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DEDD negatively regulates transforming growth factor-β1 signaling by interacting with Smad3

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ABSTRACT

Transforming growth factor-β1 (TGF-β1) regulates a wide variety of cellular responses, such as proliferation, differentiation, migration and apoptosis. Here we report that death effector domain-containing DNA-binding protein (DEDD) physically interacts with Smad3. The inhibition of Smad3 by DEDD resulted in a reduction in TGF-β1/Smad3-mediated transcription. DEDD inhibited the functions of Smad3 by preventing Smad3 phosphorylation, which led to the reduced expression of TGF-β1/Smad3-targeted genes. TGF-β1 inhibited DEDD expression, and DEDD inhibited TGF-β1-mediated invasion. Therefore, our findings suggest that through its interaction with Smad3, DEDD is a novel negative regulator of the TGF-β1 signaling pathway.

Structured summary:
MINT-7895480: *DEDD* (uniprotkb:O75618) *physically interacts* (MI:0915) with *Smad3* (uniprotkb:P84022) by *anti bait co-immunoprecipitation* (MI:0006)

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1. Introduction

The transforming growth factor-β (TGF-β) family members, which include the TGF-βs, activins, nodals and bone morphogenetic proteins, are secreted cytokines that regulate a broad array of cellular responses including proliferation, differentiation, migration and apoptosis. The TGF-β1 signaling pathway is important in metazoan biology, and its dysregulation can often result in tumor development [1]. The TGF-β1 signaling pathway has been well described [1,2]. Although TGF-β1/Smad-mediated signaling is conceptually simple, the number of Smad-interacting proteins provides a high degree of signaling specificity and versatility [2].

Apoptotic signaling is regulated and mediated by specialized proteins that often possess protein-protein interaction domains. One of these domains is the death effector domain (DED), which is predominantly found in components of the death receptor-induced signaling complex that forms at death receptor family members following their ligation. DED-containing proteins include Fas-associated death domain protein (FADD), caspase-8 and death effector domain-containing DNA-binding protein (DEDD) [3]. DEDD plays an important role in CD95-mediated apoptosis by activating caspase-3 or caspase-6 as a scaffold protein [4–6]. A recent study found that DEDD can suppress the activity of the Cdk1/cyclin B1 complex and maintain S6K1 activity, suggesting that DEDD participates in regulation of the cell cycle and inhibits cell mitosis [7,8]. However, it is unclear whether DEDD, through interactions with key signaling proteins, is involved in other signaling pathways. Additionally, the pathophysiological functions of DEDD have not been fully elucidated.

Using a yeast two-hybrid screen designed to identify Smad3-interacting proteins, we have identified an interaction between Smad3 and DEDD. We investigated the mechanism and functional significance of this interaction and found that DEDD inhibits the function of Smad3 and suppresses TGF-β-mediated invasion. DEDD itself also blocks cell invasion, suggesting that DEDD functions as a tumor suppressor. To the best of our knowledge, this is the first report demonstrating that Smad3 interacts with DED-containing proteins.

2. Materials and methods

2.1. Cell culture and transfection

HEK 293T, HepG2 and HeLa cells were obtained from American Type Culture Collection (ATCC) and were grown in Dulbecco's

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modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). HEK 293T, HepG2 and HeLa cells were transfected using Lipofectamine (Invitrogen) according to manufacturer's instructions.

2.2. Antibodies and reagents

The following antibodies were used: anti-HA-tag (F-7, sc-7392), anti-Smad2/3 (FL-425, sc-8332), anti-Smad2/3 (N-19, sc-6032), anti-DEDD (C-18, sc-27051), anti-p21 (F-5, sc-6246) and anti-β-Actin (C4, sc-47778) (Santa Cruz); anti-phospho-Smad3 (Ser423/425, #9520) (Cell Signaling Technology); anti-Flag M2 (F-3165) (Sigma); anti-Myc-tag (562-5) (MBL International); and anti-GAPDH (KC-5G4) (KangChen Biotech). Recombinant human TGF-β1 was purchased from Peprotech.

2.3. Plasmids

The expression vector encoding Flag-DEDD was a gift from Dr. Marcus E. Peter (University of Chicago, USA). The vectors encoding Flag-N-DEDD and Flag-C-DEDD were gifts from Dr. Toru Miyazaki (University of Tokyo, Japan). The HA-FADD and Flag-caspase-8 expression vectors were gifts from Dr. Sug Hyung Lee (The Catholic University of Korea, South Korea). The Flag-Smad7 expression vector was a gift from Dr. Carl-Henrik Heldin (Uppsala University, Sweden). The SBE4-luc reporter plasmid was a gift from Dr. Bert Vogelstein (Johns Hopkins University, USA), and p3TP-luc was a gift from Dr. Joan Massagué (Memorial Sloan-Kettering Cancer Center, USA). pcDNA3.1-Myc-Smad2, -Smad3, -Smad4, pcDNA3.1-HA-DEDD, -GSK3β were generated by PCR and confirmed by sequencing.

2.4. Yeast two-hybrid screening

The yeast two-hybrid screen was performed according to Clontech's GAL4-based system. The full length Smad3 was ligated into the pGBKT7 vector to create the pBD-Smad3 plasmid. The construct was used to screen a human embryonic kidney library. The transformants were selected on yeast media lacking leucine and tryptophan and then transferred to quadruple dropout media also lacking adenine and histidine. Colonies that were viable on quadruple selection media were subjected to a β-galactosidase assay. False positives were eliminated by autoactivation test and co-transformation. The remaining positive clones were then shuttled into *Escherichia coli* DH5α cells, rescued and sequenced.

2.5. Co-immunoprecipitation and immunoblot analyses

HEK 293T and HepG2 cells were grown in 10 cm dishes and transfected with the appropriate plasmids. They were washed once with ice-cold PBS and lysed in ice-cold lysis buffer. After centrifugation, the supernatants were collected, and the protein concentration was measured using the Bio-Rad Protein Assay Kit. An equal amount of protein was used for immunoprecipitation, and the precipitated proteins were subjected to SDS–PAGE in 8%, 10% or 12% polyacrylamide gels and blotted onto polyvinylidene difluoride membranes, as previously described [9].

2.6. GST pull-down assays

The GST-Smad3 fusion protein was expressed in *E. coli* and purified using glutathione–sepharose 4B beads. Equal amounts of GST or GST-fusion proteins bound to glutathione–sepharose beads were incubated overnight with cell lysates from HEK 293T cells overexpressing HA-DEDD. The bound complexes were eluted and analyzed using an immunoblot.

2.7. Luciferase reporter assays

HEK 293T and HepG2 cells were transiently co-transfected with the empty vector; the Myc-Smad3 expression vector; the indicated DEDD construct or HA-FADD; and the SBE4-luc, p3TP-Luc reporter construct. Luciferase activity was measured using a Dual Luciferase Assay System (Promega).

2.8. siRNA

SMART pool siRNA duplexes specific for DEDD and a non-targeting siRNA (siControl) were purchased from Dharmacon. HepG2 cells were transfected with the DEDD-specific or control siRNA using Lipofectamine (Invitrogen) according to manufacturer's instructions. The cells were cultured in serum-free medium for the indicated time, and TGF-β1 was added 24 h after transfection.

2.9. RT-PCR

Total RNA was extracted using Trizol (Invitrogen). Reverse transcription of the total cellular RNA was carried out using oligo-dT primers and M-MLV Reverse Transcriptase (Promega) according to manufacturer's instruction.

2.10. Cell invasion assay

In preliminary study, we found that transfection of DEDD did not affect cell proliferation in HeLa cells which were used for the invasion assay. The cell density was adjusted to 2.5×105/ml, and 200 μl of this suspension was added to the top chamber of a 24-well transwell plate (Millipore; 8-μm pore size) coated with Matrigel. The medium in the upper chamber was serum-free DMEM, and the medium in the lower chamber was supplemented with 10% fetal bovine serum. After incubation for 20 h at 37 °C, the cells that invaded through the filter were stained and counted in six random fields of view at 200× magnification. The data are expressed as the average number of cells per field of view.

2.11. Statistical analysis

The data are expressed as the mean ± S.E. Statistical analyses were performed using a paired Student's *t*-test. A *p*-value less than 0.05 was considered significant.

3. Results

3.1. Identification of DEDD as a Smad3-interacting protein

A yeast two-hybrid screening system was used to identify proteins that interact with Smad3 and 34 positive clones were identified and sequenced. Of these clones, one contained a cDNA encoding DEDD. To validate the yeast two-hybrid screen results, pAD-DEDD and pBD-Smad3 were co-transfected into yeast AH109 cells, grown in high-stringency medium (SD-Ade/-His/-Leu/-Trp) and tested for β-galactosidase activity. As shown in Fig. 1A, yeast cells expressing pAD-DEDD and pBD-Smad3 grew in high-stringency medium and showed positive galactosidase activity. To examine the in vivo interaction between DEDD and Smad3, HEK 293T cells were first co-transfected with an HA-tagged DEDD expression vector and various Myc-tagged Smad expression vectors. DEDD could be co-precipitated with Smad3, slightly co-precipitated with Smad4 and inhibitory Smad7 proteins, but not with the highly homologous Smad2 (Fig. 1B). To test whether the DED of DEDD was needed for the binding of DEDD to Smad3, DEDD

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