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RESEARCH ARTICLE

Role of the domains of human gene ZNF569 in MAPK pathway

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Abstract Mitogen-activated protein kinase (MAPK) signal pathways are important components in signal transduction connecting cell-surface receptors to critical regulatory targets within cells, mediating multiple intracellular signal cascades and phosphorylating their target proteins, such as transcriptional factors ELK-1, SRE and AP-1, and finally activating the expression of intracellular functional genes. Zinc finger genes are some of the largest gene families in humans, and Zinc finger proteins play an essential role in altering gene expression by acting as transcription factors. Zinc finger proteins are also involved in multiple cell processes, including proliferation, differentiation and development, by interacting with DNA. Here, we reported the transcriptional activities of the domains of zinc finger gene ZNF569 taking advantage of MAPK pathway. Overexpression of ZNF569 in COS-7 cells dramatically inhibited the transcriptional repressor activities of SRE and AP-1, which was also confirmed by subcellular localization analysis. Report assays indicated that the potent repression domains of ZNF569 were the KRAB and ZNF motifs. The results suggested that ZNF569 protein might act as a transcriptional repressor in MAPK signaling pathway to regulate cellular processes.

Keywords ZNF569, Zinc finger protein, MAPK, Transcription factor

1 Introduction

Mitogen-activated protein kinase (MAPK) pathways are important components in signal transduction (Treisman,1996). Previous studies showed that MAPK pathways can mediate multiple extracellular signals, such as insulin signals, epidermal growth factors, by connecting transmembrane receptors like G-protein-coupled receptors to intracellular critical regulatory targets through signal cascades and activate a number of transcriptional factors like ELK1, SRE, AP-1, MEF2 and ATF2 to regulate functional cellular gene expression (Gille et al., 1995; Kyriakis and Avruch, 2001). MAPK pathways are also involved in cells transducing a variety of extracellular growth and stress stimuli into intracellular response, such as proliferation, differentiation, damage repair mechanism and cell death (Bogoyevitch et al., 1995). Zinc finger proteins are DNA-binding proteins explored in the middle 1980s. They have repeated finger-shaped secondary structure and each finger contains 23 amino acid residues with four conserved residues combined a Zn^{2+} to form the zinc finger loop (Makoto et al., 2001; Wu, 2002). More than 600 zinc finger proteins were discovered, and each ZFP may have a different number of repeated zinc finger motifs, and each motif binds to 5 nucleotide-specific DNA sequence by inserting into the major grooves of the DNA double helix (Sauer and Jackle, 1991). Most zinc finger motifs tend to recognize GC-rich sequences, especially 5'GNN3' targets. However, some zinc finger motifs can recognize AT-rich sequences. Some zinc finger proteins can also bind to RNA and mediate interactions between proteins. For example, ZNF74 can only recognize specific RNA sequence as well as protein WT1 and p53 (Senatore et al., 1999; Gou et al., 2001; Ganss et al., 2002).

Zinc finger gene *ZNF569* encodes a predicted protein with 686 amino acids, which contains a N-terminal Krüppel-associated box (KRAB) domain and a series of 18 C2H2 zinc finger motifs. *ZNF569* is strongly expressed in adult heart, liver and placenta but weakly expressed in skeletal muscles and pancreas. *ZNF569* is also expressed in embryo heart, skeletal muscles and liver. This paper analyzed the transcriptional activities of the whole length cDNA and the truncated protein of ZNF569 in MAPK pathway. Our result showed that overexpression of ZNF569 in COS-7 cells dramatically inhibited the transcriptional repressor activities of the MAPK downstream transcriptional factors SRE and AP-1. This was also confirmed by

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ZNF569 subcellular localization analysis. Report assays indicated that the potent repression domains of ZNF569 are the KRAB and ZNF motifs. These results suggested that ZNF569 protein might act as a transcriptional repressor in MAPK signaling pathway to regulate cellular processes.

2 Materials and methods

2.1 Plasmid constructs

2.1.1 Construction of recombinant pCMV-Tag2C-ZNF569 ORF and pCMV-BD-ZNF569 ORF

The open reading frame of ZNF569 from T vector pMD18-T-ZNF569 was subcloned into the Sac I and Apa I sites of plasmid pCMV-Tag2C and pCMV-BD, respectively. The recombinants were confirmed by digestion analysis and sequencing.

2.1.2 Construction of recombinant pCMV-Tag2C-ZNF569 domains and pCMV-BD-ZNF569 domains

The full length cDNA of ZNF569 was divided into 4 fragments: fragment KRAB contains amino acids 1–95 (the whole KRAB domain), fragment 7ZFs 250—441 (7 zinc fingers), fragment 18ZFs 172–686 (the whole 18 zinc fingers) and fragment KRAB-9ZFs 1–249 plus 442–686 (including KRAB domain and 9 zinc fingers). The 4 fragments were subcloned separately into plasmid pCMV-BD to get recombinants GAL4-KRAB (aa 1–95), GAL4-7ZFs (aa 250–441), GAL4-KRAB-9ZFs (aa 1–249, 442–686), GAL4-18ZFs (aa 172–686), pCMV-Tag2C-KRAB (aa 1–95) and pCMV-Tag2c-18ZFs (aa 172–686). Fragment KRAB and 18ZFs were amplified by PCR then cloned to plasmid pMD18-T. All purified kits were from Sangon Tech. in Shanghai. The primers were the following:

PKRAB1: 5′-TGTCGACAAGAAGAGGAAATGACT-3′
PKRAB2: 5′-AAAGGGCCCTCTTAGT-3′
PZF1: 5′-CATGTCGACATAGCTCATCCCATTT-3′
PZF2: 5′-TCGGGCCCTTTCTAATGAGTATGAA-3′

2.1.3 Construction of recombinant ZNF569-pEGFP-N1

The open reading frame of ZNF569 from T vector pMD18-T-ZNF569 was subcloned into the Sac I and Pst I site of plasmid pEGFP-N1 as before.

2.2 Cell culture and transient transfection

Hele cells were cultured in monolayer in DMEM (Dulbecco’s Modified Eagle Medium; GIBCO BRL) supplemented with 10% fetal calf serum (FCS). Cells were collected after being digested with trypsin 24 hours before transfection and cultured in complete medium with a density of 1×10^5 to 4×10^5 in 35 mm Petri dishes for 20–24 hrs in a humidified atmosphere of 95% air and 5% CO_2. Medium was changed 1 hour before transfection. Twenty-five µg plasmid was mixed with 100µL 2.5 mol/L CaCl2 solution in a 5 mL plastic spinner flask. An equal volume of 2 × HEPES solution was then added to the mixture. We flipped the flask wall to mix them and allowed to stand at room temperature (25°C) for 1 min. The DNA mixture was then transferred to the monolayer cell dishes immediately. The medium was removed and cells were washed twice with 5 mL 1 × PBS solution and cultured in an incubator overnight at 37°C in an atmosphere of 5%–7% CO_2 after the addition of 10 mL fresh medium.

2.3 Subcellular localization analysis

Fourty-eight hours after pEGFP-N1-ZNF569 transfection, cells were fixed with 4% paraformaldehyde for 15 minutes and washed with PBS three times. The nucleus was then stained with 4′, 6′-diamidino-2-phenylindole hydrochloride (DAPI). Subcellular localization of the EGFP-ZNF569 fusion protein was detected using a Nikon inverted fluorescence microscope. Transfected pEGFP-N1 was used as a positive control.

2.4 Co-transfection and the luciferase activity assay

Method of co-transfection was the same as the standard transfection (Huang et al. 2004). The luciferase activity assay performed were as follows: (1) Cells for transfection were cultured for 2 days, then the medium was removed by vacuum; (2) Cells were then washed thoroughly two times with PBS, 1 mL for one well each time; (3) 80 µL lysis buffer (stored at –20°C) was added to each hole to induce cell cleavage; (4) Cells were then allowed to stand at room temperature for 15min, then at -80°C for 10 min, and finally at 37°C for 10–15min; (5) Cells were dislodged with a clean plastic plate and pelleted by centrifugation for 4 min at 12000 rpm; (6) Supernatant was transferred to a new EP tube, with 20 µL was transferred again to another EP tube for ONPG assay, and the remaining 80 µL stored at -80°C; (7) The EP tube which contained 20 µl supernatant for ONPG assay was incubated at 37°C for 30 min; (8) 50 µL 1 mol/L trisbase was added to the EP tube to end the ONPG reaction; (9) 300 µL distilled water was then added to each tube before the absorption spectrum was measured via spectrophotometry at 420 nm; (10) Relative luciferase activity was normalized for transfection efficiency by co-transfection with pCMV-lacZ and spectrophotometry analysis; (11) Each experiment was performed in triplicate and each assay was repeated at least three times. The mean of the data from three individual transfected wells were presented after normalization for β-galactosidease activity. Lex A-VP16 was used to enlarge the luciferase reporter

gene activity found in pL8G5, pSRE and pAP-1.

3 Results

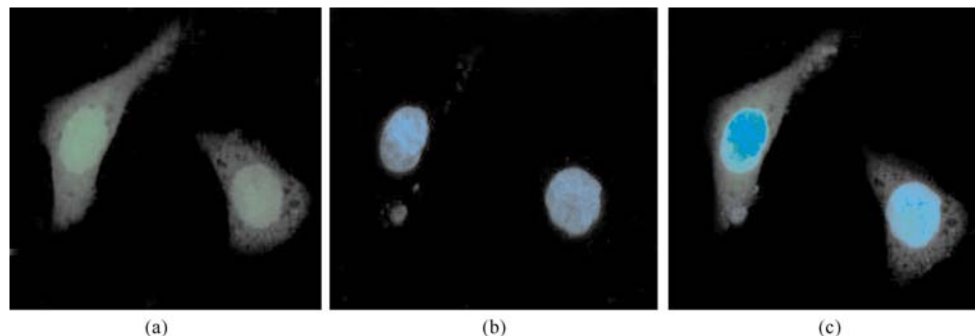
3.1 Subcellular localization analysis of ZNF569

Since ZNF569 protein contains a KRAB box and 18 C2H2 type zinc finger motif, we predicted this protein should be localized in the nucleus, and it should be a nuclear protein (Zhou et al., 2002; Luo et al., 2002). The pEGFP-N1-ZNF569 was transfected into Hele cells, and 48 hours after the transfection, the cells were studied under fluorescence microscope after being labeled with DAPI for nucleus. The EGFP-N1-ZNF569 fusion protein was clearly localized in the nucleus of Hele cells, but EGFP-N1 alone was expressed in the whole cell (Fig. 1). This result confirmed that ZNF569 was localized in the nucleus and that it served as a nuclear transcription factor.

3.2 Transcription activity analysis on the open reading frame and domains of ZNF569

In order to measure the transcription activity of ZNF569, we introduced a two-hybrid system into our research. As showed in Fig. 2, the two-hybrid system consisted of 2 plasmids. One was pL8G5-Luc, the other pCMV-BD. pL8G5-Luc contained 8 binding sites of the LexA domain, 4 binding sites of the GAL4 domain and a downstream luciferase reporter gene. ZNF569 ORF was subcloned downstream but contiguous to the GAL4-BD domain of pCMV-BD. When recombinant pCMV-BD-ZNF569 was expressed in COS-7 cells, fusion protein GAL4-BD-ZNF569ORF would be produced. This fusion protein then bound to the GAL4 binding sites of pL8G5-Luc when co-transfected, which in turn activated the expression of the luciferase reporter gene. When the positive control pLex A-VP16 was co-transfected, LexA-BD was seen to bind to its binding site on pL8G5-Luc, and the transcription activity of the reporter gene largely enhanced.

In this experiment, we constructed recombinant GAL4-ZNF569 ORF and pCMV-Tag2C-ZNF569 ORF. Transient transfection results showed that ZNF569 was a transcription repressor, which inhibited the luciferase activity of the reporter gene ten times compared with the control (Fig. 3). Co-transfection of pCMV-Tag2C-ZNF569 ORF and pSRE or pAP1 also indicated that ZNF569 could inhibit the transcription activity of SRE or AP1 about five times (Fig. 4). All these results suggested that ZNF569 acted as a transcription repressor in MAPK pathway.



(a): GFP localization; (b): DAPI stained nucleus; (c): the cofocal image of (a) and (b)

Fig. 1 Localization of EGFPN1-ZNF569 in COS-7 cells

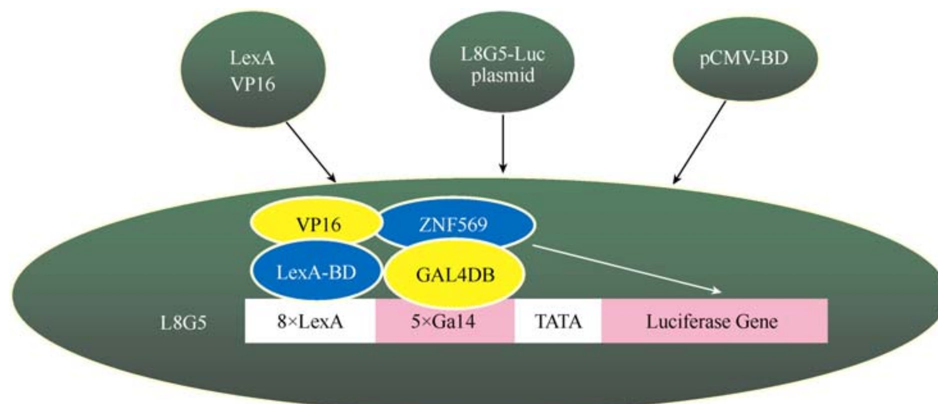


Fig. 2 Two-hybrid system

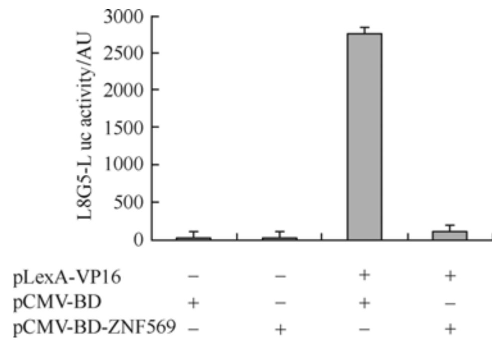


Fig. 3 The inhibition activity of ZNF569

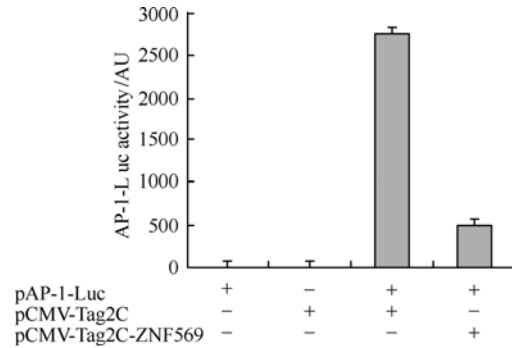
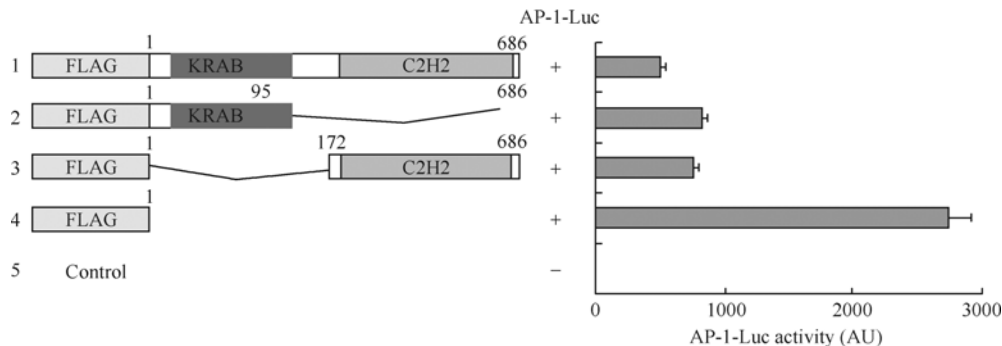


Fig. 4 ZNF569 inhibits AP-1 transcriptional activity



1: GAL4-ZNF569; 2: GAL4-KRAB; 3: GAL4-18ZFs; 4: GAL4; 5: no SRE

Fig. 5 The ORF and domains of ZNF569 inhibit SRE transcriptional activity

Which segment of ZNF569 played the role of transcription repressor? To address this question, we divided the full length cDNA of ZNF569 into 4 fragments. We co-transfected pL8G5 with GAL4-ZNF569, GAL4-KRAB, GAL4-18ZFs, GAL4-KRAB-9ZFs and GAL4-7ZFs, respectively. The results showed that GAL4-KRAB-9ZFs, which contained both KRAB and 9 zinc finger motifs, inhibited the reporter gene expression most, almost equaling to ZNF569 ORF. GAL4-7ZFs had the least inhibitory effect. GAL4-KRAB and GAL4-18ZFs had a moderate inhibition. Taken together, the repressor domains of ZNF569 were KRAB and C2H2 domain. This conclusion was identical with the experiment involving the co-transfection of pSRE and pAP-1 with pCMV-Tag2C-KRAB, pCMV-Tag2c-18ZFs and pCMV-Tag2c-ZNF569 ORF respectively, which showed that the full length open reading frame of ZNF569 inhibited the transcription activity of SRE and AP-1 five times, while KRAB domain or 18ZFs domain alone only inhibited SRE transcription activity three times, respectively.

4 Discussion

It is estimated that among all zinc finger proteins, about one-third of them are C2H2 type proteins. C2H2 zinc finger motif contains 20−30 amino acid residues, including two conserved Cys and His residue, forming a C-2-C-X-H-3-H structure. Previous studies showed that C2H2 zinc finger proteins function as transcriptional factors and play an important role in many physiological processes (Wu, 2002). Many C2H2 transcriptional factors contain other conserved domains except C2H2, such as KRAB (Collins and Stone, 2001). KRAB domain is consisted of 75 amino acid residues, usually encoded by 2 exons, tethered to the template DNA by a DNA-binding domain (Mark and Hellman, 1999; Dang et al., 2000). The exact functions of these C2H2 zinc finger proteins with KRAB domain remain unclear, but most of them were found involved in cell differentiation and development by interacting with DNA acting as transcriptional factors, and regulating target gene expression (Vissing et al., 1995). MAPK is a kind of protein kinase-joining signal transduction, which interacts with many transcriptional factors, such as AP-1 and SRE, that is involved in multiple responses like those to neurotransmitters, hormones, growth factors, cellular factors, stress stimuli and so on (Peng et al., 2000; Shaulian and Karin, 2002). In this paper, we first confirmed that ZNF569 is a nuclear protein through subcellular localization analysis, then we analyzed the transcriptional activities of the whole length ORF of ZNF569. Our results showed that overexpression of ZNF569 in COS-7 cell line significantly reduced the endogenous SRE- and AP-1-luciferase activities. The truncated protein analysis showed that the repressor domains of ZNF569 are KRAB domain and C2H2 zinc finger motif. Therefore, it is possible that, via the KRAB and zinc finger domains, ZNF569 protein is involved in MAPK pathway to regulate cellular processes.

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