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NRAGE induces β-catenin/Arm O-GlcNAcylation and negatively regulates Wnt signaling

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ABSTRACT

The Wnt pathway is crucial for animal development, as well as tumor formation. Understanding the regulation of Wnt signaling will help to elucidate the mechanism of the cell cycle, cell differentiation and tumorigenesis. It is generally accepted that in response to Wnt signals, β-catenin accumulates in the cytoplasm and is imported into the nucleus where it recruits LEF/TCF transcription factors to activate the expression of target genes. In this study, we report that human NRAGE, a neurotrophin receptor p75 (p75NTR) binding protein, markedly suppresses the expression of genes activated by the Wnt pathway. Consistent with this finding, loss of function of NRAGE by RNA interference (RNAi) activates the Wnt pathway. Moreover, NRAGE suppresses the induction of axis duplication by microinjected β-catenin in Xenopus embryos. To our surprise, NRAGE induces nuclear localization of β-catenin and increases its DNA binding ability. Further studies reveal that NRAGE leads to the modification of β-catenin/Arm with O-linked beta-*N*-acetylglucosamine (O-GlcNAc), and failure of the association between β-catenin/Arm and pygopus(pygo) protein, which is required for transcriptional activation of Wnt target genes. Therefore, our findings suggest a novel mechanism for regulating Wnt signaling.

1. Introduction

Neurotrophin receptor-interacting MAGE homolog (NRAGE), also known as MAGE-D1 or Dlxin-1, belongs to the type II melanoma antigen-encoding gene family [1]. Recent studies show that NRAGE plays an essential role in the regulation of proliferation and odontoblastic differentiation of mouse dental pulp cells mDPCs [2]. In addition, NRAGE has been shown to induce cell death and apoptotic activity via a JNK dependent pathway [3]. In a previous study, we demonstrated that NRAGE can disrupt the association of E-cadherin and β-catenin and decrease the total protein level of β-catenin [4].

O-linked *N*-acetylglucosamine (O-GlcNAc) is a novel protein modification that participates in regulating several cell-signaling pathways, such as insulin signaling and stress responses [5]. The O-GlcNAc modification is mediated by joint actions of O-GlcNAc transferase (OGT), which catalyzes the addition of O-GlcNAc, and O-GlcNAcase (OGA), which removes O-GlcNAc from proteins [6]. It has been reported that O-GlcNAcylation of β-catenin leads to reduced β-catenin transcriptional activity [7].

In the present study, we explored the effects of NRAGE on the regulation of Wnt/β-catenin signaling. Our results revealed that NRAGE induces nuclear transportation of β-catenin and enhances its DNA binding ability, but suppresses the Wnt pathway. Moreover, we found that NRAGE activity can induce the O-GlcNAcylation of β-catenin.

2. Materials and methods

2.1. Antibodies, plasmids and recombinant adenoviruses

Anti-Myc (9E10), anti-HA (F-7), anti-β-catenin (Mouse E-5, Rabbit H-102), anti-cyclin D1 (Mouse HD11) antibody were purchased from Santa Cruz Biotechnology. Anti-α-actin (Ab-1) was purchased from Oncogen. Anti-GFP antibody was purchased from Roche Molecular Biochemical. The O-GlcNAc Western blotting kit (including general anti-*O*-GlcNAc antibody 110.6) was bought from

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Pierce. WGA-agarose was purchased from Sigma.

The adenovirus carrying NRAGE cDNA was made according to the system introduced by He et al. [8]. In brief, NRAGE with a myc-tag was inserted between *Bgl*II and *Hin*d III of pShuttle and recombined with pAdEasy-1 in BJ5183 bacteria, and then the virus was packaged in 293A cells. Multiplicity of infection (MOI) was tested according to the application manual of AdEasyTM (Quantum Biotechnologies). NRAGE was also subcloned into pEGFPc3 (BD, Clontech) between *Xho*I and *Sac*II by RT-PCR using 5′ AAACTCGAGATGGCTCAGAAAATGGACT3′, as a forward primer and 5′AAACCGCCGCTCAACCCAGAAGAAACCAATG3′, as a reverse primer and confirmed by sequencing. The small RNA interference plasmid targeting hNRAGE was constructed by inserting a 19-nt fragment into pSuper-EGFP1 vector under the control of the H1 promoter. The sequence of the inserted fragment is GATGAAAGTGCTGAGATTC.

TOPFlash and FOPFlash plasmids were purchased from Invitrogen. Myc-tagged Pygo (amino acids 105 to 815) and HA-tagged Arm (amino acids 128 to 844) were gifted by Dr. Xinhua Lin. cyclin D1-luciferase reporter and cDNA of human beta-catenin were gifted by Dr. Bin Xie. cDNAs of human pygopus1 and pygopus2 were gifted by Dr. K. Basler and subcloned into pCMV-script vector (Stratagene).

2.2. Cell culture, transfection and reporter assays

HEK293 and 293A cells were cultured in DMEM. U2-OS cells were cultured in RPMI Medium 1640. Cells were supplied with 10% (v/v) fetal bovine serum (FBS) and cultured at 37 °C in humidified air with 5% CO2. For reporter assays, U2-OS, HEK293 and PC12 cells were first transfected with the indicated reporters and pCMV-β-gal using FuGENE-6 (Roche Diagnostics) or Lipofectamine (Invitrogen) for 24 h and then cultured with Ad-mycNRG or Adv as control for another 24 h. Cells were lysed and processed for measuring luciferase activity using a luciferase reporter gene assay kit (Roche); luciferase activity was normalized to β-gal activity.

2.3. Axis formation assays

The NRAGE and beta-catenin cDNAs were subcloned into the pCS2+ vector. Messenger RNAs were synthesized from linearized vectors using Sp6 RNA polymerase. The RNAs were injected into the marginal zones of early-stage Xenopus embryos. GFP mRNA was co-injected as a marker for injection sites.

2.4. Immunofluorescence

Cells were cultured on glass coverslips, fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. The coverslips were incubated with rabbit anti-β-catenin antibody (1:200) at room temperature for one hour. The secondary antibody is Rhodamine-conjugated goat anti-rabbit immunoglobulin G (1:500) (Molecular Probes). Images were acquired using an epi-fluorescence microscope (Leica).

2.5. FACS analysis

Cells were collected and fixed with cold ethanol and then resuspended in 1 ml solution containing 50 mg/ml RNase A and 50 mg/ml propidium iodide. At least 15,000 cells were collected at each treatment by FACScan and analyzed with the CellQuest program (Becton Dickinson).

2.6. Immunoprecipitation and immunoblotting

Cells cultured in 100 mm plates were lysed in 1 ml of NP-40 buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 10% glycol, 0.5 mM DTT) in the presence of protease inhibitors (Roche), scraped and centrifuged. For immunoprecipitation, 0.5 ml of cell lysates were incubated with indicated antibodies for one hour at 4 °C. The complexes were collected by binding to protein A-Sepharose beads (Roche) for 4 h or overnight at 4 °C. After three washes with lysis buffer, the immunoprecipitates were subjected to western blotting. Proteins were detected using the BM chemiluminescence western blotting kit (Roche). O-GlcNAc modified proteins were detected using the O-GlcNAc Western blotting kit (Pierce).

2.7. RNA extraction and reverse transcription PCR

RNAs were extracted using the Trizol reagent (Invitrogen). Reverse transcription was performed with oligo-dT_{15} primer using Superscript II RT (Invitrogen). PCR was performed using the Promega PCR kit. Sequences of primers specific for MMP7 and β-actin were as follows: MMP7-FOR, 5′-TGGAGTGCCAGATGTTGCAG-3′; MMP7-REV, 5′-TTTCCATATAGCTTCTGAATGCCT-3′; β-actin-FOR, 5′-CGGCATCGTCACCAACTGG-3′; β-actin-REV, 5′-GCTGGAAGGTG-GACAGCGA-3′.

2.8. Chromatin immunoprecipitation_(ChIP) and gel retardation assays

U2-OS cells were infected with indicated dose of Ad-mycNRG and Adv control for 24 h in 100 mm dishes. ChIP assays were carried out as described. Briefly, cells were fixed with 1% formaldehyde, lysed, centrifuged and cell nuclei were resuspended in nuclear lysis buffer. The resulting lysate was sonicated 4 times for 15 s each on ice and cleared by centrifugation. Samples were incubated with 2 μg anti-β-catenin antibody or normal IgG and immunoprecipitated by protein-A sepharose and then washed. Cross-linking was reversed at 65 °C for 4 h. DNA was purified with a PCR purifying kit (QIAGEN) and amplified with 32 cycles of PCR annealing at 60 °C. The following primers for amplifying indicated promoter were used: cyclin D1-FOR, 5′-GAGCCACCTCCACCTCACC-3; cyclin D1-REV, 5′-GCTGCTACTGCGCCGACA-3′; MMP7-FOR, 5′-ATCATCTTGGCCTCACTTTC-3′; MMP7-REV, 5′-CTCACCTTC-CACGTCCCTTA-3′. For gel retardation assays, extracts were prepared from intact nuclei that were washed three times to avoid contamination with cytoplasmic β-catenin. We used a double-stranded 18-nucleotide oligomer GCACCCTTTGATCTTACC as the TCF4 probe; the control probe was GCACCCTTTGGCCTTACC. Five micrograms of nuclear protein, 0.1 ng of ^{32}P-labeled probe, and 100 ng of deoxyinosine-deoxycy-tidine (dIdC) were incubated in binding buffer (60 mM KCl, 1 mM EDTA, 1 mM DTT, and 10% glycerol) with total volume of 20 μl for 30 min at room temperature and separated by 4% non-denatured PAGE.

3. Results and discussion

NRAGE, a neurotrophin receptor p75 interacting MAGE homologue, has emerged as an important regulator for cell proliferation and apoptosis [9]. For functional analysis, we generated the adenovirus carrying the human NRAGE gene with a Myc tag at its N-terminal (Ad-mycNRG). In our previous study, we found that U2-OS cells infected with Ad-mycNRG for 40 h displayed cell cycle blockage, compared with cells infected with control adenovirus (Adv). The adhesive property of NRAGE expressing cells was also altered as significant numbers of cells detached from the bottom

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