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PCNA dependent cellular activities tolerate dramatic perturbations in PCNA client interactions

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ABSTRACT

Proliferating cell nuclear antigen (PCNA) is an essential cofactor for DNA replication and repair, recruiting multiple proteins to their sites of action. We examined the effects of the PCNA^{S228I} mutation that causes PCNA-associated DNA repair disorder (PARD). Cells from individuals affected by PARD are sensitive to the PCNA inhibitors T3 and T2AA, showing that the S228I mutation has consequences for undamaged cells. Analysis of the binding between PCNA and PCNA-interacting proteins (PIPs) shows that the S228I change dramatically impairs the majority of these interactions, including that of Cdt1, DNMT1, PolD3^{p66} and PolD4^{p12}. In contrast p21 largely retains the ability to bind PCNA^{S228I}. This property is conferred by the p21 PIP box sequence itself, which is both necessary and sufficient for PCNA^{S228I} binding. Ubiquitination of PCNA is unaffected by the S228I change, which indirectly alters the structure of the inter-domain connecting loop. Despite the dramatic in vitro effects of the PARD mutation on PIP-degron binding, there are only minor alterations to the stability of p21 and Cdt1 in cells from affected individuals. Overall our data suggests that reduced affinity of PCNA^{S228I} for specific clients causes subtle cellular defects in undamaged cells which likely contribute to the etiology of PARD.

1. Introduction

Accurate DNA replication is essential prior to cell division if daughter cells are to inherit a genome free from mutation. The proliferating cell nuclear antigen (PCNA) has a central role during DNA replication, acting to recruit enzymes to the DNA replication fork, and to modify enzymatic activity and processivity. At replication sites PCNA recruits proteins required throughout the complex processes of chromosomal duplication, including (but not limited to): DNA polymerase delta (for lagging strand synthesis) [1], DNA ligase 1 (Lig1) and Flap endonuclease 1 (Fen1) (for Okazaki fragment maturation) [2–4], RNaseH2B (for ribonucleotide removal) [5–7], polymerase eta (Polη) (for translesion synthesis) [8], DNA methyltransferase 1 (DNMT1) (for DNA methylation maintenance) [9], and chromatin assembly factor 1 (for chromatin assembly) [10,11]. PCNA also regulates entry into S-phase via interactions with the cell cycle regulator p21 [12] and the pre-replication complex component Cdt1 [13]. These PCNA partners all utilise a similar peptide motif to associate with PCNA, the PCNA-interacting-protein (PIP) box. This short motif interacts via a combination of charge-pair and hydrophobic interactions with the interdomain connecting loop (IDCL) of PCNA [14,15]. Proteins that bind PCNA in this manner therefore compete for the same interaction surface, perhaps to enable dynamic associations essential for progressing through the multiple stages of chromosomal replication [16]. It has often been postulated that PCNA might coordinate DNA replication, the trimeric nature of PCNA lending itself to a "toolbelt model" where, by binding multiple proteins sequentially, PCNA ensures appropriate selection of enzymes at the replication fork [17]. However, this model has recently been challenged by work showing that PCNA with only a single interaction surface is still functional [18].

As well as its essential role in DNA replication, PCNA is also required for DNA repair. PCNA is involved in the processes of nucleotide excision repair (NER), long-patch base excision repair (BER) and mismatch repair (MMR) [16]. During these processes PCNA interacts directly with repair proteins, including (but not

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limited to): xeroderma pigmentosum A and G (XPA, XPG) (for NER) [19,20], DNA glycosylases (UNG2 and MPG), endonucleases (APE1 and APE2) and polymerase beta [21–25] (for BER), and MutS homologs 3 and 6 (for MMR) [26]. It is also possible that PCNA is required for DNA repair synthesis during homologous recombination [27].

As PCNA is a crucial component of important pathways for DNA metabolism, it is not surprising that the encoding gene is essential in yeast and mice [28–30]. The gene is highly conserved from yeast to humans; *S. cerevisiae*, *S. pombe* and *M. musculus* PCNAs are 35%, 51% and 97% identical to the human protein, respectively [EMBOSS Needle]. Site-specific mutations of the *S. cerevisiae* protein result in a variety of phenotypes, including cold sensitivity, sensitivity to DNA damaging agents and alterations to telomere position effects [31–34]. In mice the only characterised PCNA variant is the site directed mutation of lysine-164 to arginine, which results in infertility and in alterations to the somatic hypermutation spectrum due to the requirement for ubiquitination on PCNA Lys164 for the recruitment of Polη [35]. The PCNA protein is not invariant in the human population, but its variation is very low. There are only seven missense coding SNPs reported in the 1000 genomes browser (rs140522967, Ser223Pro; rs369958038, Ser228Ile; rs376351202, Met139Val; rs141842220, Ala67Thr; rs144468297, Asn65Thr; rs1050525, Ser39Arg; and rs375496467, Val15Leu) all with minor allele frequencies of less than 0.01 (where reported). Of these only one very rare allele (rs369958038, S228I) is reported pathogenic in the homozygous state [36]. We previously described four individuals from the Ohio Amish population who are homozygous for this S228I sequence alteration and affected by PCNA-associated DNA repair disorder (PARD), characterised by short stature, hearing loss, premature aging, telangiectasia, neurodegeneration and photosensitivity [36]. A further PARD affected Amish individual from Wisconsin homozygous for the same S228I founder mutation has since been identified, she presented aged 4 years with short stature, sun sensitivity, progressive gait instability and hearing concerns. On examination, there was no evidence of ocular or cutaneous telangiectasia, which appear to be a later manifestation of the disease.

We previously showed that PCNA^{S228I} protein has altered binding to a number of client proteins, in particular XPG, Lig1 and Fen1, and that cells from PARD affected individuals were more sensitive to UV damage [36]. We here show data that PCNA^{S228I} also causes repair independent consequences in cells from PARD affected inidividuals and present in-depth characterisation of the protein binding capability of PCNA^{S228I}, showing that the effect on binding varies significantly across a range of PCNA interactors, dependent on the sequence of the PIP-box. These consequences for cellular functions will shed light on the complex pathology of this disorder.

2. Material and methods

2.1. Cell lines

EBV transformed lymphoblastoid cell lines were established from four PARD affected individuals (1504, 1505, 1506, 1779) and two Amish wild type controls (0920, 0924) using the service from Public Health England. Cell lines were maintained in RPMI with 10% FBS, 2 mM glutamine (Sigma), and 1% penicillin and streptomycin (PAA). Sensitivity of lymphoblasts to T2AA [37] (T2 amino alcohol ((*S*)-4-(4-(2-amino-3-hydroxylpropyl)-2,6-diiodophenoxyphenol)) and T3 (3,3′,5-triiodothyronine) was determined by addition of T2AA or T3 to 5 ml cells at 2×10^5/ml and counting viable using trypan blue exclusion at indicated time points.

2.2. Genotyping

Genotypes were confirmed regularly for quality control purposes. Genomic DNA was extracted from lymphoblastoid cell lines using GenElute Mammalian Genomic DNA Miniprep Kit as per manufacturer's instructions. The genomic region surrounding PCNA nucleotide position 683 was amplified using standard PCR with Phusion® High Fidelity DNA Polymerase (NEB, as per manufacturer's instructions), using the following primers 5′-ATAGCTCCCTCCAAAGTGACC-3′ and 5′-CATCCTCGATCTTGGGAGCC-3′. Amplification of a single band was confirmed by agarose gel electrophoresis and samples sequenced by Sanger sequencing (GATC Biotech) using the primer 5′-ACTAACTTTTGCACTGAGG-3′.

2.3. FACS analysis of cell cycle

Exponentially growing lymphoblastoid cells were incubated with 10 μM Bromodeoxy-uridine (BrdU) for 30 min then centrifuged (1000*g*, 5 min) and fixed in 4% paraformaldehyde (PFA), 20 min. Cells were washed in PBS, treated with 0.2 mg/mL pepsin in 2 M HCl, 20 mins, then probed with anti-BrdU (1:50, 347580, BD) 1 h in PBS with 1% BSA and 0.5% Tween 20 with intervening washes with PBS. Cells were washed in PBS with 1% BSA, 0.5% Tween 20 and incubated with Alexa Fluor 633 anti-mouse (1:200, Invitrogen) for 1 h. Cells were washed as before, incubated in PBS with 0.5 mg/mL RNase A overnight at 4 °C and stained with 10 μg/mL propidium iodide before analysis using a CyAn ADP Analyzer (Beckman Coulter).

2.4. Cell manipulations

For analysis of PIP degrons, exponentially growing lymphoblasts were treated with indicated levels of 254 nm UVC light in PBS. Cells were allowed to recover in growth medium for the indicated times, then harvested at 4 °C for protein analysis. Samples were prepared in Benzonase buffer (25 mM NaCl, 50 mM HEPES pH 7.8, 0.05% SDS, 4 mM $MgCl_2$, 5x cOmplete EDTA free Protease Inhibitor Cocktail Tablets (Roche), 0.5% Benzonase (Novagen)) with incubation on ice for 10 mins. Protein levels were adjusted by dilution using Bradford Reagent and 4x Laemmli added, 95 °C, 5 mins. Proteins were separated on SDS-PAGE gels and transferred to 0.2 μm nitrocellulose.

2.5. Antibodies

The following primary antibodies were used for western blotting mouse anti-actin (A4700, Sigma Aldrich), PCNA (PC10, Millipore), rabbit anti-Cdt1 (D10F11, Cell Signalling Technology), DNMT1 (NB100-56519, Novus Biologicals), Fen1 (EPR4459(2), GeneTex), Ligase I (PA5-27820, Thermo Scientific), p21 (EPR362, abcam), Polη (ab17725, abcam), Ubiquitinated-PCNA (D5C7P, Cell Signalling Technology).

2.6. Mass spectrometry

HEK293 stably expressing StrepTagII V5 PCNA^{WT} or empty vector control were made using pEXP pcDNA3.1/StrepTagII V5 PCNA^{WT} generated from pcDNA3.1/nV5-DEST (ThermoFisher) with StrepTagII inserted at HinDIII site and PCNA inserted by LR reaction (ThermoFisher). Stable clones were selected with Neomycin and selected for level of exogenous PCNA expression by western blot. Cells were cultured and lysates collected in extract buffer (0.5% Igepal, 40 mM NaCl, 50 mM Tris pH 7.5, 2 mM $MgCl_2$, 2x cOmplete Protease Inhibitor Cocktail Tablets (Roche), 1x phosphatase inhibitor (Roche), 0.1% Benzonase (Novagen)) on ice, 10 min. NaCl was increased to 150 mM with 20 min incubation, 4 °C and lysates