PIAS1 mediates TGFβ-induced SM α-actin gene expression through inhibition of KLF4 function-expression by protein sumoylation

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Materials and Methods

Cell Culture and Transient Transfection and Luciferase Assays

BALBc3T3 cells, rat aortic SMC, and COS cells were cultured as previously described. Cells were transfected using Fugene (Roche) according to the manufacturer’s protocol. The cells were incubated 48 hours before being harvested with Passive Lysis buffer (Promega). Luciferase activity was measured with luciferase assay substrate (Promega), and was normalized to total protein (Coomassie Plus Protein Assay Reagent, Pierce) or Renilla luciferase activities using Dual-luciferase reporter assay system (Promega). Each experiment was used three samples and performed three times, respectively.

Construction of Short Interfering (si) RNA Plasmid, siRNA oligonucleotide and transfection

A plasmid-based system for production of shRNA was previously described. To generate the siRNA specific for PIAS1, an oligonucleotide (TTAAA TCCGGATCATTTTCTAGAGC TTCAAGAGAAGCTCTAGAATGATCGGAA TTTTTGGAAAAG; italic means specific sequence to rat PIAS1) was inserted downstream of an H1 promoter of a pMighty-Empty vector, and it was designated as pMighty-αPIAS1. Transfection of siRNA plasmid was carried out using Fugene
siRNA oligonucleotides (αPIAS1: UCCGGAUCAUUCUAGAGCU, αubc9: CUAUCAAUUUACUGCCAA, αEGFP: GAACGGCAUCAAGGUGAAC) were purchased from Hayashi Kasei Co., Ltd. and transient transfection of siRNA oligonucleotide was carried out using lipofectamine2000 (Invitrogen) according to the manufacturer’s protocol.

**Construction of Plasmids**

Expression plasmids for flag-tagged mouse PIAS1 in pCMV5 vector was a generous gift from Dr. Ke Shuai (Department of Medicine, University of California, Los Angeles, CA). C351S and pcDNA3-KLF4 were previously described [1, 4]. The -2555 to +2813 fragment of rat SM α-actin was subcloned into a pGL3-basic vector (Promega). Triple CArG mutants, double E-box mutants, and TCE mutant of the SM α-actin gene were described previously [1, 2, 6]. All constructs were confirmed by DNA sequencing.

**RNA extraction and Real-Time Reverse Transcription (RT)-PCR**

Total RNA was prepared from the cultured SMC using Isogen (Wako) according to the manufacturer’s protocol. One microgram of RNA was used for reverse transcription with RNA LA PCR Kit (Takara) followed by removing DNA with DNA-free kit (Ambion), and real-time RT-PCR analysis was performed using SYBR green (TOYOBO) according to the manufactures’ protocol. Each experiment was used three samples and performed three times, respectively.

Primer sequences were as follows: rat/human PIAS1-sense,
5’-TCCTGCTGTAGATACAAGCTAC-3’; anti-sense, TGCCAAAG
TGGACGCTGTGTC-3’; rat SM α-actin-sense,
5’-AGTCCGCCATCAGGAACCTCGAG-3’; anti-sense,
5’-ATCTTTTCCATGTGCTCCAGTTG-3’; human SM α-actin-sense,
5’-ATGAGGGCTATGCCTTGCCC-3’; anti-sense,
5’-CCCAGATGAAGATGGCTGGA-3’; human TGFβ-sense,
5’-GTACCTGAAACCCTGTTGCT-3’; anti-sense,
GTCCTTGCGGAGTCAATGT-3’; human BMP2-sense,
5’-CCCAGTTGGAGGAGAAACAA-3’; anti-sense,
5’-ACGTCTGAACATGGCATGA-3’; GAPDH-sense,
5’-AACGACCCCTTCCATTTGAC-3’; anti-sense, 5’-TCCACGACATCCTACGAC-3’;
human KLF4-sense, 5’-CCCACACAGGTGAGAAACCT -3’; anti-sense, 5’-
ATGTGTAAGGCAGGTGGTC- 3’

**Western blot analyses**

Whole cell extracts were prepared by using modified RIPA buffer containing
50 mM Tris-HCl (pH7.4), 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EDTA, 1 mM PMSF and 1 mg/mL aprotinin, leupeptin, pepstatin. The mixture was rotated at 4°C for 15 minutes and centrifuged at 2000 X g for 10 minutes. Western blot analysis was performed according to standard procedures using the following primary antibodies: monoclonal anti-flag antibody (M2; Sigma Chemical Co.), monoclonal anti-GAL4 DNA-BD antibody (Clontech), monoclonal anti-ubc9
antibody (BD Biosciences Pharmingen), polyclonal anti-SRF antibody (Santa Cruz Biotechnology, Inc.), monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Chemicon International, Inc)  Antigens were revealed by ECL (Amersham Biosciences) after incubation with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG.

**Mammalian two-hybrid assays**

Mammalian two-hybrid assays were performed as described previously. GAL4BD-KLF4 (full-length KLF4), VP16-PIAS1 and GAL4BD-SRF (full-length SRF) were constructed by PCR, and expression was confirmed by Western blotting. Expression plasmids for GAL4BD fusion protein and VP16 fusion protein were co-transfected into BALBc3T3 cells with pG5Luc reporter plasmid, and luciferase activity was measured. Each experiment was used three samples and performed three times, respectively.

**Immunoprecipitation analyses**

293T cells were transiently transfected with FLAG-PIAS1 and GAL4BD-KLF4 with the calcium phosphate precipitation method. Cells were harvested 72 hours after the transfection in phosphate-buffered saline (PBS) and were lysed in NETN buffer (20 mM Tris-HCl [pH 8], 100 mM NaCl, 1 mM EDTA, and 0.5% NP-40) supplemented with freshly prepared protease inhibitors (1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 2 µg/ml pepstatin A and aprotinin). Following brief sonication and the removal of cellular debris by centrifugation, KLF4
protein was precipitated with anti-GAL antibody and protein A/G sepharose beads (Amersham). The bound proteins were washed four times in NETN buffer, resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and transferred to nitrocellulose membranes. Membranes were immunoblotted with antibodies as indicated, and proteins were visualized with a chemiluminescence detection system (Millipore). Each experiment was performed three times and shown the representative data.

**In vivo sumoylation assays**

COS7 cells (1 x 10⁵ per 6-cm-diameter dish) were transfected using FuGENE 6 according to the manufacturer's instructions. After incubation, cells were lysed in 1 ml of RIPA buffer (25 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM dithiothreitol, 5 mM EDTA, 10 mM N-ethylmaleimide, 200 μM indole-3-acetic acid, and a complete protease inhibitor mixture tablet (Roche Applied Science)) for 30 minutes on ice. Cell debris was removed by centrifugation for 15 minutes. Lysates were first cleared with protein G beads for 30 min, followed by incubation with antibodies for 1 hour at 4 °C. Finally, the antibody complexes were captured with protein G beads for 1 hour. Beads were washed four times with the same buffer, and immunoprecipitates were eluted and analyzed by Western blot as described previously. Each experiment was performed three times and representative data shown.

**Immunohistochemistry**
All samples were immediately fixed in 10% buffered formalin and embedded in paraffin.

All tissues were stained with antibodies against SM α-actin (DACO) (dilution 1:200 in PBS).

**Statistical analyses**

Statistical analyses were performed using 1-way ANOVA or Student’s t test when appropriate. P values of less than 0.05 were considered statistically significant.

**References**


Figure legend

Supplemental Figure I. TGFβ did not alter expression of PIAS1 in cultured rat aortic SMC. Cultured SMC were treated with TGFβ for indicated times. mRNA expression of SMα-actin gene, PIAS1 and GAPDH was determined by real-time RT-PCR and ratios of SMα-actin and PIAS1 to GAPDH mRNA expression were calculated. An arbitrary value of 1.0 was assigned to the control. Values represent means ± standard errors of the mean. *, P of <0.05 compared with control.

Supplemental Figure II. PIAS1 interacted with KLF4 by co-immunoprecipitation (IP) assays using 293T cells.

Supplemental Figure III. Evaluation of SRF protein half-life. Cycloheximide (CHX) was added to COS cells at 48h after transfection with the indicated plasmids. Cell extracts were prepared as indicated time points and performing immunoblotting with anti-GAL4, anti-flag and anti-GAPDH antibodies. The relative amount of SRF protein was evaluated by densitometry and normalized to GAPDH. Each experiment was performed three times and the representative data were shown (n=3). An arbitrary value of 1.0 was assigned to the cells treated with cycloheximide for 0 hour.
Supplemental Figure II Kawai-Kowase K et al. ATVB 2008/172700

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IB:anti-Flag Ab (PIAS1)

IB:anti-GAL4 Ab (KLF4)