DNA Repair 11 (2012) 401–409

Contents lists available at SciVerse ScienceDirect

DNA Repair

journal homepage: www.elsevier.com/locate/dnarepair

Release from quiescence stimulates the expression of human *NEIL3* under the control of the Ras dependent ERK–MAP kinase pathway

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ARTICLE INFO

Article history:
Received 17 June 2011
Received in revised form 11 January 2012
Accepted 12 January 2012
Available online 23 February 2012

Keywords:
Base excision repair
DNA glycosylase
NEIL3
Cell cycle regulation
MAP kinase

ABSTRACT

Base excision repair (BER) is believed to be the predominant pathway for the repair of oxidative DNA damage. BER is initiated by lesion-specific DNA glycosylases that recognize and remove the damaged base. NEIL1, NEIL2 and NEIL3 are three mammalian members of the Fpg/Nei DNA glycosylase family with similar enzymatic properties. In this study we showed that both the transcription and protein levels of hNEIL3 fluctuated during the cell cycle. Based on predicted promoter elements of cell cycle-regulated genes and microarray data from various reports, we suggest that *hNEIL3* repression in quiescent cells might be mediated by the DREAM (DP1, RB p130, E2F4 and MuvB core complex) complex. Release from G0 by mitogenic stimulation showed an induction of *hNEIL3* in early S phase under the control of the Ras dependent ERK–MAP kinase pathway. In contrast, the total expression of *hNEIL1* was downregulated upon release from quiescence while the expression of *hNEIL2* was cell cycle independent. Notably, *hNEIL3* showed a similar regulation pattern as the replication protein hFEN1 supporting a function of hNEIL3 in replication associated repair. Thus, it appears that specialized functions of the NEILs are ensured by their expression patterns.

1. Introduction

To preserve genetic information the DNA must be protected from damage generated spontaneously or induced by environmental agents [1]. To counteract DNA damage, different repair mechanisms have evolved for the many types of DNA lesions. DNA glycosylases in the base excision repair (BER) pathway recognizes and excise specific base lesions leaving an abasic site [2–5]. Repair synthesis is completed by several BER enzymes executing gap filling and ligation [2]. DNA glycosylases that recognize oxidized bases have been divided into two structural families, the Nth family and the Fpg/Nei family [6,7]. Three Fpg/Nei homologues designated hNEIL1, hNEIL2 and hNEIL3 in humans and mNeil1, mNeil2 and mNeil3 in mouse respectively, have been identified in mammals [8–12]. These are bifunctional glycosylases, also cleaving the exposed backbone at the abasic site. NEIL3 is the largest member of the family, consisting N-terminally of the characteristic Fpg/Nei motifs and C-terminally of a disordered extension with unique structural features containing a Ranbp-like zinc finger motif, a putative NLS and tandem repeated GRF zinc finger motifs.

NEIL1 and NEIL2 have been extensively studied biochemically. In the case of NEIL3, only the mouse ortholog, mNeil3, has been examined thoroughly [13]. In summary, the three homologues have broad and overlapping specificity for oxidative lesions and a preference for single stranded DNA, bubble and fork structures. Hydantions such as spiroimidodihydantoin (Sp) and guanidinohydantoin (Gh) are the preferred substrates for mNeil3. These lesions have garnered much attention due to their extremely high mutagenic potential in cells which is significantly greater than 8-oxoG [14]. Altogether, a function during replication or transcription when partly unwound regions of DNA are exposed has been suggested for the NEILs. All three proteins have been reported to be localized in the nucleus, while hNEIL1 has been shown to be found also in mitochondria [10,11,15,16]. Human *NEIL1* and *hNEIL2* are ubiquitously expressed although at different levels in distinct organs [9–11]. In contrast, *hNEIL3* is expressed in thymus and testis and in multiple forms of cancer, while *mNeil3* is expressed in hematopoietic tissue and testis, during embryonic development and in stem/progenitor rich regions in the brain [11,16–20]. Thus, hNEIL3/mNeil3 might function to remove lesions from the genome in proliferating cells. During the cell cycle, the expression of *hNEIL1* has been reported to be induced in S phase [9]. Functional interactions of hNEIL1 with WRN, PCNA, FEN1 and RPA have suggested that

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doi:10.1016/j.dnarep.2012.01.007

hNEIL1 is probably involved in repairing the replicating genome [21–25]. Unlike *hNEIL1*, *hNEIL2* expression is independent of the cell cycle stage and was recently shown to be involved in repairing oxidized bases in the transcribed genes of mammalian cells [10,26].

Transition through the mammalian cell cycle requires interplay of transcription factors that together induce or repress gene expression in a temporally defined manner. The E2Fs are a large family of transcription factors that bind target promoters and regulate their expression. There are eight known members of the E2F family in mammals, and they form active DNA-binding heterodimers with either DP1 or DP2 [27–29]. E2F activity is controlled in part by interactions with members of the pRB (pocket protein) family: the RB tumor suppressor, p107 and p130 [30]. The DREAM (DP1, RB p130, E2F4, and MuvB core complex) complex represses cell cycle-dependent genes during quiescence maintaining cells in G0 phase [31]. Upon re-entry into the cell cycle, the repressive complex is relieved by CDKs (cyclin-dependent kinases) that phosphorylate RB proteins in a cell cycle dependent manner [32,33]. The activation of CDKs involves the mitogenic stimulation of receptor tyrosine kinases (RTKs) and the downstream mitogen activated protein (MAP) kinase pathways [34–36]. Activation complexes as E2F1-NF-Y, E2F1-Sp1 can then be recruited to the promoter [37–39].

In the present study we have examined the cell cycle-dependent regulation of hNEIL3 at the transcriptional and protein level in comparison with other repair and replication associated genes and in cells from different origins. Furthermore, we have identified several putative cell cycle-regulated promoter elements and demonstrated the involvement of the Ras dependent ERK–MAP kinase pathway in the induction of *hNEIL3* expression.

2. Materials and methods

2.1. Cells

Human embryonic fibroblasts (HE) were obtained from the National Institute of Public Health (Folkehelsa, Oslo, Norway). MRC-5 (human fetal lung primary fibroblasts), HaCaT (human keratinocytes) and MCF-7 (human epithelial breast cancer cell line) were obtained from ATCC. HE cells were cultured in a 1:1 ratio minimal essential medium (MEM) + Dulbecco's modified Eagle's medium (DMEM) (Gibco, Life Technologies, Carlsbad, CA, USA) ratio supplemented with 10% fetal bovine serum (Standard quality FBS, PAA lab, GmbH, Austria), 1× GlutaMax (200 mM, Gibco, Life Technologies), and 1× penicillin–streptomycin (10,000 U/ml, Lonza, Basel, Switzerland). MRC-5, HaCaT and MCF-7 cells were cultured in DMEM supplemented with 10% FBS, 1× GlutaMax and 1× Pen-Strep.

2.2. Cell cycle synchronization and analysis by flow cytometry

Synchronization of the cells in G0 phase was achieved by culturing cells as a confluent layer for 72 h followed by serum starvation (0.2% serum) for 72 h. The cells were G0 released by trypsination for 4 min at 37 °C (Trypsin-EDTA (200 mg/L), Lonza) and cultivated in standard growth medium at 25% confluence. Cells were harvested by trypsination at indicated time points, washed in ice-cold PBS and stored at −20 °C. Cells used for phase analysis were resuspended in PBS and fixed by addition of ice-cold 100% ethanol to a final concentration of 70%. The cells were stored at −20 °C. For FACS analysis the cells (about 10^6/ml) were stained with propidium iodide (50 μg/ml, Sigma–Aldrich, St. Louis, MO, USA) in 0.1 mg/ml RNaseA (Molzym GmbH & Co, Bremen, Germany)/0.1% Triton X-100 (Sigma–Aldrich)/4 mM Na-citrate buffer for 10 min at 37 °C and put on ice. Cells were subjected to flow cytometric analysis (BD LSRII flow cytometer (Becton Dickinson, San Jose, CA, USA), and the results were analyzed with the CellQuest software (Becton Dickinson).

Table 1
Primers used in Real-Time qRT-PCR.

Target	Sequence (5′ → 3′)	Acc. nr.
NEIL1	(+)-GCTGACCCTGAGCCAGAAGAT (−)-CCCCAACTGGACCACTTCCT	NM_024608.2
NEIL2	(+)-ACCTGTGACATCCTGTCTGAGAAGT (−)-TAATGATGTTCCCTAGCCCTGAGA	NM_145043.2
NEIL3	(+)-GGTCTCCACCCAGCTGTTAAAG (−)-CACGTATCATTTTCATGAGGTGATG	NM_018248.2
UNG2	(+)-GCCAGAAGACGCTCTACTCC (−)-GTGTCGCTTCCTGGCGGG	NM_080911
FEN1	(+)-AGGGAGAGCGAGCTTAGGAC (−)-GGCAACACAGAGGAGGGAT	NM_004111
GAPDH	(+)-CCACATCGCTCAGACACCAT (−)-GCGCCCAATACGACCAAAT	NM_002046.3

2.3. Kinase and ROS inhibitor treatment of HE cells

Synchronized cells were trypsinized and resuspended in growth medium without serum. The cells were preincubated with kinase inhibitor for 15 min and plated in complete growth medium with addition of inhibitor at 25% confluence. Inhibitors used: SB203580 (p38a,b,b2 inhibitor, 10 μM, Promega Corp, Madison, WI, USA), SP600125 (JNK inhibitor, 20 μM, Sigma–Aldrich), U0126 (MEK inhibitor, 20 μM, Promega) and N-acetyl cystein (NAC, ROS inhibitor, 10 mM, Sigma–Aldrich). Cells were harvested at indicated time points as described.

2.4. Total RNA isolation, cDNA synthesis and Real-Time qRT-PCR

Total RNA was extracted from cell pellets using the RNeasy kit (QIAGEN GmbH, Hilden, Germany) according to protocol. The RNA was treated with TurboDNase (Applied Biosystems, Foster City, CA, USA) and the purity controlled by absorbance (260/280 nm and 260/230 nm) measurements using Nanodrop spectrophotometer. cDNA was synthesized from 50 ng RNA in 20 μl reaction using the High-Capacity cDNA Reverse Transcription kit (Applied Biosytems). Real-Time qRT-PCR was performed in 20-μl reactions containing 2.5 ng of cDNA and 100 nM primers using the Power SYBR Green PCR master mix and the Step One Plus Real-Time PCR system (Applied Biosystems) according to the system and kit instructions. The following thermal cycle parameters were used: 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at 60 °C. All samples were run in triplicate. Melting point analyses were performed to confirm the specificity of the PCR. Relative quantitation (RQ) or fold change of gene expression was calculated using the comparative CT method described by the manufacture (Applied Biosystems) GAPDH was used as the reference gene for normalization, G0 as the reference sample for RQ calculation. Primers (Table 1) were designed using the Primer Express software version 2.0 (Applied Biosystems).

2.5. Immunoprecipitation and Western blotting

All procedures were performed at 4 °C. Cell pellets containing about 10^7 cells collected at given time points were suspended in 1 ml of lysis buffer (50 mM Tris–HCl pH 7.5, 500 mM NaCl, 1.0% NP-40, 1% Protease Inhibitor Cocktail (P8340, Sigma–Aldrich)), and disrupted by sonication. Cell lysates were centrifuged, and protein concentration was determined by the Bradford method using the DC Protein Assay Kit II (Bio-Rad, Hercules, CA, USA). 1 mg of extract was incubated with 10 μg of normal rabbit IgG (sc-2027,