DNA Repair 9 (2010) 964–975

Contents lists available at ScienceDirect

DNA Repair

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

Human RECQL5 overcomes thymidine-induced replication stress

Rachel Blundred[a,1], Katie Myers[a], Thomas Helleday[b], Alastair S.H. Goldman[c], Helen E. Bryant[a,*]

[a] The Institute for Cancer Studies, University of Sheffield, Sheffield S10 2RX, UK
[b] Gray Institute for Radiation Oncology & Biology, University of Oxford, Oxford OX3 7DQ, UK
[c] Department of Molecular Biology and Biotechnology, Krebs Institute, The University of Sheffield, Sheffield S10 2TN, UK

ARTICLE INFO

Article history:
Received 1 March 2010
Received in revised form 14 June 2010
Accepted 22 June 2010

Keywords:
Homologous recombination
Thymidine
RECQL5
Replication

ABSTRACT

Accurate DNA replication is essential to genome integrity and is controlled by five human RecQ helicases, of which at least three prevent cancer and ageing. Here, we have studied the role of RECQL5, which is the least characterised of the five human RecQ helicases. We demonstrate that overexpressed RECQL5 promotes survival during thymidine-induced slowing of replication forks in human cells. The RECQL5 protein relocates specifically to stalled replication forks and suppresses thymidine-induced RPA foci, CHK1 signalling, homologous recombination and γH2AX activation. It is unlikely that RECQL5 promotes survival through translesion synthesis as PCNA ubiquitylation is also reduced. Interestingly, we also found that overexpressing RECQL5 relieves cells of the cell cycle arrest normally imposed by thymidine, but without causing mutations. In conclusion, we propose that RECQL5 stabilises the replication fork allowing replication to overcome the effects of thymidine and complete the cell cycle.

1. Introduction

Accurate DNA replication is essential to genome integrity and the prevention of tumorigenesis. When a replication fork is perturbed multiple damage limitation pathways are activated. These pathways stabilise the replication complex and help by-pass or correct the problem. The RecQ helicase family, which is conserved from bacteria through to humans, significantly contribute to the maintenance of genome integrity during replication. *E. coli* and budding yeast express only one RecQ helicase but humans have at least five, BLM, WRN, RECQL1, RECQL4 and RECQL5. Mutations in BLM, WRN and RECQL4 cause syndromes that are associated with cancer predisposition and premature aging [1]. These disease associated helicases have been extensively studied, but the function of human RECQL5 is not well characterised.

Mutating the yeast RecQ orthologue (*SGS1*) causes phenotypes reminiscent of human RecQ deficiencies. Some of the phenotypes can be rescued by expression of dmRECQ5/QE [2]. These phenotypes include sensitivity to the replication inhibitor hydroxyurea and the alkylating agent methyl methanesulfonate, as well as elevated frequencies of sister chromatid exchange and homologous recombination (HR). Thus it is likely that RECQL5 has an evolutionary conserved function related to overcoming replication stress and suppression of HR. Further evidence for this conserved role comes from more direct studies of human RECQL5. The protein interacts with topoisomerases 3α and 3β [3] and the flap endonuclease FEN1 [4], both suggesting a possible function at replication forks. It also co-localises with PCNA foci during S-phase. The colocalisation of RECQL5 and PCNA is increased following UVC or hydroxyurea induced replication stalling [5]. RECQL5 also co-localises with regions incorporating BrdU confirming its presence at replication forks. In addition, mouse embryonic fibroblasts (MEFs) deficient in RECQL5 are sensitive to camptothecin-induced replication arrest [6].

A potential role for RECQL5 in the control of HR is suggested by RECQL5 deficient MEFs, which exhibit an approximate two-fold increase in spontaneous RAD51 foci (a marker of HR) compared to wildtype cells [7]. Furthermore, RECQL5 interacts with RAD51 in cells and purified human RECQL5 can prevent D-loop formation *in vitro* [7]. RECQL5 may therefore promote an HR-independent way of resolving replication stalling.

Here we demonstrate that RECQL5 can promote survival during thymidine-induced slowing of replication forks in human cells. Following thymidine treatment, cells overexpressing RECQL5 contain fewer RPA foci and less γH2AX activation than cells with endogenous levels of RECQL5. The CHK1 mediated signalling pathway is also down regulated and HR is reduced. It is unlikely that RECQL5 promotes survival through translesion synthesis (TLS) as PCNA ubiquitylation is also reduced. We also found that overexpress-

Abbreviations: DSB, double strand break; dsDNA, double stranded DNA; HR, homologous recombination; MEFs, mouse embryonic fibroblasts; ssDNA, single-stranded DNA; TLS, translesion synthesis.

* Corresponding author. Tel.: +44 114 2712993; fax: +44 114 2795320.
E-mail address: h.bryant@sheffield.ac.uk (H.E. Bryant).
[1] Current address: Cancer Research-United Kingdom Institute for Cancer Sciences, University of Birmingham Medical School, Edgbaston, Birmingham B15 2TT, UK.

1568-7864/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.dnarep.2010.06.009

ing RECQL5 relieves cells of the cell cycle arrest normally imposed by thymidine. We propose that RECQL5 stabilises the replication fork allowing replication to overcome the effects of thymidine and complete the cell cycle.

2. Materials and methods

Cell lines in this study were grown and treated in DMEM with 10% Foetal bovine serum, penicillin (100 U/ml) and streptomycin sulphate (100 μg/ml) at 37 °C under an atmosphere containing 5% CO_2. SPD8 cells were maintained in 5 μg/ml 6-thioguanine (6TG) to suppress spontaneous reversion. pcDNA 3.1 plasmids containing human RECQL5β cDNA and RECQL5KR (RecQL5β cDNA sequence with the helicase domain disrupted by a K58R point mutation) were a generous gift from Dr. Pavel Janscak (Institute of Molecular Cancer Research, University of Zurich). CHK1 inhibitor used was Gö6976 (Calbiochem).

2.1. Recombination assay

1.5×10^6 SPD8 cells were inoculated into 100 mm dishes, in media without 6TG, 4 h prior to a 24 h treatment with 10 mM thymidine. The cells were then rinsed three times with PBS and 10 ml media added before allowing the cells to recover for 48 h. After recovery, cells were released by trypsinisation and counted. $HPRT^+$ revertants were selected by plating 3×10^5 treated cells per dish in the presence of HAsT (50 μM hypoxanthine, 10 μM L-azaserine, and 5 μM thymidine). To determine cloning efficiency, two dishes were plated with 500 cells each without selection. The colonies obtained were stained with methylene blue in methanol (4 g/L), following 7 days (in the case of cloning efficiency) or 10 days (for reversion) of incubation. Reversion/recombination frequency = number of revertants/($3 \times 300{,}000$) × cloning efficiency.

2.2. Immunofluorescence

Cells were plated onto coverslips allowed to settle for 4 h and grown for 24 h in the presence or absence of treatments as indicated. Medium was then removed and coverslips were rinsed once in PBS at 37 °C. Cells were fixed in 3% paraformaldehyde in PBS containing 0.1% Triton X-100 for 20 min at room temperature and then extensively washed (2×15 min in PBS containing 0.1% Triton X-100 and 0.15% bovine serum albumin, 1×10 min in PBS containing 0.3% Triton X-100 and 1×15 min in PBS containing 0.1% Triton X-100 and 0.15% bovine serum albumin) prior to incubation with rabbit polyclonal anti-RAD51 antibody (H-92, Santa Cruz), rabbit polyclonal anti-RPA 34 (Calbiochem) or rabbit polyclonal anti-RECQL5 antibody (a gift from Dr. Janscak – Institute of Molecular Cancer Research, University of Zurich) at a dilution of 1:1000, 1:1000 and 1:3000 respectively for 16 h at 4 °C. The coverslips were subsequently washed (as above) followed by 1 h incubation at room temperature with Cy-3-conjugated goat anti-mouse or rabbit IgG antibody (Zymed) at a concentration of 1:500 as required and finally washed again as above. Coverslips were washed briefly in PBS, DNA stained with 1 μg/ml To Pro (Molecular Probes) and finally mounted in SlowFade Antifade (Molecular Probes).

Images were obtained with a Zeiss LSM 510 inverted confocal microscope using planapochromat 63X/NA 1.4 oil immersion objective and excitation wavelengths 488, 546 and 630 nm. Through focus maximum projection images were acquired from optical sections 0.50 μm apart and with a section thickness of 1.0 μm.

The frequencies of cells containing foci were determined in at least three separate experiments. At least 100 nuclei were counted on each slide.

2.3. Clonogenic survival assay

500–5000 cells were plated in triplicate onto 100 mm dishes 4 h prior to treatment with increasing doses of drugs as indicated. 10–14 days later, when colonies could be observed, they were fixed and stained with methylene blue in methanol (4 g/L). Colonies consisting of more than 50 cells were subsequently counted. Each colony was assumed to represent one cell surviving from the original 500 and surviving fraction for each dose calculated.

2.4. Western blotting

Cells were lysed in RIPA buffer in the presence of 1× protease and phosphatase inhibitor cocktails (Sigma). An aliquot of 50 μg total protein was run on an SDS-PAGE gel and transferred to Hybond ECL membrane (Amersham Pharmacia). This membrane was immunoblotted with rabbit anti-RECQL5 (1:1000, Abcam), anti-mouse PCNA (1:1000, Abcam), rabbit anti-phospho-CHK1 (ser 345) (1:1000, Cell Signalling), rabbit anti-CHK1 (1:1000, cell signalling), rabbit anti-γH2AX (ser 139) (1:1000, Cell Signalling), and rabbit anti-β-actin (1:2000, Sigma) antibodies, in 5% milk overnight. After addition of the appropriate HRP conjugated secondary antibody and further washes, immunoreactive protein was visualised using ECL reagents (Amersham Pharmacia) following manufacturer's instructions.

2.5. Cell cycle analysis

Cells were fixed in 70% methanol and left overnight at −20 °C. After washing in PBS cells were stained with propidium iodide/RNaseA solution (50 mg/ml PI, 100 mg/ml RNaseA) for at least 30 min. Samples were analysed by flow cytometry (Becton-Dickenson FACSort, 488 nm laser).

2.6. Fluctuation assay

1000 cells were inoculated into 6-well plates, containing media without 6TG, 4 h prior to a treatment with or without 10 mM thymidine for 24 h. Cells were then rinsed three times with PBS and 5 ml media added before allowing cells to grow to 80–90% confluency in fresh DMEM. The total number of cells in each well was then determined, and cells were re-plated at a density of 1×10^5 on 100-mm dishes for selection in 5 μg/ml 6TG. 14 days later, they were fixed and stained with methylene blue in methanol (4 g/L). The number of colonies were counted and the spontaneous mutation rate of cells in culture was determined using a published table for the estimation of the spontaneous mutation rate of cells in culture (method of the mean) [8] and using the P_0 method [9].

2.7. CldU co-immunoprecipitation of proteins present at stalled replication forks

1.5×10^6 cells were treated with 2 mM hydroxyurea or 10 mM thymidine for 24 h. Hydroxyurea and thymidine were washed away and cells were labelled with Chlorodeoxyuridine (CldU) (100 μM) for 40 min. Cells were crosslinked in 1% formaldehyde for 15 min at R/T, and treated with 0.125 M glycine for 15 min at R/T. Cells were harvested by scraping into cold PBS. Cytoplasmic proteins were removed by incubation in hypotonic buffer (10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol, 1 m MDTT, 1× protease inhibitors cocktail (Roche)) for 10 min on ice and centrifugation at 3000 rpm for 5 min. Nuclear soluble fraction was removed by incubation with nuclear buffer (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, 1× protease inhibitors (cocktail, Roche)) for 10 min on ice and centrifugation at 3000 rpm for 5 min. Pellets were resuspended in lysis buffer (10 mM HEPES pH 7, 500 mM NaCl,