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ARHGAP21 is a RhoGAP for RhoA and RhoC with a role in proliferation and migration of prostate adenocarcinoma cells

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ABSTRACT

Background: Several Rho GTPase-activating proteins (RhoGAPs) are implicated in tumor progression through their effects on Rho GTPase activity. ARHGAP21 is a RhoGAP with increased expression in head and neck squamous cell carcinoma and with a possible role in glioblastoma tumor progression, yet little is known about the function of ARHGAP21 in cancer cells. Here we studied the role of ARHGAP21 in two prostate adenocarcinoma cell lines, LNCaP and PC3, which respectively represent initial and advanced stages of prostate carcinogenesis. Results: ARHGAP21 is located in the nucleus and cytoplasm of both cell lines and its depletion resulted in decreased proliferation and increased migration of PC3 cells but not LNCaP cells. In PC3 cells, ARHGAP21 presented GAP activity for RhoA and RhoC and induced changes in cell morphology. Moreover, its silencing altered the expression of genes involved in cell proliferation and cytoskeleton organization, as well as the endothelin-1 canonical pathway. Conclusions: Our results reveal new functions and signaling pathways regulated by ARHGAP21, and indicate that it could contribute to prostate cancer progression.

1. Introduction

ARHGAP21 is a member of the RhoGAP family of proteins that has received much attention since first being described by our group in 2002 [1–3]. RhoGAPs catalyze the conversion of active GTP-bound forms of Rho-family GTPases to their inactive GDP-bound state. Rho family GTPases are key regulators of many cell functions, including actin reorganization, migration, gene transcription, survival, adhesion, and proliferation [4,5]. Several Rho GTPases are known to contribute to cancer progression [4], with either oncogenic or tumor suppressor activities [6,7]. There are 20 human Rho GTPase genes, of which the most extensively studied members are RhoA, Rac1 and Cdc42. When activated, Rho GTPases interact with several downstream effector proteins leading to activation of multiple signaling pathways which results in cellular responses until intrinsic or RhoGAP-mediated GTPase activity returns the proteins to the GDP-bound state [8].

ARHGAP21 is a protein with 1958 amino acids that contains, in addition to the RhoGAP domain, a PDZ and a pleckstrin homology (PH) domain. It has been reported that ARHGAP21 has RhoGAP activity for RhoA and Cdc42 [9,10] and interacts with several proteins, such as FAK, PKC-ζ, α-catenin, β-arrestin-1 and ARF1, mediating cross-talk between Rho GTPases and other signaling pathways [1,3,9–11]. ARHGAP21 plays a role in the vesicular trafficking of Golgi membranes, cell–cell interactions, influenza virus replication and cardiac stress [3,9,12,13], but its function in cancer cells has been poorly investigated. ARHGAP21 is overexpressed in head and neck squamous cell carcinoma, and could be a possible potential therapeutic target [14]. We found that ARHGAP21 silencing in glioblastoma cell lines increased cell migration and secretion of metalloprotease-2, as well as FAK and Cdc42 activities [11].

Other RhoGAPs have been investigated in solid tumors, with roles in cancer development and progression, including prostate cancer [15–17]. Prostate cancer is one of the leading causes of cancer-related mortality among men worldwide [18]. Although there has been progress in the last years, several challenges remain regarding diagnosis and treatment [19]. Several signaling pathways are known to be aberrantly activated in prostate cancer progression, including the endothelin-1 pathway [20,21]. However, a better understanding of the molecular mechanisms related to prostate cancer progression may lead to more effective

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therapeutic strategies. We therefore aimed to evaluate ARHGAP21 functions in prostate adenocarcinoma cell lines.

2. Materials and methods

2.1. Cell culture

LNCaP and PC3 cell lines were used as a model for human prostate adenocarcinoma in specific assays as appropriated. HEK293T cells were used for pull down assays. Cell lines were obtained from ATCC (Philadelphia, PA, USA) and cultured in appropriate medium (RPMI for LNCaP and PC3 cells and DMEM for HEK293T cells) containing 10% fetal bovine serum with penicillin/streptomycin and maintained at 37 °C, 5% CO_2. HUVECs were purchased from PromoCell (Heidelberg, Germany) and cultured according to the manufacturer's instructions.

2.2. Plasmid constructs

The plasmid pCMV containing the cDNA encoding full-length human ARHGAP21 and the empty vector were purchased from OriGene Technologies (Rockville, MD, USA). The plasmids encoding pEGFP wild type RhoA, RhoC and constitutively active Cdc42 (Q61L) were provided by Ferran Valderrama (St George's, University of London). The plasmid encoding FLAG-p190-B RhoGAP in CMV2 expression vector was a kind gift from Jeff Settleman (Massachusetts General Hospital, Cambridge, USA).

2.3. Immunofluorescence and confocal microscopy

PC3 and LNCaP cells were grown on cover slips and fixed in 4% paraformaldehyde for 20 min, permeabilized with 0.5% Triton X-100 in PBS for 10 min at room temperature and blocked in PBS containing 3% bovine serum albumin (BSA) (60 min at room temperature). The cells were then incubated overnight at 4 °C with anti-ARHGAP21 antibody (sc-98336, Santa Cruz, CA, USA), diluted in PBS (1:200) containing 1% BSA, followed by incubation with secondary antibody diluted in PBS (1:400) containing 1% BSA for 2 h at room temperature. All incubations were followed by three 5-minute PBS washes. The slides were mounted in ProLong Gold Anti-Fade Mounting Medium with DAPI (Molecular Probes). Images were generated using a confocal laser-scanning microscope (LSM 510, Carl Zeiss, Welwyn Garden City, UK).

2.4. Subcellular fractionation

PC3 and LNCaP cells were trypsinized and collected by centrifugation at 200 ×g for 5 min at 4 °C. Cells were washed in ice-cold phosphate-buffered saline and collected by centrifugation at 1500 ×g for 5 min at 4 °C. The pellets were gently resuspended in buffer 1 (10 mM Hepes pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 10 mM Na_3VO_4 and 2 mM PMSF). Cells were chilled on ice for 10 min and then lysed by the addition of 0.1% (v/v) Nonidet P-40 and homogenization by 10 passages through a 26.5-gauge needle. The extracts were centrifuged at 12.000 ×g for 10 min at 4 °C. The supernatant was used as a cytosolic and membrane fraction. The pellet was resuspended with a buffer 2 (20 mM HEPES pH 7.9, 25% Glycerol, 1.5 mM $MgCl_2$, 20 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 10 mM Na_3VO_4 and 2 mM PMSF). The homogenate was incubated on ice for 30 min at 4 °C and centrifuged at 12.000 ×g for 10 min at 4 °C. Supernatant was used as the nuclear fraction. Equal amount of proteins was used for Western blot analysis.

2.5. Transient transfections

Silencing of ARHGAP21, RhoA, RhoC and Cdc42 was performed in prostate adenocarcinoma cells using specific siRNAs from ThermoFisher Scientific (Lafayette, CO, USA), as previously described [22]. Briefly, the cells were plated at 70% confluence and transfected using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Cells were analyzed 72 h after transfection. All siRNA sequences are described in Supplementary Table S1.

Overexpression of ARHGAP21, p190-B RhoGAP, GFP-RhoA and GFP-RhoC was performed with appropriated amount of vectors and jetPEI reagent (Polyplus Transfection), according to the manufacturer's instructions.

2.6. RNA extraction

Total RNA from siARHGAP21 and siControl cells was extracted using Trizol (Invitrogen), according to the manufacturer's instructions. For microarray assays, RNA was purified with Qiagen®, RNeasyTM Micro Kit and integrity of the RNAs was analyzed with Agilent 2100 Eletrophoresis Bioanalyser (Agilent Technologies, Santa Clara, CA).

2.7. Quantitative RT-PCR analysis (qPCR)

Reverse transcription reaction was performed using RevertAid™ First Strand cDNA Synthesis Kit, according to the manufacturer's instructions (MBI Fermentas, St. Leon-Rot, Germany). Real-time detection of *ARHGAP21* amplification was performed in 7500 Real-Time PCR System (Applied Biosystems) using Power SybrGreen PCR Master Mix (Applied Biosystems) and specific primers: forward 5′-ATGCACTGTACACTCGCTTCGA-3′ and reverse 5′-CAACGACGCCAGC AAAAAC-3′. *HPRT* was used as housekeeping gene and the sequence of used primers was: forward 5′-GAACGTCTTGCTCGAGATGTGA-3′, and reverse 5′-TCCAGCAGGTCAGCAAAGAAT-3′. Relative levels of gene expression were calculated using the equation, $2^{-\Delta\Delta CT}$ [23]. A negative 'No Template Control' was included for each primer pair. Three replicas were run on the same plate for each sample.

2.8. Western blotting

Cells were lysed in ice-cold Tris–HCl buffer (100 mM Tris, pH 7.5), containing 10 mM EDTA, 10% Triton X, 100 mM NaF and phosphatase and protease inhibitors (10 mM Na_3VO_4, 10 mM $Na_4P_2O_7$, 25 mM PMSF and 0.1 mg/mL aprotinin). Equal amounts of cell lysates were subjected to SDS-PAGE and western blot analysis with specific antibodies and ECL (Amersham Pharmacia Biotech, UK Ltd., Buckinghamshire, England). Polyclonal antibodies against ARHGAP21 (sc-98336), RhoA (sc-179), RhoC (sc-12116) and monoclonal antibodies against Stathmin (sc-55531) and GFP (sc-7383) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal antibody against p190-B RhoGAP (611612) was from BD Transduction Laboratories (San Diego, CA, USA) and polyclonal anti-Cdc42 (2462) was from Cell Signaling Technology (Danvers, MA, USA). Quantitative analyses of the optical intensities of protein bands were carried out with Un-Scan-It Gel 6.1 (Silk Scientific Inc., Utah, USA) and normalized to GAPDH or actin for protein expression or total protein for pulldown assays.

2.9. Analysis of cell proliferation

Cell proliferation was measured by methylthiazoletetrazolium (MTT) assay. Twenty-four hours after transfection, 9×10^3 cells per well were plated in a 96-well plate in RPMI containing 10% FBS. To evaluate cell viability, 10 μL of a 5 mg/mL solution of MTT (Sigma-Aldrich; St. Louis, MO, USA) were added to the wells and incubated at 37 °C for 4 h. The reaction was stopped by using 100 μL of 0.1 N HCl in anhydrous isopropanol and the absorbance was measured at 570 nm, using an automated plate reader. All conditions were tested in six replicates.

2.10. TUNEL assay

Twenty-four hours after transfection, a total of 3×10^4 cells per well were plated in a 12-well plate in RPMI containing 10% FBS. The