PCNA interactions with TLS DNA Polymerases; as a target for the development of more effective therapies to avoid Chemoresistance.

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Abstract

In response to the continuous modifications of DNA by endogenous and environmental genotoxic agents, cells have evolved mechanisms of DNA damage response (DDR). During DDR, cellular pathways are activated (homologous recombination, nucleotide excision repair, mismatch repair etc.) that lead to either DNA repair or DNA damage tolerance. Cells have the ability to tolerate a certain amount of DNA damage during replication via specific mechanisms that allow them to copy over DNA lesions and postpone it to a latter moment of the cell cycle. In this pathway, low-fidelity translesion synthesis (TLS) polymerases that can replicate damaged DNA gain access to replication forks via interactions with proliferating cell nuclear antigen (PCNA). PCNA is a DNA-sliding clamp that acts as a processivity factor for the replicative DNA polymerase in eukaryotic cells. PCNA binds to a variety of DNA replication and repair proteins and coordinates their actions at replication forks. Alongside of PCNA, another scaffold protein involved in the DNA damage tolerance pathway is a Y-family DNA polymerase REV1. REV1 is involved in bypass replication across sites of DNA damage and postreplicational gap filling. In the process of TLS, high-fidelity replicative DNA polymerases stalled by DNA damage are replaced by error-prone TLS enzymes; a process mediated by PCNA and REV1. Because TLS enzymes are responsible for the majority of mutagenesis in eukaryotic cells, their inhibition may provide therapeutic strategies to prevent mutagenesis in tumors and avert acquired chemoresistance. Using NMR spectroscopy, we have studied the binding of human PCNA and REV1-PAD domain with the long term goal to develop inhibitors of this interaction that block TLS polymerase access to DNA holding promise as new anti-cancer therapeutics.

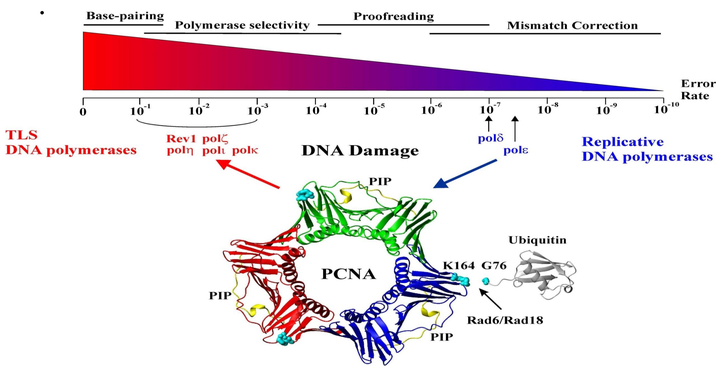

Figure. 1. Fidelity of DNA replication and switching of replicative to TLS polymerases in response to DNA damage.

Introduction

- DNA replication is very important and also fragile; errors during this process can alter and compromise genomic integrity. (ex: Damaged DNA that cannot be replicated by the polymerase can lead to stalled replication forks, forks breakdown and chromosome instability)
- In DDR (DNA Damage Response); there are two pathways: the translesion pathway and the template switch pathway.
- Activation of TLS: The activation of TLS and polymerase switch reaction is thought to be mediated by modifications of PCNA.
- Mechanisms that are thought to be involved in DDR at replication forks are mediated by a series of protein-protein interactions as well as protein-DNA interactions.
- PCNA serves as a binding platform for various proteins that are also involved in DDR. (**Figure1**)
- Monoubiquitination of Lysine 164 PCNA by RAD6/RAD18 proteins triggers DNA damage tolerance by TLS.
- Most Y-family TLS DNA polymerase possesses PCNA Interacting Box motifs (PIP box) that enhance their interaction with ubiquinitnated PNCA.

Methodology

- Use of different media the grow PCNA and REV1-PAD in *E.Coli*.
  - M9 Media for tagged growth of bacteria.
  - LB media for untagged growth of bacteria.
- Protein Purification: GST-tagged or His-tagged proteins
  - Use of columns and beads that will bind the protein of interest. For GST-tagged proteins, we used Glutathione agarose and for His-tagged proteins we used Ni-NTA agarose.
- Use of Bradford Reagent to monitor protein outcome.
- Use of proteases Thrombin or TEV to cleave the tag off the protein.
- FPLC: Fast protein liquid chromatography (FPLC), is a form of liquid chromatography that is often used to analyze or purify mixtures of proteins.
  - Mobile phase : buffer and Stationary phase: resin beads in the column.
  - Separation by size: PCNA trimer 28.8 Kda and REV1-PAD 13.6 Kda.
  - Fraction Collection allows us to isolate our protein of interest and use SDS

Page for further studies.

- Sodium Dodecyl Sulfate SDS gel Electrophoresis (SDS Page): Technique used to linearize proteins by imparting an overall negative charge on them. Proteins are separated by size and my migration rate through the matrix.
- Nuclear Magnetic Resonance (NMR) Spectroscopy: we used NMR spectroscopy to study proteins structures through HSQC's and protein interactions by peaks shift.

Results

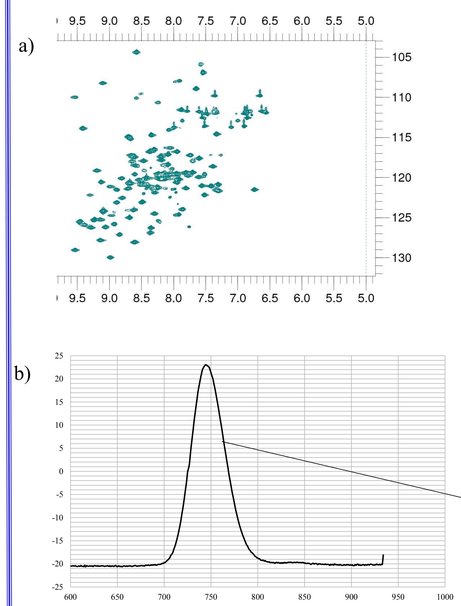

Figure 2: a) HSQC shows that ^{15}N labeled-REV1 –PAD, is a stable and complex in structure at pH 7 in PBS buffer. **b)** Shows a chromatograph from FPLC of REV1, showing that it elutes at around 75mL. **c)** SDS-Page shows PCNA and Rev1 and elucidates their differences in size.

-HSQC is obtained by NMR spectroscopy . Each residue of a protein except proline has an amide proton attached to a nitrogen in the peptide bond. HSQC provides correlation between the nitrogen and amide proton; each amide yields a peak in HSQC.

-After protein purification, REV1-PAD was isolated by FPLC. Mobile phase of that chromatographic separation was a Phosphate Buffer (Na_2HPO_4, NaH_2PO_4, NaCl and DTT) at pH 7; protein was collected and ran on SDS-Page gel.

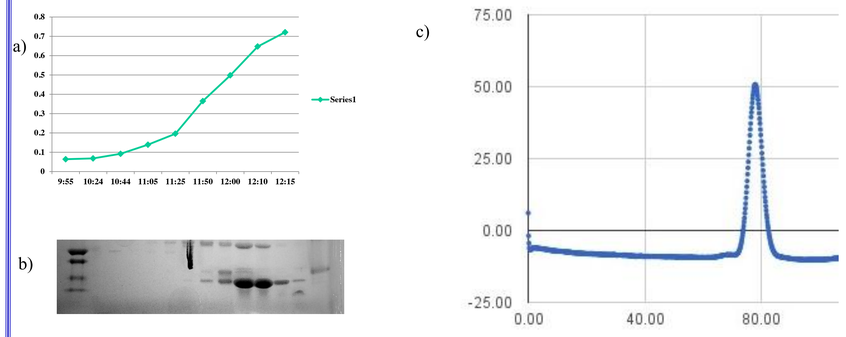

Figure 3: a) Growth curve of PCNA; PCNA was grown in bacteria on LB/KAN plates and Kanamycin (KAN) was used as the antibiotic during the cell culture. PCNA was untagged and grown in LB broth. IPTG was used as a protein inducer when OD_{600} reached about 0.7.Overnight growth at about 20 degrees.
b) Shows PCNA on SDS Page before and after FPLC. Before proteins are ran on FPLC they should be cleaved using a protease. Thrombin was used to cleave the protein, and the picture shows the slight size difference between the two.
c) Shows a chromatograph from FPLC of PCNA, showing that it elutes at around 80mL . To study the binding of PCNA and REV1, the proteins are ran in the column using the same buffer at pH 7.0.

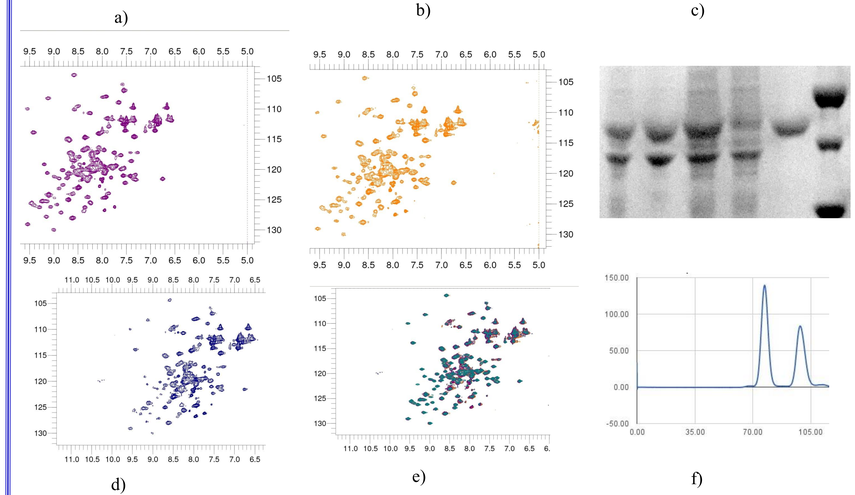

Figure 3: a), b), d), and e) are HSQC's of PCNA and REV1-PAD at different mole raitos and concentrations. a) Hsqc shows PCNA and REV1-PAD: 0.25:1 ratio. b) Hsqc shows PCNA and REV1-PAD: 0.5:1 ratio. d) Hsqc shows PCNA and REV1-PAD: 0.75:1 ratio. e) Represents and over lap of ^{15}N-labeled Rev1-PAD with the 3 Hsqc's at different mole ratios. PCNA was unlabeled. c) SDS Page gel of coeluted PNCA and REV1-PAD recovered from FPLC; showing separate elution. f) FPLC of PCNA and REV1-PAD; coelution in Phosphate buffer. Hints that the two proteins may not interact in those conditions.

Conclusion

PCNA and REV-PAD (708-824) proteins are involved in DNA damage tolerance pathways. Finding their interaction site would be beneficial for drug design purposes for avoidance of chemoresistance. To do so, we had to purify each protein, and using titration techniques we utilized NMR spectroscopy to obtain HSQC's. We used ^{15}N-labeled REV1-PAD as the control, with unlabeled PCNA to observe peak shifts that would in turn indicate binding. In Figure 3.e), we observe very little peak shifts; furthermore, FPLC and SDS-Page (Figure 3.c &3.f) support the hypothetical conclusion that there is no binding. Another easily arguable position would suggest that the conditions that we conducted the experiments in weren't ideal for binding, or even that the construct of REV1 that we used also wasn't ideal.

Future Work

- Use the longer construct of REV1-PAD (708-842)
- Try different concentrations, mole ratios and salt gradients.
- Use ^{15}N-labeled PCNA as the control, with unlabeled REV1-PAD and observe for peak shifts.
- We conducted our experiment at pH 7, try working in more acidic conditions. Ex: pH 5.

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References: I) Philip A. Knobel and Thomas M. Marti (2011) *Translesion DNA Synthesis in the context of Cancer Research* II)Yulia Pustovalova, Mark W. Maciejewski and Dmitry M. Korzhnev (2013) *NMR Mapping of PCNA Interactions with TLS DNA Polymerase REV1 mediated by REV1-BRCT domain*