Negative Regulation of a Protein Tyrosine Phosphatase by Tyrosine Phosphorylation

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Supporting Information

Methods

Preparation of the LMPTP B Constructs— DNA encoding the full-length human LMW-PTP B and the truncated version LMW-PTP B (1-123) were amplified from a LMW-PTP containing plasmid kindly provided by Dr. Tomas Mustelin. The full-length construct was amplified with *Nde*I and *Sma*I restriction digestion sites at the 5' and 3' ends by PCR and cloned into a pTYB2 vector (New England Biolabs). The full-length LMPTP was expressed from this vector as C-terminal fusion protein with the Vent-intein followed by a chitin binding domain (CBD). The *SmaI* site at the 3' terminus of the full length LMW-PTP B gene was deleted by QuikChange site-directed mutagenesis (Stratagene). QuikChange was also performed to mutate the Gln-124 to Cys, which served as a control in protein activity assays. The LMW-PTP B gene encoding the first 123 amino acids with *NdeI* and *XhoI* sites at each end was cloned into a pTXB1 vector (New England Biolabs), which contains a smaller Gyr Intein and CBD in frame. The extra *XhoI* site was deleted by QuikChange mutagenesis and the resulting plasmid was used to generate all ligated proteins. All constructs were confirmed by DNA sequencing of the entire open reading frames of the LMPTP B gene.

Preparation of Fmoc-Pmp(OBz)$_2$-OH[1]— The synthesis of Fmoc-Pmp(OBz)$_2$-OH was performed as the following:[1] 1.8 mmol Fmoc-Pmp-OH (Advanced ChemTech) was added at room temperature to a stirred solution of O-benzyl-N,N'-dicyclohexylisourea (5.4 mmol) in a mixture of toluene (36 ml) and DMF (18 ml). The reaction mixture was heated at 85-90 °C for 6 h before it was stirred at room temperature overnight. After

filtration, the reaction mixture was concentrated under reduced pressure. The oily intermediate was dissolved in a mixture of methanol (22.5 ml) and water (7.5 ml), and was treated at 0 °C with LiOH (380 mg). The reaction mixture was stirred at 4 °C overnight and then neutralized at 0 °C with trifluoroacetic acid, and concentrated under reduced pressure. The resulting residue was dissolved in an aqueous solution of 10% sodium carbonate (17.3 ml) and treated drop-wise with a solution of Fmoc-OSu (3.03g, 9 mmol) in 1,4-dioxane (14 ml) at room temperature. The reaction mixture was stirred at room temperature for 5 h before water (140 ml) was added and the excess Fmoc-OSu was extracted away with diethyl ether (2x280 ml). The aqueous phase was acidified at 0 °C with 6N HCl to pH ~2 before being extracted with ethyl acetate. The resultant organic phase was concentrated under reduced pressure and the resulting oily material was subjected to flash column chromatography (silica gel: acetone/hexanes (1/10-1/3), affording 360 mg of the product as viscous oil. The molecular weight of the synthesized compound was 662 ($[M+H]^+$) in agreement with the assigned structure of the previously prepared compound.

Peptide Synthesis— Standard Fmoc-protected amino acids, peptide synthesis reagents, and Wang resins were purchased from Novabiochem. All peptides were synthesized on Wang resin using the standard Fmoc strategy on a Rainin PS-3 peptide synthesizer. Pmp containing peptides were prepared by incorporating the corresponding non-natural amino acids during the coupling reactions. Fmoc-phosphonomethylene-L-phenylalanine (Fmoc-Pmp-OH) was purchased from Advanced ChemTech and used to generate Pmp-131 or Pmp-132 containing peptides without further modification. To increase the yield of the

Pmp131/132 peptide, commercially available Fmoc-Pmp-OH was side-chain protected with two benzyl groups as described above. Crude peptides were purified by reversed-phase high performance liquid chromatography on a preparative or semipreparative C18 column. Secondary purification was performed if needed. The purity (>95%) of the peptides was established by reversed-phase analytical high performance liquid chromatography and the molecular weights were confirmed by both MALDI and electrospray mass spectrometric analysis.

Recombinant protein expression— Escherichia coli Recombinant proteins were expressed and purified as described previously.[2] Briefly, BL21(DE3) cells transformed with the full length or Q124C LMW-PTP construct were grown at 37 °C until A_{595}=0.6–0.8, followed by treating with 0.2 mM isopropyl-1-thio-beta-D-galactopyranoside (IPTG). After 20 h growth at 16°C, the cells were harvested and lysed by French press. The proteins in the supernatant were purified by chitin beads and eluted by incubation with 50 mM DTT at room temperature for 24 h. Finally the proteins were concentrated and stored at -80°C.

Generation of thioester protein[2-4]*— Escherichia coli* BL21(DE3) cells were transformed with the pTXB1 -LMPTP (1-123) vector and 1Liter of the transformants were grown at 37 °C until A_{595}=1.0-1.2. The expression was induced by the addition of IPTG to 1.0 mM for 5 hours at 37°C. The cells were harvested, lysed and centrifuged at 12,000 g for 30 min at 4°C. The supernatant was discarded, the inclusion bodies were washed once with 100 ml of buffer containing 50 mM Tris, pH 7.5, 1% Triton X-100 (v/v), 1 mM EDTA, 2

M urea, and solubilized in 200 ml of buffer containing 50 mM Tris, pH 7.5, 1% Triton X-100 (v/v), 1 mM EDTA, 8 M urea. After 2 h at room temperature with gentle stirring, the non-dissolved pellet was removed by centrifugation at 12,000 g for 10 min at room temperature. The supernatant was refolded by dialysis against 4 L of refolding buffer (50 mM Tris, pH 7.5, 0.2 M NaCl, 1% Triton X-100 (v/v), 1 mM EDTA) at room temperature for 4 h, any precipitation was removed by centrifugation. The dialysis and centrifugation were repeated an additional time. The proteins in the supernatant were then loaded onto 20 ml of pre-equilibrated chitin beads and incubated for 30 min at room temperature with gentle stirring. The chitin column was then washed with 400 ml of buffer (50 mM Tris, pH 7.5, 0.2 M NaCl) to remove any unbound impurities. Desired LMW-PTP (1-123) thioester protein was generated by incubation with 20 ml of buffer containing 200 mM 2-mercaptoethanesulfonic acid (MESNA), 50 mM Tris, pH 7.5 and 0.2 M NaCl for 24 hours at room temperature with gentle stirring. Once the proteins were cleaved off the fusion proteins, they precipitated immediately onto the chitin beads. After MESNA cleavage, the chitin column was washed with 800 ml of ddH_2O to remove MESNA and Triton X-100. The proteins were eluted off the column by a solution containing 50% 2,2,2-trifluoroethanol, 0.1% trifluoroacetic acid (TFA) in H_2O and dried on a lyophilizer. As confirmed by 15%SDS PAGE and MALDI (data not shown), the proteins eluted from the chitin column were comprised of approximately equal amount of LMW-PTP (1-123)-Gyr Intein-CBD fusion protein, Gyr Intein-CBD protein and LMW-PTP (1-123) thioester protein. The mixture was used for protein ligation without further purification.

Protein ligation[3]—10 mg of proteins eluted from the chitin column and 5.0 mg SDS were dissolved in 5 ml of 50% TFE; 1.3 mg of synthesized peptide was dissolved with 1.0 mg SDS in 1 ml of 50% TFE. The above solutions were lyophilized overnight, dissolved in 0.6 ml of 100 mM sodium phosphate, pH=7.6, 2% thiophenol and mixed at 37°C with gentle stirring for 48 h.[3]

Protein refolding and purification after ligation—The ligation reaction mixture was diluted with 6 ml of refolding buffer (50 mM Tris, pH 7.5, 0.25 M NaCl, 10 mM EDTA) and refolded by dialysis against 2 L refolding buffer overnight at 4°C followed by buffer exchange and another 4 h of dialysis. The refolded proteins were loaded onto 20 ml of pre-equilibrated chitin beads to remove the remaining Gyr Intein-CBD and fusion protein, which were bound to the chitin beads through the CBD domain. The flow-through containing ligated protein was concentrated to ~2 ml. Solid urea was added to the protein solution to make the final concentration of urea 8 M. The solution was then incubated at 37°C for 1 h to fully denature the protein. The denatured protein was centrifuged, filtered and purified by reverse-phase HPLC using a C4 column. HPLC fractions containing the desired protein identified by MALDI were lyophilized, dissolved in 5 ml of 50% TFE and dialyzed against 2 L of refolding buffer twice and the 1 L of storage buffer (50 mM Tris, pH 7.5, 0.25 M NaCl, 10 mM EDTA, 10 mM DTT, 10% glycerol) once. Proteins were then concentrated and stored at -80°C. Proteins used for cell microinjection were passed over Extracti Gel D Detergent Removing Gel (Pierce) after chitin purification, dialyzed intensively, concentrated to about 1 mg/mL and stored at -80°C in microinjection storage buffer (20 mM Tris, pH 7.4, 20 mM NaCl, 1 mM

$MgCl_2$, 0.1 mM EDTA, 5 mM BME, 150 mM KCl, 10% glycerol)[4]. The identity of the proteins was confirmed by SDS-PAG and MALDI-TOF (Figure S1)

Phosphatase Assays with para-Nitrophenyl Phosphate —Reactions were carried out with 0.2 µM LMW-PTP protein and incubated with pNPP (Fluka) in 50 µl of reaction buffer containing 50 mM Tris, pH 7.4, 0.2 mg/ml bovine serum albumin, 150 mM NaCl, 1 mM EDTA, 10 mM dithiothreitol at 25 °C for 30 min. 950 µl of 1 N NaOH was added to quench the reactions and the turnover rates of the proteins were calculated from the amount of the released *p*-nitrophenolate, which was determined from its spectrophotometric absorbance at 405 nm.[2]

Phosphatase Assays with phenyl phosphate and phosphotyrosine containing-peptides[5] -
A coupled assay was used to monitor the phosphate release from the peptide-substrates and phenyl phosphate.[5] All components were provided by the EnzCheck Phosphate Assay Kit (E-6646) from Molecular Probes. Reactions were carried out in 50 mM Tris, pH 7.5, 1 mM $MgCl_2$, 0.1 mM sodium azide (component C of EnzCheck kit) in the presence of 200 µM 2-amino-6-mercapto-7-methylpurine riboside (MESG, component A of EnzCheck kit) and 2 units/mL of Purin nucleoside phosphorylase (PNP, component B of EnzCheck kit). A reaction volume of 60 µL was pre-incubated for 10 min at 25°C, containing all components mentioned above plus various concentrations of substrate peptides or phenyl phosphate. After pre-incubation the reaction-mixture was transferred into a quartz cuvette and the reaction was initiated by addition of 0.5 µM YY, 3.33 µM Pmp131, 1.94 µM Pmp132 or 2µM PMP131/132 LMW-PTP semisynthetic proteins. The reaction was monitored at 360 nm in a Beckman DU 640 Photometer for 5 min taking a

measurement every 5 s. Each assay was performed at least in duplicate with less than 10% of initial substrate utilization. The amount of released phosphate was calculated from a calibration curve measured with phosphate standards and k_{cat}/K_M values were determined by fitting the data sets either to the Michaelis-Menten equation or applying a linear fit (when $K_m >> S$).

Microinjection Experiments

REF-52 cells were cultivated in Dulbecco's Modified Eagle Media (DMEM) supplemented by 10% Fetal Bovine Serum (v/v) at 37°C in presence of 5% CO_2. Prior to microinjection the cells were seeded onto glass coverslips and incubated to 60-70% confluence. For microinjection one 10 µL aliquot of protein solution in microinjection storage buffer was thawed and diluted 2-fold into phosphate buffered saline (PBS). Microinjection was performed at RT using an Eppendorf 5171 micromanipulator, Eppendorf 5246 transjector and Zeiss Axiovert 100 microscope. Following microinjection the cells were incubated 1 h at 37°C in presence of 5% CO_2. In each experiment at least 50 cells were microinjected and each experiment was repeated at least three times.

Immunocytochemistry

After microinjection and incubation, the cells were fixed with 3.7% formaldehyde in PBS at room temperature for 1 h. The coverslips were washed with PBS and the cells permeabilized for 5 min with 0.2% Triton X-100 in PBS. Coverslips were washed and incubated for 40 min with 10% (w/v) bovine serum albumin (BSA). Afterwards the cells

were stained with 5 µg/ml Sheep IgG Anti- LMW-PTP antibody (Exalpha) in PBS + 5% (w/v) BSA for 1 h at 37°C. Secondary antibody staining was performed with rabbit anti-sheep FITC-IgG (Santa Cruz Biotechnology), 5 µg/ml in PBS + 5% (w/v) BSA for 90 min at 37°C. The cover slips were mounted on Microscopy slides using Fluoromount G (Electron Microscopy Science). Photographs of positive cells were taken on Zeiss Axiovert 100 microscope. By using the IPLab software, the fluorescence intensity of all positive cells was quantified. The relative fluorescence intensity was calculated by the sum of fluorescence, normalized by the exact concentration of injected proteins and multiplied by the ratio of positive cells to injected cells.

Confocal microscopy

For confocal microscopy cells were treated as described above. After microinjection the cells were incubated for 30 min, followed by fixing and staining as described above.

The mounted cells were analyzed on a Perkin Elmer UltraVIEW Spinning Disk Confocal Microscope.

Supporting Figures

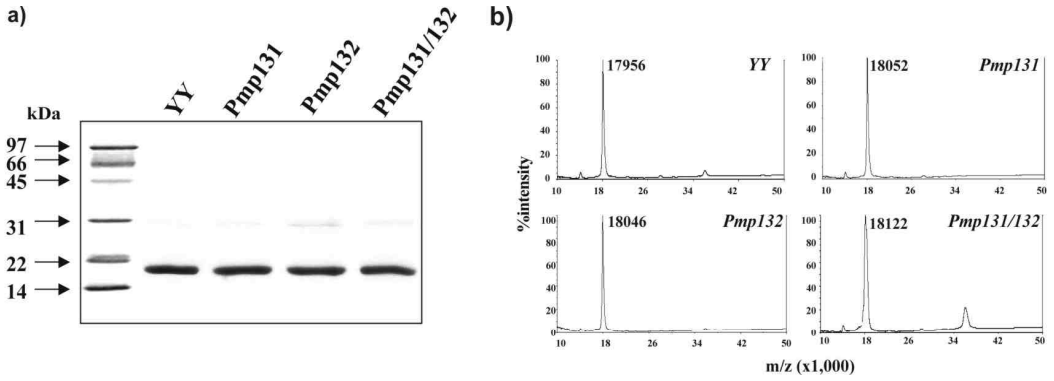

Figure S1 a) SDS-PAGE of the semi-synthetic LMW-PTPs after synthesis and purification. b) MALDI-TOF spectra of the semisynthetic LMW-PTPs. YY-LMW-PTP, m/z: 17956 (calculated: 17976); Pmp131-LMW-PTP 18052 (calculated: 18054); Pmp132-LMW-PTP, m/z 18045 (calculated: 18054); and Pmp131/132-LMW-PTP, m/z: 18122 (calculated: 18130)

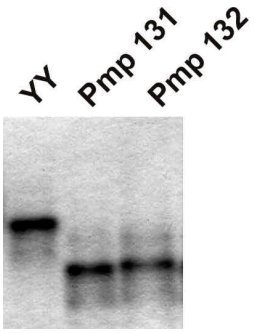

Figure S2 Native gel of semisynthetic LMW-PTPs. The proteins were stained with Coomassie blue. The introduction of Pmp in position 131 or 132 lead to a difference in the migration of the proteins during electrophoresis. The native gel was run at pH 8.3.

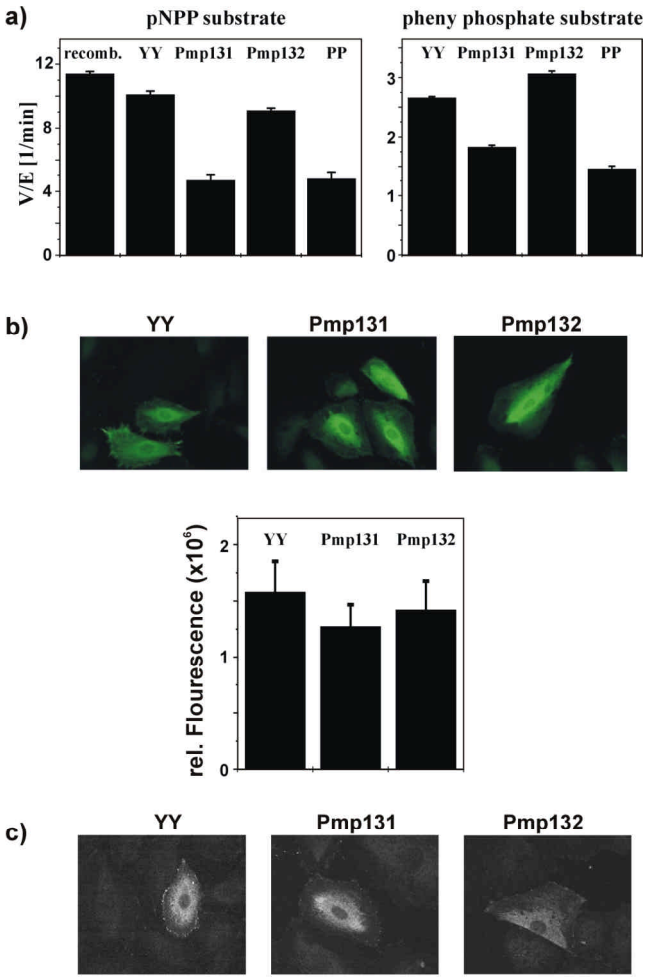

Figure S3 Activity with phenyl phosphate substrates and cellular stability of semisynthetic LMW-PTPs. a) Kinetic assays with semisynthetic LMW-PTPs and pNPP as substrate were carried out with 2 mM pNPP at 25°C, pH 7.4 monitoring the release of nitro-phenol. Phenyl phosphate (1 mM) was used as a substrate like the peptides monitoring the phosphate release with the EnzCheck-assay. The bars represent the standard errors. b) Microinjection experiments with semisynthetic LMW-PTPs. After microinjection and incubation REF-52 cells were fixed and stained with LMW-PTP antibody. The relative fluorescence was quantified, the bars represent the standard error. c) Confocal image of a Z-slice through the microinjected cells.

References Supporting Material

(1) Hoffmann, M. A. *Synthesis* **1988**, *1988*, 62-64

(2) Zhang, Z.; Shen, K.; Lu, W., Cole, P.A. *J. Biol. Chem.*, **2003**, *278*, 4668-4674

(3) Valiyaveetil, F. I.; MacKinnon, R.; Muir, T. W. *J. Am. Chem. Soc*. **2002**, *124*, 9113-9120

(4) Lu, W.; Gong, D.; Bar-Sagi, D.; Cole, P. A.; *Mol. Cell*. **2001**, *8*, 759-769.

(5) Webb, M. R. *Proc. Natl. Acad. Sci. U S A*., **1992**, *89*, 4884-4887