated in medium containing 1% FBS overnight and then in serum-free medium for 30 minutes before subjecting to PDGF stimulation. The cells were then harvested at 0, 15, 30 minutes for immunoblot analysis. Some cells were used for LMW-PTP immunoprecipitation by anti-LMW-PTP antibody followed by LMW-PTP activity assay.

Immunoblot analysis

Cells were washed two times with ice-cold PBS and lysed on plate with 180 l lysis buffer (containing 100 mM NaCl, 50 mM Tris-HCl, pH 7.4, 50 mM NaF, 5 mM EDTA, 20 M Na_3VO_4, 40 mM alpha-glycerophosphate, 6 g ml^{-1} chymostatin, 1 g ml^{-1} leupeptin, 1 g ml^{-1} pepstatin A, 1 mM PMSF and 30% M-Perzwïtterionic detergent) on ice and rotated for 15 minutes at 4°C. The supernatant of the cell lysate was collected after centrifuging at 13,000 rpm for 15 minutes at 4°C and stored at −80°C pending analysis. Protein concentration was determined by using BCA reagent [19].

Cell lysates containing equal amount of proteins were applied on 10% of SDS-PAGE and the resolved protein bands were transferred to Hybond™ ECL™ membrane and probed with antibodies against human LMW-PTP, PDGFRβ, phospho-PDGFRβ, phosphor-Akt or phosphor-ERK1/2. The corresponding protein bands were detected and visualized using a SuperSignal® west pico chemiluminescent substrate.

Immunoprecipitation and LMW-PTP activity assay

Cells were harvested in buffer (containing 50 mM Tris-HCl, pH7.5, 150 mM NaCl, 2mM EDTA, 1 mM PMSF, 1 g ml^{-1} Leupetin) and were sonicated on ice. Cell lysates were clarified by centrifugation. The supernatant of cell lysates containing 500 g total protein were immunoprecipitated for 4 hours at 4°C with 5 g antibody against human LMW-PTP. Immune complexes were collected on protein A/G agarose. Then PTP activity was measured in anti-LMW-PTP immunoprecipitates. The results were normalized on the basis of total protein content.

PTP Activity Assay

PTP activity was analyzed by using p-nitrophenyl phosphate (pNPP) as the substrate, following the published method [13]. In brief, the enzyme was assayed in a total volume of 0.9 ml, containing 0.1 M acetate buffer (pH 5.5) and 5 mM pNPP, and incubated for 30 minutes at 37° C. The reaction was terminated by adding 100l of 1N NaOH, and the absorbance of the reaction product (p-nitrophenol) was read at 405 nm in a spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the hydrolysis of 1 mol of pNPP per min under the described conditions.

Cloning of Human Lens Low Molecular Weight PTP (LMW-PTP)

Total RNA was isolated from HLE B3 cells. RT-PCR was performed under standard conditions, using primers designed based on human red blood cell acid phosphatase gene sequence (GenBank accession number NM_004300) and supplied by Integrated DNA Technologies, Inc. (Coralville, Iowa): 5′-GCC GCC ATG GCG GAA CAG GCT ACC-3′ as forward primer and 5′-AGA AAT GCA GGA CCT CAG G-3′ as reverse primer. PCR products were purified from agarose gel and cloned in pCR3.1 vector. Plasmid DNA was prepared and sequenced at the DNA Sequencing Facility (Iowa State University). The sequences were compared with published LMW-PTP sequences.

Overexpression and Purification of LMW-PTP2

Human lens LMW-PTP2 cDNA was cloned into expression vector pET23a(+) with His-tag in the C-terminal of recombinant protein. The resultant plasmid was transformed into BL21 (DE3). The positive clones were examined for fusion protein expression. When cell culture reached an optical density of 0.6, expression was induced by adding 0.4 mM IPTG. After 6 hours, the cell culture was harvested by centrifugation and cell pellet was resuspended in BugBuster reagent. After 10-minute incubation at room temperature, the suspension was centrifuged at 13,000 rpm for 15 minutes at 4°C and clear supernatant was saved for further purification. His-Bind column was used to purify His-tag LMW-PTP2 according to manufacture's protocol. The eluate from the column was dialyzed in 0.1 mM Tris-HCl buffer (pH7.5) containing 150 mM NaCl and 2 mM EDTA. The resultant supernatant was checked for PTP activity, run on 10% SDS-PAGE and subjected to immunoblot analysis.

Inactivation of purified LMW-PTP2

As purified LMW-PTP2 lost its activity spontaneously during purification, it is necessary to reactivate LMW-PTP2 with DTT before conducting the inactivation experiments with various LMW-PTP inhibitors or oxidants. To reactivate LMW-PTP2, the purified enzyme was incubated with 10 mM DTT at 30°C for 30 minutes in 0.1 M Tris-HCl buffer, pH7.5. Excess DTT was removed using PD-10 column and the eluate containing LMW-PTP2 was used immediately for various experiment. To test the effect of inhibitors or oxidants on enzyme activity, the experiment was performed at 25°C for 30 minutes in 0.1 M Tris-HCl buffer, pH7.5. The incubation mixture (60 l) contained 1 g of DTT-reactivated LMW-PTP2 and various inhibitors or oxidants. The residual activity was immediately assayed as described above.

Regeneration of spontaneously inactivated LMW-PTP2

The regeneration experiment of oxidized LMW-PTP2 was performed at 30°C for 30 minutes in 0.1 M Tris-HCl buffer, pH 7.5. The incubation mixture (60 l) contained 1 g of inactive LMW-PTP2 and regenerating agent of GSH (10 mM) and thioltransferase (1.5 M). Ten mM DTT was also used under same conditions and served as positive control. PTP activity was immediately assayed as described above.

Determining oxidation state of cysteines in LMW-PTP2 using Nano-Liquid Chromatography Mass Spectrometry-Mass Spectrometric (LCMS MS) Analysis

LMW-PTP2 alone, LMWPTP2 with thioltransferase (1.5 M) plus GSH (10 mM), or LMW-PTP2 with DTT (10 mM) was incubated at 30°C for 60 minutes. Then all of these protein samples were treated with iodoacetamide (40 mM) in the presence of guanidine-HCl (6M) to alkylate free or reduced cysteines. After salt was removed with ZipTip C_4 (Millipore), protein was digested with trypsin 50:1 (protein:trypsin) at 37°C for overnight. The digested samples were then injected using FAMOS autosampler into LC Packings C18 PepMap100, 75 m × 15 cm, 3 m column. Tryptic peptides were then separated using a gradient elution consisting of water (0.3% Formic Acid in H_2O) as mobile phase A and acetonitrile (0.3% Formic Acid in Acetonitrile) as mobile phase B. The gradient was begin at 10% B, followed by linear increase to 15% B in 10 minutes, followed by linear increase to 40% B in 30 minutes and then to 80% B in 10 minutes.

The column is then washed with 80% B for 10 minutes before returning to initial conditions. A flow rate of 170 nano-Liter/min was generated using Ultimate LC Packing Systems. The MS/MS analysis of tryptic peptides was performed using quadrupole time of flight mass spectrometer (Q-Star XL, Applied Biosystems Inc.) equipped with nano-ESI source. The parent ions for tryptic peptides were scanned in positive ion mode from 250–1250 amu (atomic mass units) in 1s. Information dependent acquisition (IDA) was performed using Analyst QS

software. The IDA criteria was set to perform collisionally activated dissociation (CAD) fragmentation (MS/MS) on all the parent ions containing 2–4 charge state with area count above 60 cps (count per second). The daughter ions generated from the fragmentation were accumulated for 4 s and scanned from 50–1900 amu (atomic mass units). The resolution of masses for this experiment was about 13000 and the mass accuracy was about 5 ppm. The observed masses for peptides were both verified by mass accuracy as well as MS/MS fragmentation pattern. The observed multiply charged masses of peptides generated by electrospray ionization source (ESI) are converted to molecular masses of peptides using Baysian Peptide Reconstruct option in Bioanalyst 1.15 software. The parent ion mass of the tryptic peptides, its daughter ion and the modifications were searched by in-house MASCOT search engine.

Effect of H_2O_2 on cellular PTP activity in HLE B3 cells

HLE B3 cell was cultured as described above. For H_2O_2 treatment, cells (3.2×10^6) were plated into 100-cm plate and were gradually deprived of serum before exposing to a bolus of H_2O_2 (0.1 mM). Cells were harvested and lysed in 0.1M Tris-HCl buffer (pH7.5) containing NaCl, EDTA, and protease inhibitors. After centrifugation at 13,000 rpm for 10 minutes, clear supernatant was analyzed for total PTP activity. For LMW-PTP activity assay, the enzyme was immunoprecipitated from the cell lysates and then measured PTP activity following the method described above.

Results

Detection and cloning of low molecular weight PTP (LMW-PTP) from Human lens epithelial cells

Human LMW-PTP is known to present in two isoforms originating by an alternative splicing process of a common primary transcript of a single gene. To examine its presence in human lens epithelial cells, we performed both immunoblot analysis and RT-PCR experiments. LMW-PTP protein was detected as one single band by anti-human LMW-PTP polyclonal antibody in both human lens epithelial cell lines, B3 and SRA 01/04 cells (Figure 1A). We chose to use B3 cell line for rest of our studies since many biochemical parameters of the B3 cells have been well-established.

LMW-PTP cDNA was amplified from HLE B3 cells using RT-PCR and visualized as two major bands on the agarose gel (Figure 1B). Both DNA sequence and deduced amino acid sequence (Figure 1C) revealed that there were three isoforms of LMW-PTP present in B3 cells (Figure 1D). All three isoforms, designated as LMW-PTP1, LMW-PTP2 and LMW-PTP3, contained the same active site CLGNICR. The isoform 1 & 2 included a different variant domain (either exon 3 or exon 4) as observed previously [20]. The third isoform, LMW-PTP3, lacked the variant domain and thus both its cDNA and deduced protein were smaller than the other two [10].

Purification and characterization of human lens LMW-PTP2

To obtain a large quantity of LMW-PTP for the in vitro studies, human lens LMW-PTP2, which was identical to human RBC acid phosphatase HCPTP-B, was overexpressed in *E. coli* and purified. LMW-PTP2 was first cloned into pET23a(+) vector with C-terminal His-tag and then this His-tag LMW-PTP2 was purified to homogeneity. As shown in Figure 2A, the purified LMW-PTP2 is visualized as a single band with expected size of 18 kDa on SDS-PAGE. It was positively reacted with anti-human LMW-PTP antibody (data not shown). The purified enzyme was spontaneously inactivated almost completely either during purification or in storage, but the activity could be restored upon DTT treatment. This property suggests that lens LMW-PTP2 is extremely sensitive to oxidation.

The effect of some PTP inhibitors on the activity of purified LMW-PTP2 was examined using DTT-reactivated LMW-PTP2 after removal of the excess DTT. This included vanadate, which resembles phosphate intermediates in structure and is a potent inhibitor of protein tyrosine phosphatase, NaF, which is known as a potent inhibitor for high molecular weight acid phosphatases, and EDTA, which is a classical inhibitor for phosphoserine/threonine protein phosphatases. As shown in Figure 2B, vanadate completely inhibits LMW-PTP2 activity at a concentration as low as 100 nM. In contrast, both NaF and EDTA were ineffective even at concentration as high as 10 mM (Figure 2C). Iodoacetamide, a SH blocker, also inhibited LMW-PTP2 almost completely at 1 mM (Figure 2D), implying that free SH groups are essential for the full activity of this enzyme. All these results confirm the known properties of LMW-PTP from other cell types, thus lens LMW-PTP2 can be considered as an enzyme in the class of low molecular weight tyrosine-specific phosphatases.

Inactivation of purified LMW-PTP2 activity by oxidation

The possible mechanism of oxidation-induced inactivation of LMW-PTP2 was studied using DTT-reactivated purified enzyme and treated with various oxidants under the same experimental conditions. As shown in Figure 3A, oxidized GSH (GSSG) at 10 mM can decrease LMW-PTP2 activity by 10% in 30 minutes, while cystine decreased 20% of PTP activity under the same conditions. It is likely that cystine and GSSG may each attack the SH group at the active site (cysteine residue) forming either LMW-PTP2-S-S-glutathione or LMW-PTP2-S-S-cysteine mixed disulfides, which may attenuate the catalytic activity. However, it is striking that LMW-PTP2 was extremely sensitive to H_2O_2, which was able to directly inactivate LMW-PTP2 at concentrations of micromolar range (Figure 3B). H_2O_2 inactivated LMW-PTP2 30% at 0.1 M, 50% at 1 M and total inhibition at 10 M. This finding is consistent with the observations that LMW-PTP is easily inactivated by intracellular ROS generated during growth factor stimulation [21].

Reactivation of LMW-PTP2 activity

We used purified but spontaneously inactivated LMW-PTP2 to investigate if physiological reductants or redox regulating systems other than the chemical reductant DTT could regenerate the phosphatase activity. The TTase/GSH system was chosen because of its ability to reduce oxidized thiols, such as protein-SSG mixed disulfide. The regeneration of LMW-PTP2 activity was compared with 10 mM DTT restoration (positive control). As shown in Figure 3C, GSH alone at 10 mM restores only a small fraction (5%) of the LMW-PTP2 activity in comparison with that of the DTT regenerated control. TTase alone could not recover any phosphatase activity (data not shown), but together with its cofactor GSH (10 mM), TTase regenerated 30% of the activity in comparison with the control. Reactivation of LMW-PTP2 was TTase concentration-dependent. The higher the activity and amount of TTase used in the reaction, the more the recovery of LMW-PTP2 could be achieved. The 30% activity recovery shown in Figure 3C was obtained by using 0.1 g (or 1.5 M) TTase while in a separate experiment, 0.68 g (or 12.2 M) TTase reactivated 66% of LMW-PTP2 activity (data not shown).

Mass Spectrometric analyses of the oxidation status at the active site of LMW-PTP2

Tryptic digestion of LMW-PTP2 yielded a peptide fragment identified by MS/MS to be SVLFVCLGNICR (Figure 4). This tryptic fragment of amino acid residues from 8–19 containing the active site of LMW-PTP2 (CLGNICR) has a theoretical mono isotopic mass of 1322.6839. However, if both cysteines at 13 and 18 (they are equivalent to C12 and C17 as reported by Chiarugi et al., [21]) make intramolecular disulfide bond with each other, the mass of two hydrogens will be subtracted and the monoisotopic mass of oxidized peptide would be decreased to 1320.6683. Since the protein was treated with IAM after reduction with either DTT or TTase, the free thiol would be carbamidomethylated with additional 57.0215 mass per

each cysteine modification. Thus, the peptide with single reduced cysteine should be modified to yield 1377.6898 amu while both reduced cysteines should be modified to yield 1436.73 amu.

Our results showed that when LMW-PTP2 was treated with IAM and analyzed with LCMS/MS after tryptic digestion, a mass of 1320.67 (observed doubly charged mass 661.3472, see Figure 5A) was found, indicating that a disulfide bond was formed between C13 and C18 with no reduced cysteine at these two positions. As expected, when LMW-PTP2 was treated with DTT and modified with IAM, both cysteines at positions 13 and 18 were modified with carbamidomethyl that resulted in a mass of 1436.73 amu (observed doubly charged mass 719.3772, see Figure 5B) and no disulfide bond was detected between C13 and C18 (data not shown). When LMW-PTP2 was treated with TTase/GSH followed by modification of IAM, a mixed population of oxidized cysteines (disulfide bond between C13 and C18) and carbamidomethylated cysteines (reduced C13 and C18) was found in the same sample.

The amino acid sequence of oxidized peptide SVLFVCLGNICR derived from tryptic digestion of LMW-PTP2 before TTase treatment was confirmed with MS/MS fragmentation pattern (data not shown). The modification of C13 and C18 with carbamidomethyl in the peptide after TTase treatment was also confirmed with MS/MS analyses (Figure 4). The intensity of the doubly charged masses for intramolecular disulfide bonded peptide at m/z 661.35 was 239 counts (Figure 5A) while the intensity of the doubly charged mass of both cysteines 13 and 18 modified with carbamidomethyl at m/z 719.37 was 116 counts (Figure 5B). The intensity counts for both of these masses were generated using selected ion chromatogram (SIC). As both of these intensities are representing the oxidized or reduced forms of cysteines 13 and 18, both intensities are added up to calculate the percentage of oxidized and reduced forms. The relative comparison of intensities of oxidized (67%) and carbamidomethyl modified (reduced) cysteines (33%) in the same sample suggesting that TTase partially reduced C13 and C18 at the active site. This observation is consistent with the activity assay performed with TTase in the same reaction mixture used for the MS study showed 30% recovery of LMW-PTP2 activity compared to DTT (Figure 3C). This mass spectrometry data showed a direct correlation between the percentage of reduced cysteines 13 and 18 (33%) to the activity of LMW-PTP2 (30%) after TTase treatment shown in Figure 3C.

Oxidation- induced inactivation of intracellular PTP in human lens epithelial cells

Because there is no specific assay for LMW-PTP, we first examined the total PTP activity loss in HLE B3 cells exposed to H_2O_2. Serum-free cells were treated with a bolus of 0.1 mM H_2O_2 and the cell lysates were assayed for PTP activity at 0, 5, 10, 15, 30, 60, 120 and 180-minute time intervals. As shown in Figure 6A, the total cellular PTP activity is transiently inactivated with quick recovery. Inactivation of the phosphatase activity occurred within 5–10 minutes with a maxima activity loss of 25% at 15 minutes, after which the activity slowly recovered to 90% and maintained at this high level during the next 60–180 minutes when H_2O_2 in the media was completed detoxified by the cells (see Figure 6A).

To closely study the in vivo LMW-PTP inactivation under similar oxidation conditions, LMW-PTP was immunoprecipitated from the cells treated with or without H_2O_2 (0.1 mM) for 20 minutes. As shown in Figure 6B, only 25% of LMW-PTP activity remains after H_2O_2 treatment compared to that without H_2O_2 treatment. The inactivated LMW-PTP was recovered over 70% in samples treated with DTT (10 mM), indicating that LMW-PTP appears to be more oxidation sensitive in comparison with other PTPs.

Evidence of the TTase-dependent LMW-PTP function in mouse lens epithelial cells

To clarify the role of LMW-PTP in PDGF-induced cell signaling and if TTase is important to the cellular function of LMW-PTP, TTase knock-out (KO) and wild-type (WT) mouse lens

epithelial cell cultures were used for the in vivo studies. As shown in Figure 7A, TTase was detected only in WT cells by Western blot, not in the TTase KO cells, confirming the absence of TTase in this mutant primary cell culture. However, deleting the TTase gene did not affect LMW-PTP expression as the levels of LMW-PTP were the same in both WT and TTase KO cells (Figure 7A). To examine the functional difference of LMW-PTP in WT and KO cells, we stimulated the cells with PDGF, which is known to involve LMW-PTP in its redox signaling-mediated mitogenic action in other cell types [22], and examined the status of LMW-PTP activity using immunoprecipitation. As shown in Figure 7B, during the 60 minutes of PDGF stimulation of the WT cells, the LMW-PTP activity was transiently decreased to 53% of the original activity at 15 minutes followed by fully restored activity at 60 minutes. In contrast, the PDGF-stimulated KO cells showed a gradual loss of LMW-PTP activity during the entire 60 minutes, indicating that without the presence of TTase in the cells, the activity of LMW-PTP was not recovered and the cellular function of LMW-PTP would be compromised.

The phosphorylated active site at Tyr857 of PDGF receptor and the downstream molecular signaling are known as the target substrates for LMW-PTP [22], thus we further compared the status of PDGF-induced cell signaling in WT and KO cells. As shown in Figure 7C, even though the levels of PDGF receptor β (PDGFRβ) are the same in both WT and KO cells during the 0, 15 and 60 minutes of PDGF stimulation (top panel), the degree of phosphorylation at the active site (Tyr857) of the receptor is different in those two cell types. Tyr857 phosphorylation (P-Tyr857) or activation was transiently increased in WT cells during the 60 minutes of PDGF stimulation with P-Tyr857 reached maximum at 15 minutes and returned to near basal level at 60 minutes (Figure 7C, 2nd panel from the top). P-Tyr857 in PDGF-stimulated KO cells was highly elevated at 0-time and remained high throughout the 60 minutes, indicating that the active site of PDGFRβ was constantly being activated. Similar contrast in the phosphorylation/activation of Akt (P-Akt), a downstream signaling pathway for cell survival in PDGF mitogenic action, was observed between the WT and KO cells. Akt in PDGF-stimulated WT cells was transiently activated in a similar pattern as that of Tyr857 at PDGFRβ but Akt activation in KO cells was already elevated at 0-time, and further enhanced and remained at this high intensity during the next 15–60 minutes (Figure 7C, 3rd panel from the top). Phosphorylated ERK1/2 (P-ERK1/2), the downstream signaling pathways for cell proliferation induced by PDGF, showed an extensive activation during the next 15–60 minutes in the WT cells but a sustained over-activation in the KO cells (Figure 7C, bottom panel).

Discussion

This paper provided the evidence for the cloning and purification of the recombinant LMW-PTP from the human lens for the first time. Furthermore, we confirmed that all three isoforms of LMW-PTP generated from one single gene by alternative splicing were present in HLE B3 cells. Both isoforms LMW-PTP1 and LMW-PTP2 were identical to those cloned from human RBC [9], placenta [23] and Jurkat T leukemia cells [10]. The third isoform LMW-PTP3 was identical to the smaller protein found in T cells [10] but different from the one isolated from the placenta [23]. LMW-PTP1 and LMW-PTP2 were catalytically active while LMW-PTP3 was inactive possibly due to lack of both exons 3 and 4. However, LMW-PTP3 was expressed in a significant amount in HLE B3 cells (Figure 1) as well as in T cells and other tissues [10]. The significance of these isozymes remains to be further studied.

The physiological role of LWM-PTP has been studied extensively and the enzyme displayed various functions depending on the cell types. One of the probable functions of LMW-PTP is its role in regulating PDGF mitogenic action as overexpression of dominant-negative (DN) LMW-PTP in NIH3T3 cells displayed higher levels of PDGF-induced phosphorylation of the PDGF receptor and the downstream signaling components [22]. LMW-PTP was suggested to be a negative regulator for insulin signaling in NIH3T3 cells [24]. Some studies showed that

LMW-PTP appeared to regulate cell confluence and cell differentiation [25]. Recent report showed that LMW-PTP could control cell adhesion through the regulation of the cytoskeleton reassembly because inactivation of LMW-PTP was required to allow the key adhesion component of Focal Adhesion Kinase (FAK) to be phosphorylated and activated during cell focal adhesion [26]. In human megakaryocytic cells, LMW-PTP was associated with the signal transducer and activator of transcription-5(STAT-5) at its C-terminal to regulate its activity [27]. In some studies, LMW-PTP displayed oncogenic activities through EphA2 kinase in human breast epithelial cells [28]. Different LMW-PTP isoforms exerted distinct roles in vascular smooth muscle cells and endothelial cells in DNA synthesis and cell migration [29]. Up until now there was no study carried out in understanding the function of LMW-PTP in ocular cells. Based on the high structural homology of lens LMW-PTP with those from other mammalian cell types, it is likely that the human lens LMW-PTP may have similar physiological functions described above. Further studies are needed in these areas.

The mechanism of LMW-PTP function in cells is not quite understood. It has been demonstrated recently that during PDGF-mediated signaling, LMW-PTP exhibited transient inactivation by oxidation followed by GSH-dependent regeneration of its activity [21]. The same study also showed that both cysteines in the active site were essential for this reversible inactivation. Our current study confirmed that lens LMW-PTP2 was extremely sensitive to oxidation in vitro. It was spontaneously inactivated during purification and storage, in agreement with the report of Umeda et al. [13]. Our mass spectrometric data clarified that the inactivation of LMW-PTP2 was due to the intramolecular disulfide bond formation in the active site. The fact that S-thiolation of LMW-PTP2 with either GSSG or cystine to yield protein-S-S-GSH or protein S-S-cysteine, respectively showed little effect on the catalytic activity of this phosphatase, and that mass spectrometry did not detect other forms of thiol modification besides the disulfide bonds between C13 and C18, indicating that the formation of disulfide crosslink between C13 and C18 is likely the main cause for the LMW-PTP inactivation.

As expected the inactivated enzyme could be easily reactivated by a reductant such as DTT. When searching for the in vivo factors responsible for the endogenous reactivation of LMW-PTP, we used TTase system as TTase is known to be an important thiol regulator in many cells types [30], including human lens epithelial cells [31]. Our study showed that the GSH-dependent TTase could restore 30–66% of the catalytic capability of this enzyme in vitro, depending on the amounts of TTase used. Although TTase system favors PSSG as its substrate [30], however, the fact that TTase system could reduce the intramolecular disulfide bond in the active site of LMW-PTP and restored its activity suggests a new function for TTase system. However, unlike the studies of Chiarugi et al. [21], we did not find GSH effective in reducing LMW-PTP, but GSH was required for the TTase system to reduce LMW-PTP. These studies suggest that regulation of the reversible LMW-PTP activity by antagonistic oxidation and reduction is likely the mechanism of the physiological role of LMW-PTP.

The most interesting finding in our study is the extreme sensitivity of LMW-PTP2 to H_2O_2, which as low as 10 M could completely inactivate the phosphatase activity. H_2O_2 is known to produce in many cell types stimulated by growth factors or cytokines during cell signaling [3]. The intracellular level of H_2O_2 generated is likely at the micromolar range as exogenous H_2O_2 at 10–20 M could mimic the action of PDGF in stimulating human lens epithelial cell growth [32]. Therefore, the high sensitivity of the recombinant LMW-PTP to H_2O_2 seen in our study strongly suggests that the intracellular LMW-PTP may also be rapidly oxidized and inactivated by H_2O_2 generated during growth factor-induced cell signaling. Indeed we observed such PDGF-induced transient inactivation of LMW-PTP in the mouse lens epithelial cells (Figure 7B). The transient inactivation of LMW-PTP in the mouse lens cells coincided with that of the PDGF-stimulated transient ROS generation and the activation of the downstream signaling components in human lens epithelial cells [32]. This is in agreement

with the studies of several PTPs in other cell types (LMW-PTP, PTP1B, cdc25 phosphatase, PTEN, etc), in which the enzyme could be transiently inactivated during signaling, concomitantly with H_2O_2 generation [5,33] and that intramolecular disulfide bond was formed between the catalytic cysteine and an adjacent cysteine in some oxidized PTPs.

We speculate that TTase is one of the endogenous enzymes that control LMW-PTP reactivation so that the phosphatase can switch off the activated target signaling components by dephosphorylation. The importance of TTase in LMW-PTP function was demonstrated in the TTase KO cell studies, in which TTase gene deletion did not affect the expression of LMW-PTP but it did strongly attenuate the catalytic activity and the biological function of LMW-PTP during PDGF-signaling (see Figure 7B). The TTase KO cells were unable to re-activate LMW-PTP during the 60-minutes of PDGF stimulation, resulting in a constantly activated or phosphorylated Tyr857 at the active site of PDGFRβ, and a concomitantly over-activated and uncontrolled downstream Akt and ERK1/2 cascading signaling pathways (Figure 7C).

In summary, our current results provided evidence for the reversible redox regulation of LMW-PTP, a property that is essential for redox signaling. The fact that DTT-activated human lens recombinant LMW-PTP was easily inactivated by low levels of H_2O_2 (<10 M), and that the inactivated enzyme was reactivated by a thiol regulating system strongly suggest that LMW-PTP is redox regulated during cell signaling, making it a potential target for the ROS generated during growth factor signaling. Figure 8 depicts the hypothetical role of LMW-PTP in PDGF-mediated mitogenic action in lens epithelial cell, in which upon PDGF binding, PDGF receptor is autophosphorylated and ready for signaling transduction. At the same time, PDGF-induced ROS can oxidize and inactivate LMW-PTP to prevent the dephosphorylation of its target substrates, including the phosphorylated PDGF receptor. This action would allow protein tyrosine kinase to proceed in phosphorylating and activating the downstream signaling components. The unique reversible oxidant-regulated LMW-PTP in turn is then reduced and reactivated by either TTase or other reducing systems or both so that LMW-PTP can resume its ability to dephosphorylate and inactivate the target proteins, allowing the completion of cell signaling. Our hypothesis is in full agreement with the observation that in resting cells, the PTPs are abundant with activities far exceeding that of the protein tyrosine kinases (PTKs). However, during cell stimulation, the activities of PTPs are transiently and severely suppressed while PTKs are activated [34].

Acknowledgements

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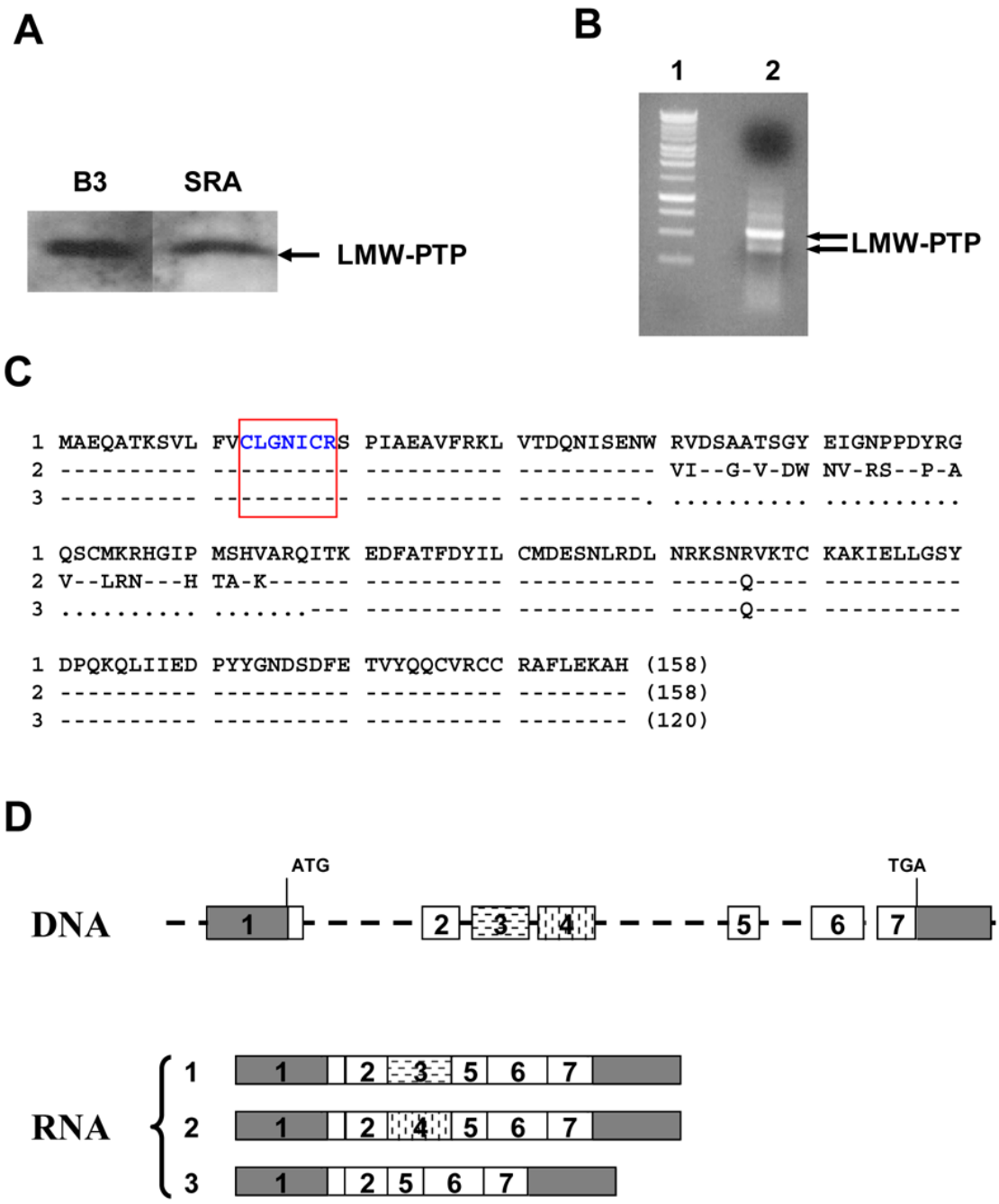

Figure 1. Detection and cloning of low molecular weight PTP from human lens epithelial cells

(A) Immunoblot analysis of LMW-PTP from HLE B3 and SRA 01/04 cells. Cell extract containing 30 g total proteins was resolved on 10% SDS-PAGE gel. LMW-PTP was detected using anti-human-LMW-PTP antibody as primary antibody.

(B) RT-PCR amplification of human lens LMW-PTP cDNA from HLE B3 cells. Total RNA of HLE B3 cell was used as a template for RT-PCR with the primers designed based on the sequence of human red cell acid phosphatase.

Lane 1: 100-bp DNA ladder;

Lane 2: LMW-PTP fragment.

(C) Sequence alignment of three isoforms of human lens LMW-PTP. In box: amino acids of the catalytic loop.
(D) Proposed splicing mechanism producing three LMW-PTP mRNA.

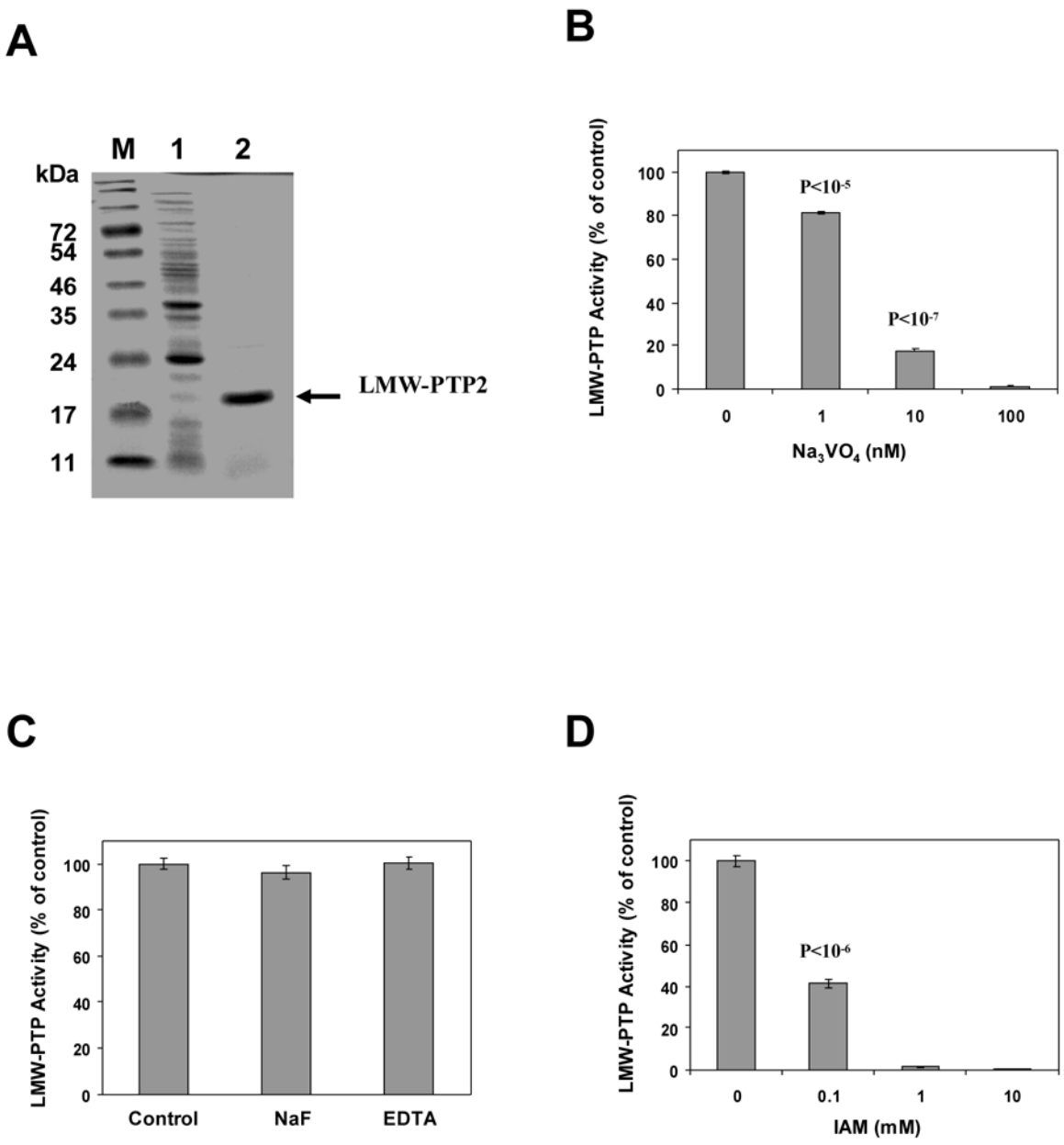

Figure 2. Purification and characterization of human lens LMW-PTP2

(A) SDS-PAGE analysis of purified LMW-PTP2.

Lane M: Prestained protein ladder;

Lane 1: Crude E. coli cell lysate (20 g);

Lane 2: Purified LMW-PTP2 (7 g).

(B) – (D) Purified LMW-PTP2 was reactivated with DTT (10 mM) and the excess DTT was removed with a PD-10 column. Reactivated LMW-PTP2 (1g) was incubated with the following reagents at room temperature for 30 minutes: Na_3VO_4 (0, 0.1, 1, 10, and 100 nM), NaF (10 mM), EDTA (100 mM), iodoacetamide (0, 0.1, 1, and 10 mM). PTP activity was analyzed and expressed as means ± SD, with n = 3.

(B) Inhibitory effect of Na_3VO_4 on LMW-PTP2.
(C) Effect of NaF and EDTA on LMW-PTP2.
(D) Inactivation of LMW-PTP2 by iodoacetamide (IAM).

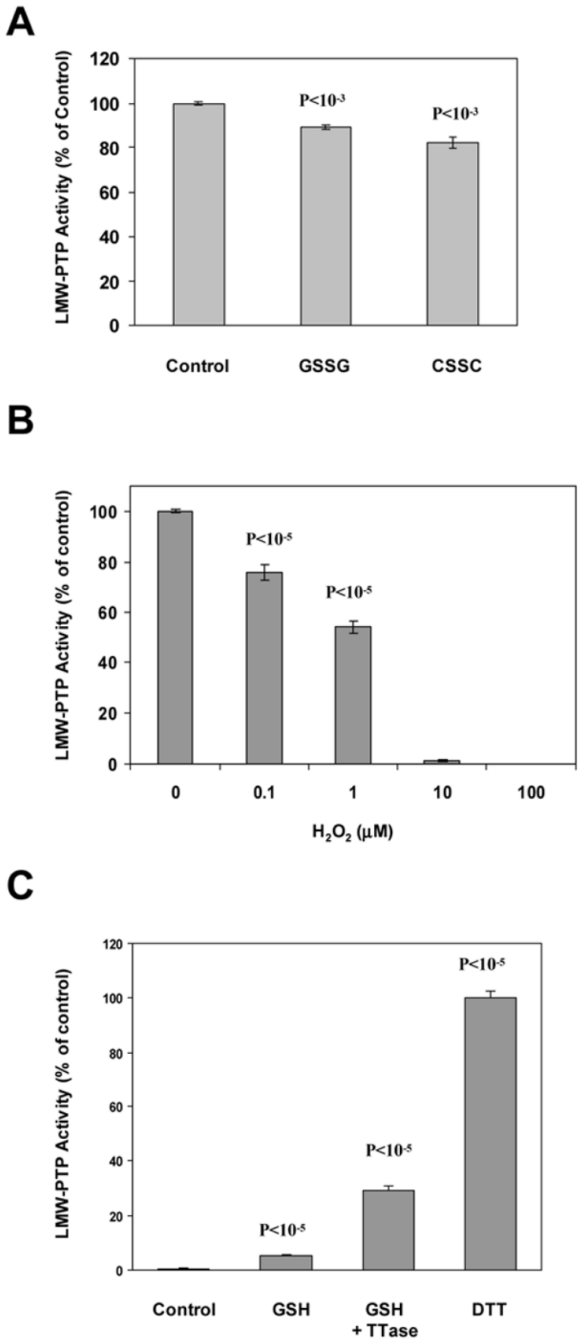

Figure 3. Reversible oxidation of purified LMW-PTP2 in vitro

Inactivation ofLMW-PTP2 by GSSG and CSSC (A) and by H_2O_2 (B) was carried out with conditionsdescribed below. Reactivated LMW-PTP2 (1g) was incubated with the followingoxidants at room temperature for 30 minutes: oxidized GSH (GSSG, 10 mM), cystine (CSSC, 10 mM), H_2O_2 (0, 0.1, 1, 10 and 100 M). PTP activity was analyzed and expressed as means ± SD, with n = 3. **(C)** Regeneration of spontaneously inactivatedLMW-PTP2 by the thioltransferase systems. Spontaneously inactivated LMW-PTP2 (1g) was incubated with the following reagents at 30 °C for 30 minutes: GSH (10 mM), thioltransferase (TTase, 1.5 M) plus GSH (10 mM), and DTT (10 mM). DTT was used as a positive control and standardized

to 100% over the untreated inactive LMW-PTP2 (control). PTP activity was analyzed and expressed as means ± SD, with n = 3.

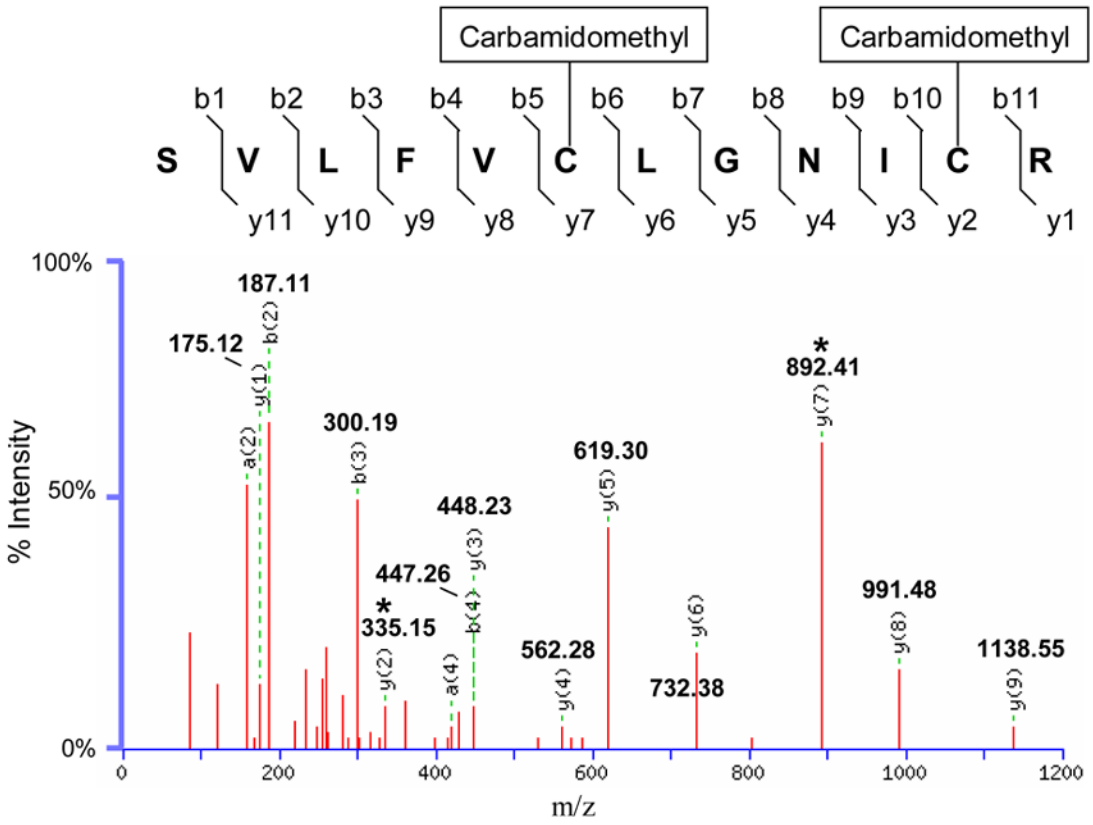

Figure 4. MS/MS of the reduced LMW-PTP2

MS/MS fragmentation pattern of doubly charged mass (719.37) of tryptic peptide 8–19 with labeled masses for b and y ions fragments. The peptide y ion fragments that contain carbamidomethylations are labeled with asterisks sign.

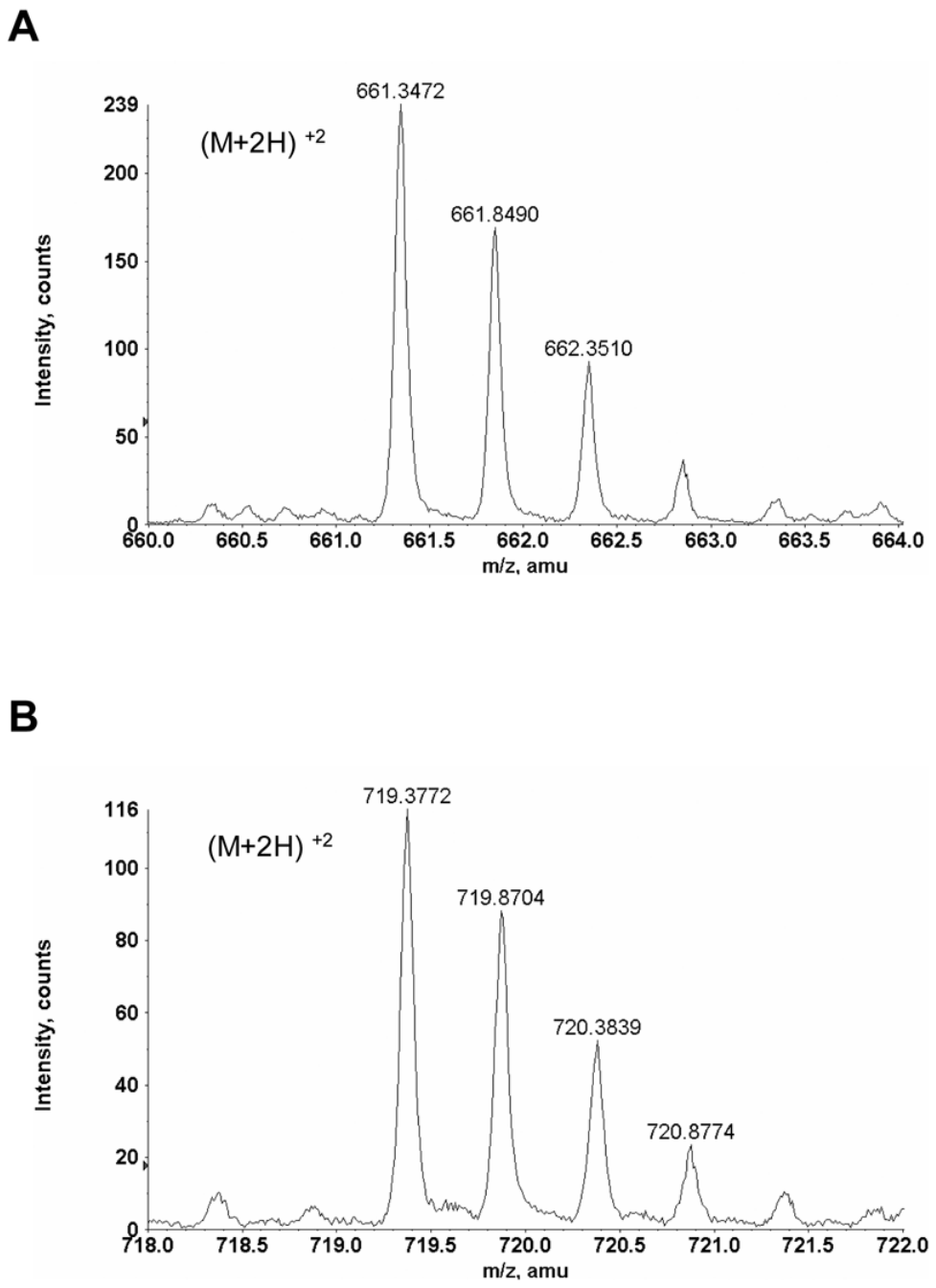

Figure 5. MS analysis of partially reduced LMW-PTP2 by thioltransferase system

The comparison of intensity counts of doubly charged masses for tryptic peptide 8–19 of LMW-PTP2 in oxidized and reduce form of C13 and C18.

(A) The intensity counts 239 found at m/z 661.3472 represents the oxidized form of cysteines 13 and 18 making intramolecular disulfide bond.

(B) The intensity counts 116 found at m/z 719.3772 represents the reduced form of cysteines 13 and 18 modified with carbamidomethyl

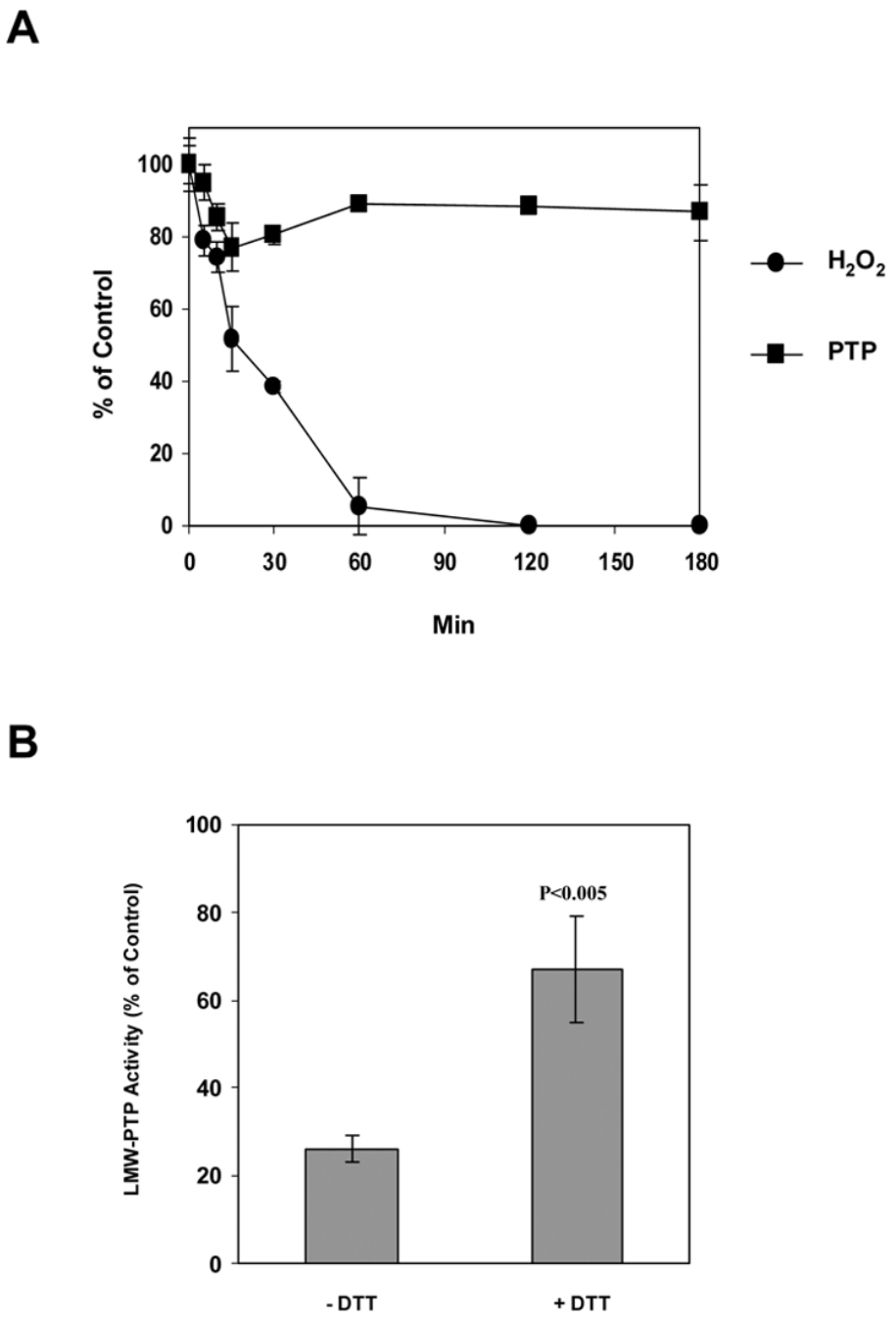

Figure 6. Oxidation-induced inactivation of cellular PTP in HLE B3 cells treated with H_2O_2

(A) Transient inactivation of cellular PTP by oxidation. HLE B3 cells were treated with a bolus of 0.1 mM H_2O_2 for a total of 3 hours. Culture medium was removed at 0, 5, 10, 15, 30, 60, 120 and 180 minutes for H_2O_2 assay. Cells were harvested and homogenized to assay PTP activity. Data are expressed as means ± SD, with n = 3. H_2O_2 in the medium (●), total cellular PTP activity (■).

(B) Oxidation-inactivated cellular LMW-PTP. After treated with H_2O_2 for 15 minutes, B3 cells were harvested and homogenized. LMW-PTP was immunoprecipitated for activity assay with or without DTT reduction (10 mM, 30 minutes, 30 °C).

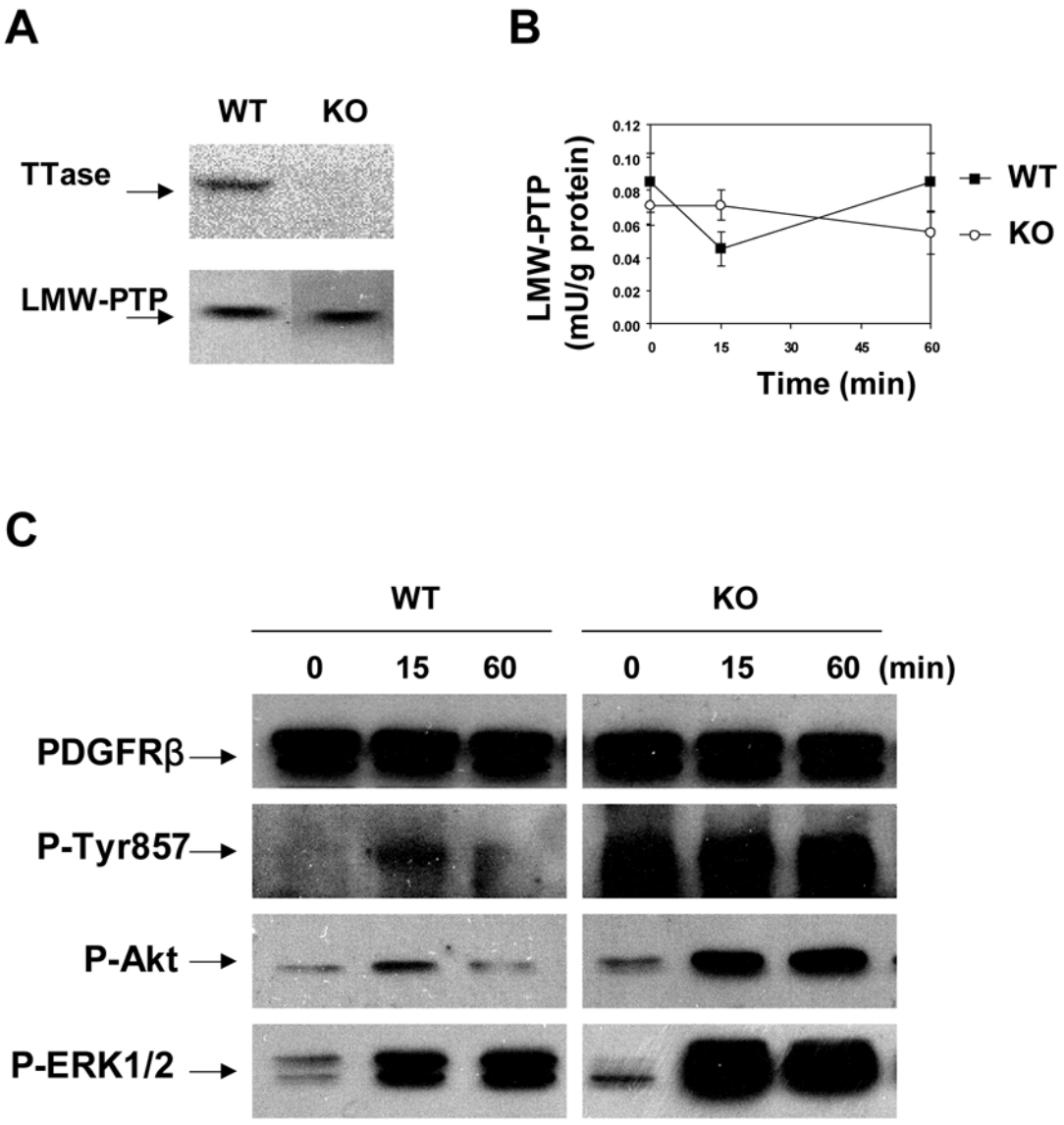

Figure 7. Evidence for the TTase-dependent LMW-PTP function in the mouse lens epithelial cells

(A) Immunoblot analysis of TTase and LMW-PTP from wild-type (WT) and TTase knock-out (KO) mouse lens epithelial cells. Cell extract containing 30 g total protein was resolved on 10% SDS-PAGE gel. TTase and LMW-PTP were probed with antibodies to TTase and LMW-PTP, respectively. The arrow indicates the protein band either reacted positively to antibodies to LMW-PTP or to TTase.

(B) PDGF-induced inactivation of LMW-PTP in wild-type (WT) (■) and TTase knock-out (KO) (○) mouse lens epithelial cells. Serum-deprived cells were stimulated with PDGF (1 ng ml^{-1}) for 0, 15 and 60 min, harvested and lysed in lysis buffer. LMW-PTP was immunoprecipitated for activity assay. Data are expressed as means ± SD, with n = 3.

(C) Comparison of the PDGF-induced activation of PDGFRβ, Akt and ERK1/2 in wild-type (WT) and TTase knock-out (KO) mouse lens epithelial cells. Serum-deprived cells were stimulated with PDGF (1 ng ml^{-1}) for 0, 15 and 60 min, harvested and lysed in lysis buffer. Cell lysate (50 g proteins) was applied on 10% SDS-PAGE gel, transferred to membrane and probed with antibodies to PDGFRβ (internal control), P-PDGFRβ(Tyr857), P-Akt and P-

Erk1/2, respectively. The Western blots shown are the representative patters from three separate experiments.

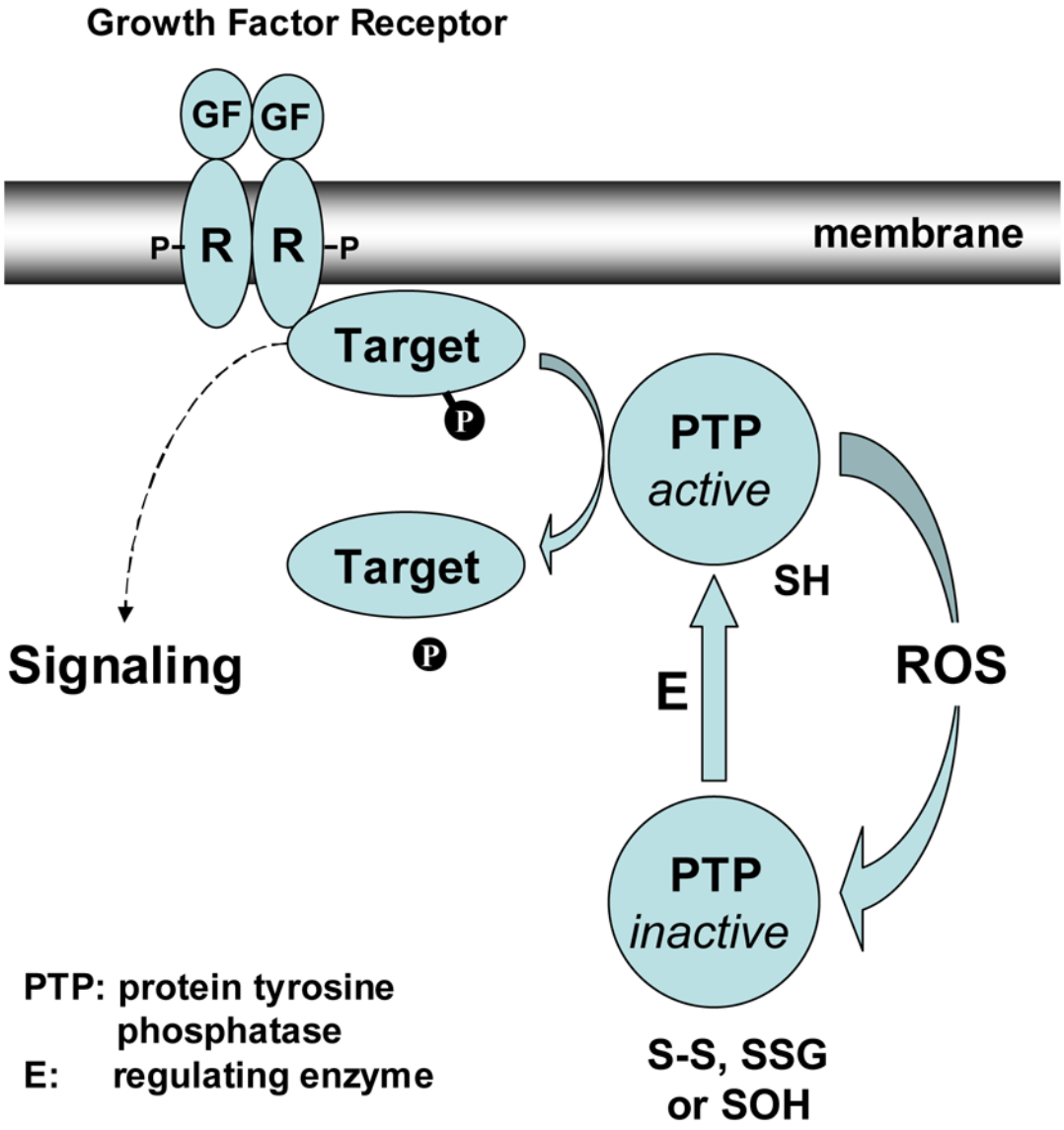

Figure 8. Hypothesis on the function and redox regulation of LMW-PTP in human lens epithelial cells