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The catalytic triad of testes-specific protease 50 (TSP50) is essential for its function in cell proliferation

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ABSTRACT

Testes-specific protease 50 (*TSP50*) is a novelly identified pro-oncogene and it shares a similar enzymatic structure with many serine proteases. Our previous results suggested that TSP50 could promote tumorigenesis through degradation of IκBα protein and activating NF-κB signaling, and the threonine mutation in its catalytic triad could depress TSP50-mediated cell proliferation. However, whether the two other residues in the catalytic triad of TSP50 play a role in maintaining protease activity and tumorigenesis, and the mechanisms involved in this process remain unclear. Here, we constructed and characterized three catalytic triad mutants of TSP50 and found that all the mutants could significantly depress TSP50-induced cell proliferation and colony formation in *vitro* and tumor formation in *vivo*, and the aspartic acid at position 206 in the catalytic triad played a more crucial role than threonine and histidine in this process. Mechanistic studies revealed that the mutants in the catalytic triad abolished the enzyme activity of TSP50, but did not change the cellular localization. Furthermore, our data indicated that all the three mutants suppressed activation of NF-κB signal by preventing the interaction between TSP50 and the NF-κB:IκBα complex. Most importantly, we demonstrated that TSP50 could interact with IκBα protein and cleave it directly as a new protease in *vitro*.

1. Introduction

Serine proteases contain a large family of enzymes that are characterized by possessing an active serine in their catalytic site, and they play a critical role in numerous physiologic processes including digestion, blood coagulation, complement activation, fibrinolysis, reproduction, embryonic development, protein processing and tissue remodeling [1–6]. Serine proteases maintain a strictly conserved active site geometry among their catalytic Ser, His and Asp residues; this shared catalytic structure suggests that common architectural motifs are likely to be found in the molecular designs of active sites utilizing a Ser-His-Asp triad [5]. The substitution of either His57 or Ser195 to Ala is sufficient to completely disable the catalytic triad, and the substitution of Asp102 to Asn in trypsin decreases k_{cat}/K_m by 10^4 at neutral pH [7]. Increasing evidences indicates that some serine proteases, which have been traditionally viewed as degradative enzymes, are also signaling molecules that regulate multiple cellular functions by activating specific receptors [8–10], protease receptors such as PAR-1, PAR-3 and PAR-2, were cleaved and activated by thrombin and trypsin-like enzymes respectively [11–13].

TSP50 is a testis-specific gene encoding a protein that is homologous to serine proteases, and it shares two critical catalytic triads, histidine and aspartic acid at positions 153 and 206, respectively. However, the most crucial triad, serine, at position 310 was replaced by threonine and it possessed enzymatic activity [14,15], suggesting that TSP50 might represent a novel type of protease. When the catalytic threonine residual of TSP50 is replaced by alanine, it was unable to cleave β-casein [15]. Our recent results showed that TSP50 T310A mutation significantly depressed TSP50-induced cell proliferation, colony formation, resisting apoptosis, cell migration and cell invasion in *vitro* and abolished the oncogenicity of TSP50 in *vivo* [16]. These results strongly indicated

Abbreviations: Bcl-2, B cell lymphoma/leukemia-2; BrdU, 5-bromo-2′-deoxyuridine; CHO cell, Chinese-hamster ovary cell; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HEK 293T, human embryonic kidney 293T cells; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-κB, nuclear factor κB; PMSF, phenylmethanesulphonyl fluoride; PMA, phorbol myristate acetate; RT-PCR, reverse transcription-PCR;TSP50, testes-specific protease 50.

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that the threonine in the catalytic triad of TSP50 is essential to its function in tumorigenesis. However, whether this is correlated to protease activity of TSP50, the role of the two other residues in catalytic triad of TSP50 in protease activity and tumorigenesis, and the mechanisms involved in this process remain unclear.

In addition to expression in normal testes, *TSP50* was abnormally expressed at high level in many breast cancer and colorectal carcinoma biopsies tested [17–19]. Previously, we revealed that overexpression of *TSP50* efficiently promotes cell proliferation and tumor formation through the activation of the NF-κB signaling pathway, and TSP50 can promote the degradation of κBα proteins by binding to the NF-κB: IκBα complex [20]. However, whether TSP50 can interact with IκBα directly and cleave it as a protease is unknown.

In this paper, we mainly studied the effects of the histidine and aspartic acid mutation in the catalytic triad of TSP50 on its function in tumorigenesis and the mechanisms involved this process. We provided evidences that the mutations in the catalytic triad of TSP50 could significantly depressed TSP50-mediated cell proliferation and tumor formation, and aspartic acid at positions 206 played a much more important role in maintaining the function of TSP50.

2. Materials and methods

2.1. Antibodies and reagents

Polyclonal antibodies against p65, IκBα, Histone1, C-Myc, COX-2, cyclinD1, Ki-67, β-actin and Bcl-2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibody against GAPDH was purchased from Kangcheng Biotech (Shanghai, China). Anti-TSP50 monoclonal antibody was prepared in our laboratory [21]. The site-directed Gene Mutagenesis Kit and the Calcium Phosphate Cell Transfection Kit were purchased from Beyotime (Shanghai, China). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The 5-bromo-2′-deoxyuridine (BrdU) labeling and detection ELISA kit was purchased from Roche Diagnostics (Mannheim, Germany). The Pierce Crosslink IP Kit was bought from Thermo Scientific (no: 26147).

2.2. Cell lines and cell culture

CHO cells (Chinese hamster ovary cells) and HEK 293T cells (human embryonic kidney 293T cells) were obtained from the Chinese Academy of Sciences Shanghai Institute for Biological Sciences-Cell Resource Center, which had characterized the cell lines by short tandem repeat profiling, cell morphology and karyotyping assay. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Invitrogen, USA), which was supplemented with 10% fetal bovine serum (TBD Science, Tianjin, China), 100 U/mL penicillin and 100 μg/mL streptomycin (Ameresco, USA), at 37°C with 5% CO_2.

2.3. Plasmid constructs and transfection

The EGFP fusion protein plasmids and the pNF-κB-luc plasmid were prepared in our laboratory. The three point mutation constructs of TSP50, T310A, D206A and H153A originated from the construct pcDNA3.0-TSP50 were achieved using Sit Directed Mutagenesis Kit. The primer sequences used were as follows: TSP50 T310A-For: 5′-GTTCTGCTATGAGCTAGCTGGAGAGCCCTTGGTC-3′, Rev: 5′-GACCAAGGGCTCTCCAGCTAGCTCATAGCAGAAC-3′, TSP50 D206A-For: 5′-GTGGGCCAGGCCAACGCCATCGGCCTCCTCAAG-3′, Rev: 5′-CTTGAGGAGGCCGATGGCGTTGGCCTGGCCCAC-3′, TSP50 H153A -For: 5′-GGTGCTGACTGTGGCCGCCTGCCTGATCTGGCGTG-3′, Rev: 5′-CACGCCAGATCAGGCAGGCGGCCACAGTCAGCACC-3′. The *TSP50* and its point mutation constructs were cloned into the pcDNA3.0 basic vector. Cells were transfected with the T310A, D206A, and H153A plasmids per well using Lipofectamine™2000 (Invitrogen), according to the instructions of the manufacturer. Transfected cells were incubated in the presence of G418 for 2 weeks and the stable transfected cell lines were selected.

2.4. RNA extract and RT-PCR

Total RNA was prepared from cultured cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The RT-PCR kit was bought from transGgenbiotech. Total RNA (3 μg) was reverse transcribed into cDNA by incubating at 50 °C for 60 min. TSP50 mRNA was amplified from cDNA templates by RT-PCR using the primers 5′-CGGATCCATGCAGGGGAAGCC-3′ (sense) and 5′-GCTCTAGAAGTCAGAGGGCAG-3′ (antisense), β-actin primers 5′-TCGTGCGTGACATTAAGGAG-3′ (sense) and 5′-ATGCCAGGGTACATGGTGGT-3′ (antisense). PCR was performed for 30 cycles (each cycle consisting of 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 30 s). The PCR products were analyzed by electrophoresis on a 1% agarose gel stained with ethidium bromide and visualized under UV light.

2.5. Western blotting assay

Cytosolic and nuclear extracts were prepared and Western blotting was performed as described previously [22].

2.6. MTT assay

Cell growth was determined by MTT assay. The cells were plated at 1×10^4 cells per well in 96-well microtiter plates. After incubation for the indicated time, MTT (5 mg/mL) was added to each well and incubated for 4 h. The absorbance was recorded on a microplate reader at a wavelength of 570 nm.

2.7. Colony formation in soft agar

One milliliter base agar (1.2% agar plus 2 × DMEM in equal volumes) was added to six-well plates. Twenty four hours prior to initiating the soft agar assay, 4×10^3 cells/well were added in 1 mL of top agar (0.7% agar mixed with 2× DMEM in equal volumes) to each well. Plates were maintained at 37 °C for 14 days before colonies were counted.

2.8. BrdU incorporation assay

DNA synthesis was assessed by measuring the incorporation of BrdU into newly synthesized strands. Cells were replated at 1×10^3 cells/well on 96-well plates. Twenty-four hours after plating, BrdU labeling was initiated by adding the labeling solution at a final concentration of 10 μM to the culture medium. After the cells were incubated for 6 h, labeling was stopped and BrdU uptake was measured according to the protocol of the manufacturer.

2.9. Analysis of luciferase activity

Firefly luciferase activity was measured 48 h after transfection. Analysis of luciferase activity was performed as described previously [23].

2.10. Immunofluorescence detection

The localization of p65 and Ki-67 staining were carried out in accordance with previously described procedures [24].

2.11. Co-immunoprecipitation

Co-immunoprecipitation experiments were performed by using the Pierce Crosslink Immunoprecipitation kit from Thermo Scientific according to the standard protocol of the manufacturer.