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Title: Protein tyrosine phosphatase PTPN21 acts as a negative regulator of ICAM-1 by dephosphorylating IKKβ in TNF-α-stimulated human keratinocytes

Author's name: Young-Chang Cho¹, Ba Reum Kim¹, Sayeon Cho¹,*

Affiliation: ¹College of Pharmacy, Chung-Ang University, Seoul 06974, Republic of Korea

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Corresponding Author's Information: Tel.: +82 2 820 5595, Fax: +82 2 816 7338
E-mail address: sycho@cau.ac.kr
1. MATERIALS AND METHODS

Cell culture, plasmids, and reagents

The human HaCaT keratinocyte cell line was purchased from Cell Lines Service (Eppelheim, Germany). HEK 293 cells were obtained from ATCC (Manassas, VA, USA). Cells were maintained in Dulbecco’s modified Eagle's medium (DMEM; GE Healthcare, Milwaukee, WI, USA) containing 10% fetal bovine serum (FBS; GE Healthcare), 50 units/ml penicillin, and 50 μg/ml streptomycin (GIBCO BRL, Grand Island, NY, USA) at 37 °C in humidified air containing 5% CO₂. FLAG-PTPN21 WT was generously provided by Dr. A. Feliciello (Federico II University, Italy). FLAG-PTPN21 C1108S catalytically inactive mutant was generated from FLAG-PTPN21 WT using a QuikChange II Site-Directed Mutagenesis Kit (Agilent Technology, Santa Clara, CA, USA). Rabbit anti-PTPN21 antibody (cat no. ab133812) was purchased from Abcam (Cambridge, U.K.). Rabbit anti-p-IkBα (Ser32/36; cat no. sc-101713), mouse anti-IκBα (cat no. sc-1643), mouse anti-ICAM-1 (cat no. sc-8439), rabbit anti-IKKα/β (cat no. sc-7607), and rabbit anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; cat no. sc-25778) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-p-IKKα/β (p-Ser176/180 of IKKα and p-Ser177/181 of IKKβ; cat no. 2697) was purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). Mouse anti-FLAG antibody was from Sigma-Aldrich (St. Louis, MO,
USA). Recombinant human TNF-α protein was from R&D Systems (Minneapolis, MN, USA). Calf intestine phosphatase (CIP) was purchased from Takara Bio, Inc. (Otsu, Japan). AccuZol reagent and AccuPower PCR Master Mix were from Bioneer (Daejeon, Korea) and TOPscript cDNA synthesis kit was from Enzymomics (Daejeon, Korea).

**RNA preparation and cDNA (complementary DNA) synthesis**

HaCaT cells were seeded on 6-well plates (2×10⁵ cells/well) and incubated at 37 °C overnight. Cells were treated with TNF-α (10 ng/ml) in the absence or presence of PTPN21 plasmids for the indicated time periods according to experimental settings. Total RNA was prepared from cells using Accuzol reagent and reverse-transcribed into cDNA using a TOPscript cDNA synthesis kit as manufacturer’s protocols.

**Polymerase chain reaction (PCR)**

PCR primer sequences of PTPs used in this study are listed in Supplementary Table 1. The primer sequences of ICAM-1 and GAPDH were designed as previously described (27, 28). The PCR was run for 17–25 cycles of 94 °C (30 s), 60 °C (30 s), and 72 °C (30 s) on a Bioer thermal cycler (Bioer Technology Co., Hangzhou, China). After amplification, 10 μl of the PCR products was separated on 1.5% (w/v) agarose gels, which were then stained with ethidium bromide.

**Preparation of total cell lysates**

HaCaT cells (1×10⁶ cells/ 60-mm dish) transfected with PTPN21 plasmids were incubated with TNF-α for the indicated time periods. After incubation, total cell lysates were prepared as previously described (29). Cells were washed 3 times with ice-cold phosphate-buffered saline. Lysis buffer, containing 0.5% IGEPAL CA-630, 0.5% Triton X-100, 150 mM NaCl,
20 mM Tris-HCl (pH 8.0), 1 mM ethylenediaminetetraacetic acid, 1% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM NaF, and 1 mM Na₃VO₄, was added to the cells and incubated for 10 min. The supernatants were collected after centrifugation at 15,814 × g for 30 min at 4 °C.

**Luciferase activity assay**

HaCaT cells (5×10⁶ cells) were seeded on 100-mm dishes (70% confluence on the day of transfection) and transfected with pNF-κB-luc cis-reporter plasmids (Agilent Technology) and gWIZ-green fluorescent protein (GFP; internal control for transfection efficiency). Transfected cells were split into 12-well plates, incubated overnight, and then transfected with FLAG-PTPN21 plasmids. After 24 h of incubation, TNF-α (10 ng/ml) was added and cells were incubated for additional 24 h. Luciferase activity assay was performed as described previously (30). Briefly, cells were lysed in cell culture lysis reagent (Promega Corporation, Fitchburg, WI, USA) and luciferase activity was measured using VivoGlo Luciferin (Promega) as a substrate. GFP fluorescence was detected at an excitation wavelength of 485 nm and an emission wavelength of 525 nm.

**Immunoblotting analysis**

Immunoblotting analysis was carried out as described previously (31). Briefly, aliquots of each boiled sample (20 μg) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. After blocking with 5% non-fat dried milk, each membrane was incubated overnight at 4 °C with primary antibody. Each membrane was then incubated for an additional 1 h with secondary peroxidase-conjugated IgG antibody (1:5,000). The proteins were detected using enhanced chemiluminescence reagent. Protein levels were quantified by scanning the immunoblots and
analyzing them with LabWorks software (UVP Inc., Upland, CA, USA).

**Knockdown of PTPN21**

For RNA interference of PTPN21, HaCaT cells (5×10^5 cells/ 6-well plate) grown to 40% confluence were transfected with 50 nM of scrambled negative control siRNA or 50–100 nM of PTPN21 siRNAs [#1: 5′-CUC UGU CAG UGG AAU CGA A(dTdT), #2: 5′-GAG AAG AGC UUU AGG UAC U(dTdT), or #3: 5′-GAG AAG AGC UUU AGG UAC U] (Bioneer) using Neon Transfection System (Invitrogen, Carlsbad, CA, USA). The negative control siRNA used was purchased from Bioneer. After 48 h of transfection, cell lysates were prepared and subjected to immunoblotting analysis with an anti-PTPN21 antibody.

**Endogenous protein binding assay**

Total lysates from HaCaT cells were incubated with mouse anti-IκB antibody, mouse anti-IKKα/β, or normal mouse immunoglobulin G (IgG) for 3 h at 4 °C and then further incubated with protein A/G beads for 1 h at 4 °C. To clear the immunoprecipitates, unbound proteins were discarded from immunoprecipitates by extensive washing (5 times) with lysis buffer. Following that, the cleared immunoprecipitates were mixed with 1× sample loading buffer, boiled at 100 °C for 5 min, and then subjected to immunoblotting analysis.

**Purification of the bacterial His-tagged proteins**

After *Escherichia coli* BL21 (DE3)RIL was transformed with pET28a-His-PTPN21 (a.a. 839-1174) WT or pET28a-His-PTPN21 C1108S, cells were grown on LB medium containing kanamycin and 0.2 mM isopropyl-β-D-1 thiogalactopyranoside at 18 °C for 16 h. Cells were harvested, resuspended in lysis buffer [50 mM Tris-HCl (pH 7.5), 500 mM NaCl, 1 mM PMSF, 4 mM 2-mercaptoethanol, and 5% (v/v) glycerol], and then lysed by sonication. The
cell extracts were centrifuged at 15,814 × g for 50 min and the supernatant was subjected to Ni-NTA agarose affinity chromatography. The PTPN21 phosphatase bound to the affinity gel was eluted by imidazole gradient method and frozen at −80 °C in a buffer containing 25 mM Tris-HCl (pH 7.5), 200 mM NaCl, 10 mM 2-mercaptoethanol, and 5% (v/v) glycerol until use in enzyme assay. Phosphatase activities of His-PTPN21 WT and C1108S were measured using the substrate 3-O-methylfluorescein phosphate (OMFP; Sigma-Aldrich). The amount of 3-O-methylfluorescein was determined by the absorbance change at 490 nm or fluorescence change of excitation at 485 nm and emission at 525 nm.

**In vitro protein binding assay**

HEK 293 cells (5×10⁶ cells/ 100-mm dishes) were transfected with FLAG-tagged IκBα or IKKβ expression plasmid (5 μg) for 48 h. Total cell lysates were pulled down with anti-FLAG M2 agarose beads for 3 h and the pulled-down proteins were subjected to extensive washing to purify FLAG-fusion proteins by excluding any bound proteins in the pulled-down complexes. To determine whether PTPN21 directly binds to IκBα or IKKβ, each anti-FLAG bead-bound protein was mixed with His-PTPN21 WT (2 μg) in 1 ml of PTP reaction buffer [100 mM Tris-HCl (pH 7.5), 40 mM NaCl, and 1 mM DTT] and incubated for 3 h at 4 °C. After incubation, the beads were washed with binding buffer 5 times and 1× sample buffer was added and boiled for 5 min at 100 °C. The samples were subjected to immunoblotting analyses using appropriate antibodies.

**In vitro phosphatase assays**

Each anti-FLAG bead-bound protein was mixed with His-PTPN21 WT or C1108S (0.1 μg) in 20 μl PTP reaction buffer [100 mM Tris-HCl (pH 7.5), 40 mM NaCl, and 1 mM DTT] and reaction mixtures were incubated at 30 °C for 30 min. CIP was used to prove the bands
detected by each antibody, which recognizes specific phosphorylation sites, are phospho-specific bands. Phosphatase reaction was stopped by adding 5× sample buffer. The beads were then resolved on SDS-PAGE and analyzed by immunoblotting using specific antibodies.

**Statistical analysis and experimental replicates**

The data are represented as the mean ± standard error of the mean (SEM). Differences between experimental conditions were assessed by Student’s t-test. p < 0.05 was considered statistically significant. In all instances, the means of data from three independent experiments were analyzed.

2. **SUPPLEMENTARY DATA LEGENDS**

**Supplementary Fig. 1.** Knockdown of PTPN21. After transfection with control or PTPN21 siRNAs (#1, #2, and #3), PTPN21 knockdown was confirmed by immunoblotting using anti-PTPN21 and anti-GAPDH antibodies.

**Supplementary Table. 1.** List of PTP primers used in this study

**REFERENCES**

1. Cho YC, Ju A, Kim BR and Cho S (2015) Anti-inflammatory effects of Crataeva nurvala Buch. Ham. are mediated via inactivation of ERK but not NF-kappaB. J Ethnopharmacol 162, 140-147
2. Seo WY, Youn GS, Choi SY and Park J (2015) Butein, a tetrahydroxychalcone, suppresses pro-inflammatory responses in HaCaT keratinocytes. BMB Rep 48, 495-500
3. Bak MJ, Truong VL, Ko SY et al (2016) Induction of Nrf2/ARE-mediated cytoprotective
genes by red ginseng oil through ASK1-MKK4/7-JNK and p38 MAPK signaling pathways in HepG2 cells. J Ginseng Res 40, 423-430

4. Dilshara MG, Kang CH, Choi YH and Kim GY (2015) Mangiferin inhibits tumor necrosis factor-alpha-induced matrix metalloproteinase-9 expression and cellular invasion by suppressing nuclear factor-kappaB activity. BMB Rep 48, 559-564

5. Park H, Ahn KJ, Lee Kang J and Choi YH (2015) Protein-protein interaction between caveolin-1 and SHP-2 is dependent on the N-SH2 domain of SHP-2. BMB Rep 48, 184-189
Supplementary Fig. 1

| siRNA          | Control siRNA | PTPN21 siRNA #1 | PTPN21 siRNA #2 | PTPN21 siRNA #3 |
|----------------|---------------|-----------------|-----------------|-----------------|
| α-PTPN21       | 130           |                 |                 |                 |
| α-GAPDH        | 40 (kDa)      |                 |                 |                 |

lysate
| PTPs   | primer sequence                        | PTPs   | primer sequence                        |
|--------|---------------------------------------|--------|---------------------------------------|
| ACP1   | Sense: 5'-GAG GGT CTG CAC CGA AAC ATG-3' | DUSP28 | Sense: 5'-CAC GCT GTG CGT CAA CGT C-3' |
|        | Antisense: 5'-CTG ACA GCT CTT GGG TCT GGG-3' |        | Antisense: 5'-GAC GCC GTT CTT GCA GTA G-3' |
| Cdc14A | Sense: 5'-GAA CCA AGC ACT TCC AGG C-3' | DUSP3  | Sense: 5'-GAG GGA GGG CAG GTC TCT CA-3' |
| Cdc14B | Sense: 5'-AGG GCA AGT TGT TCT CCC TG-3' | DUSP4  | Sense: 5'-CCA CCA TCT GCC TGG CCT AC-3' |
| Cdc25A | Sense: 5'-GGA AGC AGC ACT ACC ACA AAG G-3' | DUSP5  | Sense: 5'-GAA CCA AGT TGT TCC TGG ACG-3' |
| Cdc25C | Sense: 5'-GCC CCC AGT GTC AGG GAA AC-3' | DUSP6  | Sense: 5'-CTC GGG CTG TCT CTC AAG-3' |
| DUSP1  | Sense: 5'-CAC CCC TGA CAT CGA GAA CG-3' | DUSP7  | Sense: 5'-CCA ATG TGG AGA GCC CAC C CA C-3' |
| DUSP10 | Sense: 5'-GCC TGG CAG ATG AGA AG-3'    | DUSP9  | Sense: 5'-GCC TTC ATG GTC TGG TGG AGC-3' |
| DUSP13A| Sense: 5'-GCC CCA TGA AGC CAC AAG C-3' | hSSH-1L| Sense: 5'-CCA ATT TGG AGA GCC TGG AGG C3-3' |
| DUSP15 | Sense: 5'-CAC CCC AGA CCT CTG TGG GAG C-3' | KAP1   | Sense: 5'-GCC TGG CAG TGG ATG AGA C-3' |
| DUSP16 | Sense: 5'-GCC CCA TGA AGC CAC AAG C-3' | MTM1   | Sense: 5'-CCA CAG CAC CCG TGG CAG-3' |
| DUSP2  | Sense: 5'-GTG CCT GGT TCC AGG AGG C-3' | MTM2   | Sense: 5'-AAC CAG CAA CAA CTA AAG TCT A-3' |
| DUSP22 | Sense: 5'-TGTC TGA TGG CAA CCA AAG C-3' | MTMR2  | Sense: 5'-GCC AGT CAA TCG TGG GGT TGG T-3' |
| DUSP23 | Sense: 5'-GAC GCC GAC TAC CGT GAT C-3' | MTMR7  | Sense: 5'-CCA GGA CCA CAC CAA CGT GGG-3' |
|        | Antisense: 5'-GGT GTC GGT TCC AGG AGG C-3' | PALD1  | Sense: 5'-GCC GTC TGG CAG TGG ATG GGG C-3' |

Supplementary Table 1. List of PTP primers used in this study.
| PTPs  | primer sequence          | PTPs  | primer sequence          |
|-------|--------------------------|-------|--------------------------|
|       | Sense: 5'-CCA CAT GGC CTG ACT TTG GAG-3'  |       | Sense: 5'-TTA CTG GCC CAT TTC TCT GCT AAT G-3'  |
| PTP1B | Antisense: 5'-GGT AGG AGA AGC GCA GCT CTG GG-3'  | PTPNR | Antisense: 5'-CTG AGG CAG GAG TGC CAT-3'  |
|       | Sense: 5'-GGT GCA CCA CCA TCC AAC C-3'  | PTPRB | Sense: 5'-GGT GTG GCC AGA CCA TGG-3'  |
| PTP4A1 | Antisense: 5'-GAA TCT TTG AAA CGC AGC CGC-3'  | PTPRG | Antisense: 5'-GGT GTC AGA GCC CAT GTG AAG CTG GG-3'  |
|       | Sense: 5'-GGT CTA CCA CTG TGG TGG CTG G-3'  |       | Sense: 5'-GGT CAA GCC AAT TG-3'  |
| PTP4A3 | Antisense: 5'-GAA TCT TTG AAA CGC AGC CGC-3'  | PTPRG | Antisense: 5'-GAA TCT TTG AAC CAC AAA CCA TG-3'  |
|       | Sense: 5'-GGG CTA CCA CTG TGG TGC G-3'  |       | Sense: 5'-GAG TCT GGG AAA CCA CAG GT-3'  |
| PTPMT1 | Antisense: 5'-GAA TCT TTG AAA CGC AGC CGC-3'  | PTPRO | Antisense: 5'-GAA TCT TTG AAC CAC AAA CCA TG-3'  |
|       | Sense: 5'-GGC AGG TAG ATG GTC CCA GA-3'  | PTPRQ | Antisense: 5'-GGA GAA TGC GGG AAA CCA CAG G-3'  |
| PTPN12 | Antisense: 5'-GAG CTG CTT GCT GTT GAT GGC-3'  |       | Sense: 5'-GGT GTC ATG TGG CTC CCT GCT TG-3'  |
|       | Sense: 5'-GGC AGG TAG ATG GTC CCA GA-3'  | PTPRT | Antisense: 5'-GGA GAA TGC GGG AAA CCA CAG G-3'  |
| PTPN14 | Antisense: 5'-GAG CTG CTT GCT GTT GAT GGC-3'  | PTPRZ | Antisense: 5'-GGT GTC ATG TGG CTC CCT GCT TG-3'  |
|       | Sense: 5'-GAG CAT CCT CTG CTC CCT GAG G-3'  | SHP-1 | Sense: 5'-GGG ACA AGG ATG TGG CAA GGA G-3'  |
| PTPN18 | Antisense: 5'-GAG CAT CCT CTG CTC CCT GAG G-3'  |       | Sense: 5'-GGG ACA AGG ATG TGG CAA GGA G-3'  |
|       | Sense: 5'-GGT GCA CCA CCA TCC AAC C-3'  | PTPRZ | Antisense: 5'-GGG ACA AGG ATG TGG CAA GGA G-3'  |
| PTPN2 | Antisense: 5'-GAG CAT CCT CTG CTC CCT GAG G-3'  |       | Sense: 5'-GGT GTC GTG GTC CAA AAT GGT G-3'  |
| PTPN21 | Sense: 5'-GGT GCA CCA CCA TCC AAC C-3'  |       | Sense: 5'-GGT GTC GTG GTC CAA AAT GGT G-3'  |
|       | Antisense: 5'-GAG CAT CCT CTG CTC CCT GAG G-3'  | PTPZ | Antisense: 5'-GGT GTC GTG GTC CAA AAT GGT G-3'  |
| PTPN22 | Sense: 5'-GCT CTA CAG CCA GCC GCA GA-3'  |       | Sense: 5'-GGT GTC GTG GTC CAA AAT GGT G-3'  |
|       | Antisense: 5'-GCT CTA CAG CCA GCC GCA GA-3'  | STNS  | Antisense: 5'-GGT GTC GTG GTC CAA AAT GGT G-3'  |
| PTPN3 | Sense: 5'-GCT CTA CAG CCA GCC GCA GA-3'  |       | Sense: 5'-GGT GTC GTG GTC CAA AAT GGT G-3'  |
| PTPN7 | Antisense: 5'-GCT CTA CAG CCA GCC GCA GA-3'  |       | Sense: 5'-GGT GTC GTG GTC CAA AAT GGT G-3'  |

Supplementary Table 1. List of PTP primers used in this study (continued)