DNA Repair xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

DNA Repair

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

Aberrant C-terminal domain of polymerase η targets the functional enzyme to the proteosomal degradation pathway

Sana Ahmed-Seghir[a], Caroline Pouvelle[a], Emmanuelle Despras[a], Agnès Cordonnier[b], Alain Sarasin[a], Patricia L. Kannouche[a,*]

[a] Université Paris-Sud, CNRS-UMR8200, Equipe labellisée Ligue Contre le Cancer, Gustave Roussy, Villejuif, France
[b] CNRS-FRE3211, IREBS, BP 10413, F-67412 Illkirch-Cedex, France

ARTICLE INFO

Article history:
Received 11 November 2014
Received in revised form 14 February 2015
Accepted 16 February 2015
Available online xxx

Keywords:
Translesion synthesis
XP-V
Xeroderma pigmentosum variant
Bortezomib
MG132
Ubiquitin/proteasome system
Ultraviolet

ABSTRACT

Xeroderma pigmentosum variant (XP-V) is a rare genetic disease, characterized by sunlight sensitivity and predisposition to cutaneous malignancies. XP-V is caused by a deficiency in DNA polymerase eta (Polη) that plays a pivotal role in translesion synthesis by bypassing UV-induced pyrimidine dimers. Previously we identified a new Polη variant containing two missense mutations, one mutation within the bipartite NLS (T692A) and a second mutation on the stop codon (X714W) leading to a longer protein with an extra 8 amino acids (721 instead of 713 AA). First biochemical analysis revealed that this Polη missense variant was barely detectable by western blot. As this mutant is extremely unstable and is nearly undetectable, a definitive measure of its functional deficit in cells has not been explored. Here we report the molecular and cellular characterization of this missense variant. In cell free extracts, the extra 8 amino acids in the C-terminal of Polη^{721} only slightly reduce the bypass efficiency through CPD lesions. *In vivo*, Polη^{721} accumulates in replication factories and interacts with mUb-PCNA albeit at lower level than Polη^{wt}. XP-V cells overexpressing Polη^{721} were only slightly UV-sensitive. Altogether, our data strongly suggest that Polη^{721} is functional and that the patient displays a XP-V phenotype because the mutant protein is excessively unstable. We then investigated the molecular mechanisms involved in this excessive proteolysis. We showed that Polη^{721} is degraded by the proteasome in an ubiquitin-dependent manner and that this proteolysis is independent of the E3 ligases, CRL4^{cdt2} and Pirh2, reported to promote Polη degradation. We then demonstrated that the extra 8 amino acids of Polη^{721} do not act as a degron but rather induce a conformational change of the Polη C-terminus exposing its bipartite NLS as well as a sequence close to its UBZ to the ubiquitin/proteasome system. Interestingly we showed that the clinically approved proteasome inhibitor, Bortezomib restores the levels of Polη^{721} suggesting that this might be a therapeutic approach to preventing tumor development in certain XP-V patients harboring missense mutations.

1. Introduction

Most types of DNA damage block replication fork progression during DNA synthesis because replicative DNA polymerases are unable to accommodate altered DNA bases in their active sites. To overcome such blocks, eukaryotic cells employ a specialized set of DNA polymerases that carry out translesion synthesis (TLS) past DNA lesions [8]. These polymerases, mostly belonging to the Y family, have a more open conformation and can accommodate different damaged bases in their active sites [37]. While these catalytic sites are conserved between different family members and are typically contained within the N-terminal 432 amino acids, the 250 C-terminal amino acids are poorly conserved. This C-terminal region is mainly involved in the cellular localization and regulation of these TLS polymerases [18,19,25,34]. The best characterized of these Y-family polymerases is DNA polymerase (Polη), which participates in genome stability through its capacity to accurately bypass the most abundant photoproduct, the TT-CPD [13,24]. Mutations in the human *POLH* gene encoding Polη are responsible for the cancer-prone xeroderma pigmentosum variant (XP-V), a human recessive disorder characterized by a high sensitivity to solar light and a dramatic predisposition to cutaneous tumors. XP-V cells present a replication defect and an elevated mutagenesis upon UV exposure [23,32].

Because Polη is error-prone on undamaged DNA, it has to be tightly regulated in cells (for review [28]). A critical step in the

* Corresponding author. Tel.: +33 1 42 11 40 30; fax: +33 1 42 11 50 08.
E-mail address: patricia.kannouche@gustaveroussy.fr (P.L. Kannouche).

http://dx.doi.org/10.1016/j.dnarep.2015.02.017
1568-7864/

regulation of TLS is the post-translational modification of proliferating cell nuclear antigen (PCNA), the replicative sliding clamp that plays an essential role in DNA replication [11]. Upon UV irradiation, PCNA is monoubiquitinated on its Lys-164. This event is considered to facilitate the TLS process since Polη preferentially interacts with monoubiquitinated PCNA through its ubiquitin-binding domain (UBZ) as well as its PCNA interacting peptide (PIP)-box [2,20,36].

Recent works showed that the activity of Polη can also be regulated by its monoubiquitination [3]. It was hypothesized that, Polη is mono-ubiquitinated on one of the four C-terminal lysines at the end of the protein to inhibit its interaction with PCNA in undamaged cells [3]. Other studies highlight different mechanisms to promote the degradation of Polη. In *Caenorhabditis elegans* (*C. elegans*), the GEI-17 SUMO E3 ligase protects PolH protein from degradation mediated by $CRL4^{Cdt2}$ pathway [21]. It has been reported that in human cells, the RING-H2 type E3 ligase (Pirh2) promotes Polη degradation in an ubiquitin-independent manner [16]. In addition, the Murine double minute-2 (MDM2) can also serve as an E3 ligase for Polη polyubiquitination and proteosomal degradation in response to ultraviolet irradiation [17].

The vast majority of XP-V patients harbor *POLH* mutations giving rise to a truncated protein. However, some XP-V patients have missense mutations producing full-length Polη proteins that are generally undetectable by western blot [26]. We recently identified a new Polη variant containing two missense mutations, one mutation within the bipartite NLS (T692A) and a second mutation on the physiological stop codon (X714W) leading to a longer protein with an extra 8 amino acids (721 instead of 713 AA [26]). As the Polη missense variant is extremely unstable and is nearly undetectable, a definitive measure of its functional deficit in cells has not been explored.

In the current work, we report the characterization of the Polη variant, $Polη^{721}$ at the molecular and cellular level. We found that $Polη^{721}$ is functional *in vitro* and *in vivo* and that the patient displays a XP-V phenotype because the mutant protein is excessively unstable. We also demonstrated that the extra 8 amino acids of $Polη^{721}$ do not act as a degron but rather induce a conformational change of the Polη C-terminus exposing its bipartite NLS as well as a sequence close to its UBZ to the ubiquitin/proteasome system. Interestingly we showed that a clinically approved proteasome inhibitor, Bortezomib fully restores the levels of $Polη^{721}$ suggesting that this might be a therapeutic approach to preventing tumor development in certain XP-V patients harboring missense mutations.

2. Materials and methods

2.1. Cell culture and treatments

Normal and XP-V primary diploid fibroblasts (405VI and XP872VI respectively), SV-40 immortalized fibroblasts XP-V (named XP30RO) and SV-40 immortalized human fibroblasts MRC5 were cultivated in Minimal Eagle Medium (MEM; Gibco) supplemented with 10% fetal calf serum (FCS), L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin under 5% CO_2. HEK293FT cells (Invitrogen) were cultivated in D-MEM (Dulbecco's Modified Eagle medium) containing sodium pyruvate, penicillin/streptomycin and 10% FCS under 5% CO_2. Cycloheximide and MG132 purchased from Sigma were used at 25 μg/ml and 10 μM respectively. Bortezomib was purchased from Calbiochem and used as indicated. Global and local UV-C irradiations were performed as previously described [18].

2.2. Constructions and transfections

All constructs for mutant analysis were generated using standard molecular biology techniques and were verified directly by DNA sequencing. The parent plasmid for expression of $Polη^{721}$ and its various mutant derivatives was pCDNA-POLH [32], which contains a full-length *POLH* cDNA under the control of the CMV promoter. Point mutants were generated by PCR-based methods using a QuikChange® site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. Details on all constructs are available upon request.

Transient transfection was performed using Exgen500 (Fermentas) according to the manufacturer's protocol, and cells were analyzed 24–48 h later. For stable transfection, XP30RO fibroblasts were transfected using FuGENE 6 (Roche) and stable $Polη^{721}$ transfectants were isolated and further propagated in medium containing 100 μg/ml zeocin (InvivoGen). Stable XP30RO cell lines expressing wild-type Polη ($Polη^{wt}$) or $Polη^{D652A}$ have been previously described ([7,32], respectively).

siRNA were purchased from Eurogentec: siRNA PCNA [GCCGAGAUCUCAGCCAUAU], and Dharmacon: non-targeting siRNA [ON-TARGETplus non target pool], siRNA Cdt2 [ON-TARGETplus SMART pool] and siRNA Pirh2 [CCAACAGACUUGUGAAGAA]. Cells were transfected with 10 nM of siRNAs using INTERFERin reagent (Polyplus) according to the manufacturer's instruction, and analyzed 72 h later.

2.3. Antibodies

Antibodies used for western blotting and immunofluorescence were purchased from Abcam (Polη ab17725, Cdt2, GAPDH, GFP), Santa Cruz (Chk1, Pol H-300, Lamin A/C, PCNA, Nbs1,), Bethyl (pS33-RPA 32, Cdt1, Polη), Sigma (β-actin), BD biosciences (β-catenin), Calbiochem (RPA32), Cell signaling (Ps345-Chk1), and Novus biological (Ps343-NBS1).

2.4. Cell lysates and western blot

For analysis of phosphorylated proteins, cells were scrapped directly in NETN buffer (150 mM NaCl, 1 mM EDTA, 50 mM Tris pH 7.5, 0.5% NP-40, antiproteases, antiphosphatases (PhosSTOP, Roche)) and incubated 30 min on ice. Extracts were sonicated 15 s at amplitude 30%. Whole cell extracts were prepared in lysis buffer (20 mM NaCl, 1 mM $MgCl_2$, 50 mM Tris pH 7.5, 0.1% SDS, protease inhibitor cocktail and 50 U/ml benzonase, Novagen). Proteins were separated by electrophoresis in 8–10% SDS-PAGE gels and analyzed by western-blotting.

2.5. Immunoprecipitation, His pull-down and cell fractionation

Immunoprecipitations of PCNA were performed as previously described [20]. For His pull-down, HEK 293FT cells were lysed in denaturing conditions (100 mM NaH_2PO_4, 10 mM Tris–Cl, 8 M urea, 20 mM imidazole, pH 8), centrifuged and supernatant was incubated with Ni-NTA-agarose (Quiagen) during 45 min at RT before washes and a denaturation by heating.

To obtain chromatin-bound proteins cell fractionation was performed as already described [7]. Nuclear/cytosolic fractionation was performed using 1 volume of isotonic buffer (50 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 15 mM $CaCl_2$, 1.5 mM sucrose and 0.05% NP40). After incubation, extracts were centrifuged. The supernatant (cytoplasmic fraction) was then transferred in a new tube and kept at −80 °C and nuclei were resuspended in 3 volumes of Nuclear protein extraction buffer (20 mM Hepes, 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA and 25% glycerol) during 30 min at 4 °C then sonicated. After centrifugation, the nuclear soluble fraction was heat-denaturated.

Please cite this article in press as: S. Ahmed-Seghir, et al., Aberrant C-terminal domain of polymerase η targets the functional enzyme to the proteosomal degradation pathway, DNA Repair (2015), http://dx.doi.org/10.1016/j.dnarep.2015.02.017