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The direct p53 target gene, FLJ11259/DRAM, is a member of a novel family of transmembrane proteins

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Abstract

The tumor suppressor p53 regulates diverse biological processes primarily via activation of downstream target genes. Even though many p53 target genes have been described, the precise mechanisms of p53 biological actions are uncertain. In previous work we identified by microarray analysis a candidate p53 target gene, FLJ11259/DRAM. In this report we have identified three uncharacterized human proteins with sequence homology to FLJ11259, suggesting that FLJ11259 is a member of a novel family of proteins with six transmembrane domains. Several lines of investigation confirm FLJ11259 is a direct p53 target gene. p53 siRNA prevented cisplatin-mediated up-regulation of FLJ11259 in NT2/D1 cells. Likewise in HCT116 p53+/+ cells and MCF10A cells, FLJ11259 is induced by cisplatin treatment but to a much lesser extent in isogenic p53-suppressed cells. A functional p53 response element was identified 22.3 kb upstream of the first coding exon of FLJ11259 and is shown to be active in reporter assays. In addition, chromatin immunoprecipitation assays indicate that p53 binds directly to this element *in vivo* and that binding is enhanced following cisplatin treatment. Confocal microscopy showed that an FLJ–GFP fusion protein localizes mainly in a punctate pattern in the cytoplasm. Overexpression studies in Cos-7, Saos2, and NT2/D1 cells suggest that FLJ11259 is associated with increased clonal survival. In summary, we have identified FLJ11259/DRAM as a p53-inducible member of a novel family of transmembrane proteins. FLJ11259/DRAM may be an important modulator of p53 responses in diverse tumor types.

Keywords: FLJ11259; DRAM; p53; Target gene

1. Introduction

An important area of investigation is to unravel the complex network of target gene regulation surrounding the tumor suppressor p53 [1,2]. p53 is involved in the regulation of cell cycle arrest and apoptosis following DNA damage or cellular stress. Other p53 regulated biological processes include maintenance of genomic stability, inhibition of angiogenesis, DNA repair and cytoskeletal function [3,4]. p53 regulation and subsequent downstream target gene activation may be cell type-specific and context-dependent adding to the difficulty in identifying p53 transcriptional targets. Computational methods predict that thousands of genes possess p53 DNA binding elements. However, functional p53 enhancer identification requires experimental validation [5].

Our prior work utilizing microarray analysis uncovered a p53-dominant transcriptional response to cisplatin in the human embryonal carcinoma derived cell line, NT2/D1 [6]. In addition to many well-known direct p53 target genes, a previously uncharacterized open reading frame designated FLJ11259 was uncovered as the fifth-highest gene induced with cisplatin [6]. Here we confirm that FLJ11259 is a direct p53 target gene. FLJ11259 is highly conserved throughout species and predicted to have six transmembrane domains.

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FLJ11259 is regulated by DNA damaging agents in various cell lines in a p53-dependent manner, and p53 directly binds to an upstream element in the FLJ11259 gene. FLJ11259 was observed to localize in a punctate pattern throughout the cytoplasm. Stable overexpression of FLJ11259 in Cos-7, Saos2 and NT2/D1 cells resulted in increased clonagenicity, demonstrating that FLJ11259 may play a role in cell survival under certain conditions.

2. Materials and methods

2.1. Cell culture and drug treatments

NT2/D1, Cos7 and Saos2 cells (ATCC) were cultured in DMEM (Gibco) with 10% FBS supplemented with glutamine and antibiotics. HCT116 colon cancer cell lines, p53+/+ and p53−/− were a gift from Dr. B. Vogelstein (Johns Hopkins University). HCT116 cells were cultured in McCoys 5A media (Gibco) supplemented with 10% FBS, antibiotics and glutamine. MCF10A immortalized breast epithelium cells and the MCF10A/Δp53 cell line stably transfected with p53 shRNA were previously described and generously provided by Dr. A. Eastman (Dartmouth Medical School) [7]. MCF10A cells were cultured in DMEM/F12 supplemented with 10% FBS, 8 μg/ml insulin, 20 ng/ml epidermal growth factor and 500 ng/ml hydrocortisone. The MCF10A/Δp53 cell line was cultured in the same media in the presence of G418 which was removed prior to experimental conditions. Cisplatin (Bristol Laboratories) treatments were performed in the respective media at the concentrations and time points indicated.

2.2. siRNA

Two independent siRNAs designated p53 siRNA-1 and p53 siRNA-2 were designed to human p53, targeting sequences AAGACUCCAGUGGUAAUCUAC and CGGCAUGAACCGGAGGCCCAU, respectively. The siRNA control was the Scramble II sequence (Dharmacon). A final concentration of 75 nM siRNA was transfected using OligofectAMINE reagent (Invitrogen), as described previously [8].

2.3. Northern, RT-PCR and western analysis

Total RNA was harvested using TriReagent (Invitrogen). Northern hybridizations were performed with 5 μg RNA as previously described [9]. Expression levels of FLJ11259 were measured by semi quantitative RT-PCR. The cDNA was synthesized using Superscript II RT from 5 μg total RNA. PCR analysis was performed with Taq polymerase (Invitrogen) to varying cycle numbers within the linear range as previously described [9]. RT-PCR expression analysis was also performed on normal (Human II MTC panel, cat #636743) and tumor (Human multiple tumor panel, cat #K1422-1) cDNA samples (BD Bioscience). Primer sequences are available on request. For Western analysis cells were lysed in RIPA buffer and protein concentrations determined by Bradford technique. Proteins were analyzed by SDS-PAGE as previously described [10]. A GFP antibody was employed (JL-8 from BD Biosciences).

2.4. Chromatin immunoprecipitation assays (ChIP)

Cells were plated at a density of 3×10^6 per 15 cm dish. Cisplatin treatment of NT2/D1 cells was 2 μM for 6 h prior to harvesting 16 h later. In HCT116 cells the duration of 24 h at 20 μM cisplatin treatment was employed. Subsequent steps were in accordance with the Chromatin Immunoprecipitation kit (Upstate Biotech). Briefly, cells were fixed with 1% formaldehyde for 10 min at 37 °C. Following cell lysis in the presence of protease inhibitors, cells were sonicated for 3 × 10 s pulses at setting 3.5 on a Vibra Cell sonicator (Danbury, CT). Diluted samples were then pre-cleared with agarose beads. Lysates were incubated overnight with antibodies specific for p53 (DO-1, Santa Cruz and DO-7, Neomarkers) and a control antibody for the estrogen receptor (Ab-10, Neomarkers). Antibodies were used at a concentration of 2 μg/ml of lysate. Following incubation with agarose beads, and subsequent washing steps, the antibody complexes were eluted and incubated at 65 °C for 4 h to reverse cross-linkages. DNA was eluted with the Qiagen PCR purification kit (according to the manufacturer's directions) prior to PCR amplification. PCR primers surrounding the p53 response element of FLJ11259 were GGGAGGTCTGCACTGTTGAATT (sense) and CCAGCTTGCTTTA-GGCAGACA (anti-sense). Primers surrounding the p53RE in p21 were CGAGGCAGGCCAAGGG (sense) and GCAGAGGATGGATTGTTCA (anti-sense).

2.5. Expression constructs

A clone of FLJ11259 in pCMV-SPORT6 was purchased from Open Biosystems. Restriction digestion with *Eco*R1 and *Sal*1 removed the entire coding sequence which was inserted into pcDNA 3.1 Myc-His (−) (BD Bioscience) containing a G418 resistance cassette. To facilitate in-frame ligation into the pEGFP-N2 vector (BD Bioscience), *Xho*1 and *Kpn*1 sites were engineered at the 5′ and 3′ ends respectively of the FLJ11259 insert by PCR. The 3′ primer was designed to mutate the stop codon of FLJ11259 to allow continuous translation between FLJ and GFP to create a fusion protein. Sequence analysis confirmed wild-type sequence and the correct codon usage of the FLJ–GFP fusion. A 600-bp fragment of the FLJ11259 gene containing the identified p53 response element was isolated from BAC clone RP11-9F22 by PCR and cloned into pCR 2.1-TOPO vector. Restriction digestion with *Xho*1 and *Kpn*1 facilitated insertion of this DNA fragment into the pGL3-promoter luciferase reporter vector (Promega). A version of FLJ-luc in which the p53 was mutated from AGGCATGTTTAGGCAAGCTC to AGGaATaTTTAG-GaAAaCTC was constructed using QuikChange Site-Directed Mutagenesis (Stratagene), and was DNA sequence confirmed. The tk-luc vector was a kind gift from Dr. J. DiRenzo (Dartmouth Medical School). Oligonucleotides corresponding to the FLJ p53RE (sense-AGGCATGTTTAGGCAAGCTC) were annealed and ligated into the tk-luc vector to generate a 5× p53RE construct designated FLJp53RE-luc. The dominant-negative p53 (DNp53) construct has been previously described [10].

2.6. Confocal microscopy

Cos-7 cells were plated on coverslips at a density of 0.125×10^6 cells per well of a 6 well dish. Transfection of 1 μg DNA per well was performed with Fugene according to the manufacturer's directions. The presence of GFP was detected 24 h later using fluorescent microscopy. For mitochondrial staining, Mitotracker Deep Red 633 (Molecular Probes) was prepared in DMEM at a working concentration of 500 nM and cells were incubated at 37 °C for 30 min. Cells were washed with PBS, fixed with 3.7% formaldehyde at 37 °C for 15 min prior to mounting with prolong containing DAPI (Molecular Probes). The plasma membrane was stained with 5 μg/mL Alexa Fluor 594 wheat germ agglutinin (Molecular Probes) in Hank's balanced salt solution (HBSS, Gibco) for 10 min at 37 °C. Cells were washed with HBSS and fixed in 4% formaldehyde for 15 min at 37 °C prior to mounting with prolong/DAPI. Endoplasmic reticulum (ER) was stained with ER-Tracker Red (glibenclamide BODIPY TR) at a final concentration of 1 μM in HBSS for 30 min at 37 °C. Cells were then washed with PBS and fixed for two min at 37 °C with 4% formaldehyde. Following washing cells were mounted with prolong/DAPI. Confocal microscopy was performed on the Zeiss-LSM-510 META point scanning confocal microscope. Cells were imaged using a 63× oil objective and a pinhole of 1 airy unit was the standard for image capture.

2.7. Clonal growth assay

Cos-7, Saos2 or NT2/D1 cells were plated at 0.175×10^6 cells per well in triplicate in 6 well dishes. Transfection with Fugene was performed according to the manufacturer's directions with either empty pcDNA 3.1, or pcDNA 3.1 containing the FLJ11259 insert (FLJ-1 and FLJ-2). In the p53 null, Saos2 cell line, transfection of a p53 expression vector (pC53-SN3) and empty vector control was also performed. Following transfection, 2×10^5 cells were replated