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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

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Supplementary information :

Supplementary file 1 : Supplementary information.

Supplementary file 2 : Supplementary figure 1.

Supplementary file 3 : Supplementary table 1.

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% CO_2. 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-IκBα (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 Enzynomics (Daejeon, Korea).

RNA preparation and cDNA (complementary DNA) synthesis

HaCaT cells were seeded on 6-well plates (2×10^5 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 (1×10^6 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 Na_3VO_4, 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 (5×10^6 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 (5×10^6 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

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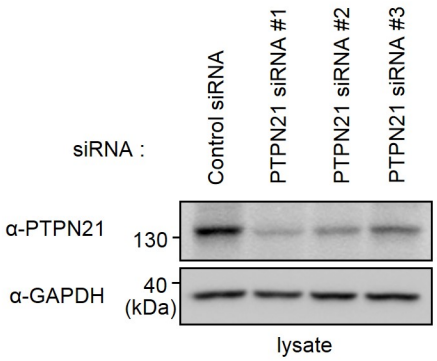
Supplementary Fig. 1



PTPs	primer sequence	PTPs	primer sequence
ACP1	Sense: 5'-GAG GGT CTG CAC CGA AAC ATG-3' Antisense: 5'-CTG ACA GCT CTT GGG TCT GGG-3'	DUSP28	Sense: 5'-CAC GCT GTG CGT CAA CGT C-3' Antisense: 5'-GCG GCC GTT CTT GCA GTA G-3'
Cdc14A	Sense: 5'-GAC CCC AGC AGC ACT TCC TG-3' Antisense: 5'-AAG GCA CGT AGT TTG TCT CCC TG-3'	DUSP3	Sense: 5'-GAG GGA GGG CAG GTC CTT CA-3' Antisense: 5'-CCA GGA AGC CAT CGT TGG G-3'
Cdc14B	Sense: 5'-GCC AGC AGC TCC TGG ACA G-3' Antisense: 5'-GGC AGG GGC TGG GTA GAG G-3'	DUSP4	Sense: 5'-CCA CCA TCT GCC TGG CCT AC-3' Antisense: 5'-GAA GAC GAA CTG CGA GGT GG-3'
Cdc25A	Sense: 5'-GGC TTC GTG GAC CTT CTC G-3' Antisense: 5'-GGC CCC AGA CAT GCT CTT C-3'	DUSP5	Sense: 5'-GAG GCA AGG TCC TGG TCC AC-3' Antisense: 5'-GCC TCC CCT TGG CAG GAG-3'
Cdc25C	Sense: 5'-GCC AAC CGT GTC AGG GAA AC-3' Antisense: 5'-GGG TGT CCA AAG GGA CGA TG-3'	DUSP6	Sense: 5'-CTC GGG CTG CTG CTC AAG-3' Antisense: 5'-GCT GGC TGT TGG ACA GCG-3'
DUSP1	Sense: 5'-CCT GTG GAG GAC AAC CAC AAG G-3' Antisense: 5'-GCT GGC CCA TGA AGC TGA AG-3'	DUSP7	Sense: 5'-CCT GCC CTA CCT CTA CCT CGG-3' Antisense: 5'-CAC CAC ACT TCT TGG AGC GG-3'
DUSP10	Sense: 5'-CAC CCC TGA CAT CGA GAA CG-3' Antisense: 5'-GCC TGG CAG TGG ATG AGA AG-3'	DUSP9	Sense: 5'-CCA ATT TGG AGA GCC TGG CC-3' Antisense: 5'-GCT TCT GCA TGA GGT AGG CCA C-3'
DUSP13A	Sense: 5'-CAG CCC ACG ACC TCC CTG-3' Antisense: 5'-GCC TCG GTT GGG GAA GAC C-3'	hSSH-1L	Sense: 5'-CAG GCT GGA GGC CAG CAT C-3' Antisense: 5'-CTC CGG GTC AGG TTG GAG C-3'
DUSP15	Sense: 5'-CCC AAC CCA GGC TTT AGG C-3' Antisense: 5'-CCC TCG GAG GCT GCT GAG-3'	KAP1	Sense: 5'-CGC AGA TGG AGG GAC TCC TG-3' Antisense: 5'-CCG GAT CCT CTT AGG TCT CGC-3'
DUSP16	Sense: 5'-CGG AGC AGA CTC CCG AAA CC-3' Antisense: 5'-CAG GCC AGC AGA CTT CGT GAG-3'	MTM1	Sense: 5'-CAA CAG CCG AAT CCA GTG GAG-3' Antisense: 5'-GAG CTC TAA TGC GGT GCC AGG-3'
DUSP2	Sense: 5'-GTG CCT GGT TCC AGG AGG C-3' Antisense: 5'-CTC AGT GAC ACA CGA CCT GGG-3'	MTMR2	Sense: 5'-GGC CAT GGA GAT AAG AACACAT GC-3' Antisense: 5'-GCG CAT GCT GGC TAC TGG-3'
DUSP22	Sense: 5'-TGG TGA TCG CAT ACA TCA TGA C-3' Antisense: 5'-CAG TCT TCT GAG AAA GGC CCA G-3'	MTMR7	Sense: 5'-CAC AGC CAG ACT CAG GGA ACC-3' Antisense: 5'-CTG ACC TGG ATG GGG TTG TG-3'
DUSP23	Sense: 5'-CGG GCC GAC TAC CTG AAT CC-3' Antisense: 5'-GGT AGT GAG GGT CCA GCA GCA G-3'	PALD1	Sense: 5'-CCA GGG CCG TAC CAC AAC TG-3' Antisense: 5'-GGC GTC ATG GTC TCG CTG-3'

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

PTPs	primer sequence	PTPs	primer sequence
PTP1B	Sense: 5'-CCA CAT GGC CTG ACT TTG GAG-3' Antisense: 5'-GGT AGG AGA AGC GCA GCT GG-3'	PTPNR	Sense: 5'-TTA CTG GCC CAT TTC TCT GAA G-3' Antisense: 5'-CTG AGG CAG GAG TGC CAT-3'
PTP4A1	Sense: 5'-GGT GCA CCA CCA TCC AAC C-3' Antisense: 5'-GAA TCT TTG AAA CGC AGC CGC-3'	PTPRB	Sense: 5'-GGT GTG GCC AGA CCA TGG-3' Antisense: 5'-CTG GAC CAT GTG AAC CCT GTG-3'
PTP4A3	Sense: 5'-GGG CTA CCA CTG TGG TGC G-3' Antisense: 5'-GAG CTG CTT GCT GTT GAT GGC-3'	PTPRD	Sense: 5'-CAC CAA GCT GCG TGA AAT GG-3' Antisense: 5'-CCG GAC TTT GGC ACT CCT TG-3'
PTPMT1	Sense: 5'-CCT CCA TGG ATT CAG GGA AGG-3' Antisense: 5'-CTC CCT GGT GTG CTA CAA TCC C-3'	PTPRG	Sense: 5'-CCT TTC GTC CTC CGG GAC C-3' Antisense: 5'-TCA TGC AGA CGC TGC TGT GG-3'
PTPN12	Sense: 5'-GTG ATC ATC CAG CGG GAG G-3' Antisense: 5'-GGC AGG TAG ATG GTC CCA GA-3'	PTPRG	Sense: 5'-CGT GCG GCA AGT CAA GTC C-3' Antisense: 5'-GGC TTC CAG GAT CGC ATC G-3'
PTPN14	Sense: 5'-CAG AGG AGG AGG GTG GAC G-3' Antisense: 5'-GAA CAT CCT CTG CTC CCT GAG G-3'	PTPRO	Sense: 5'-GCC GCC AAC GAC AAA CTC C-3' Antisense: 5'-GGC TCA CTC CAG CCA TGC AG-3'
PTPN18	Sense: 5'-CCA GCT ACA GTA TAT GTC CTG GCC-3' Antisense: 5'-CCT GTA CTG CTC CTC TGT CTG CAC-3'	PTPRQ	Sense: 5'-GGA GAA TGG TGT GGG AAA CCA G-3' Antisense: 5'-GGT GTC ATG TGC CCT GCT TG-3'
PTPN2	Sense: 5'-GGC GCT CTG GCA CCT TCT C-3' Antisense: 5'-CAT CTG CTG CAC CTT CTG AGC-3'	PTPRT	Sense: 5'-GGG ACA AGG ATG TGG CAA GG-3' Antisense: 5'-CCC CAA ACA GAG CCC ACA TC-3'
PTPN21	Sense: 5'-GGT CTA CAG CCA GCC CGA GA-3' Antisense: 5'-GCT CGC TGA CCT CCT GCA G-3'	PTPRZ	Sense: 5'-GCC AAG CGC CAT GCA GT-3' Antisense: 5'-CCC TTG ATC TTT CCA CAG GGA TG-3'
PTPN22	Sense: 5'-CTT CTC CCC CAC CTC CTC TCC-3' Antisense: 5'-CTG CAG GCT TGT TTG GTG GG-3'	SHP-1	Sense: 5'-GGC TGG CTT CTG GGA GGA G-3' Antisense: 5'-CCT GGC TGG CGA TGT AGG TC-3'
PTPN3	Sense: 5'-CGA GGA CGC CAG CCA GTA CTA C-3' Antisense: 5'-CTC CTG ATC ACC AGG GCC AG-3'	STNS	Sense: 5'-CAA GGA AGA CGC CGA GGA C-3' Antisense: 5'-CCT CCC ATT TGT AAG CTC CCA TC-3'
PTPN7	Sense: 5'-CAT CGC CAC GCG AAT TG-3' Antisense: 5'-GTC AGG GGC TGG GTT CCT C-3'		

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