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Replication Protein A (RPA) Targeting of Uracil DNA Glycosylase (UNG2)

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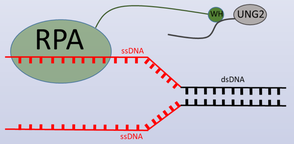
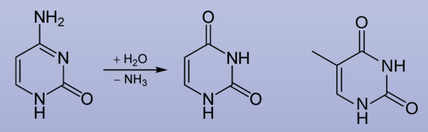
Introduction

Replication Protein A (RPA) is a single stranded DNA binding protein which stabilizes ssDNA for replication and repair. One function of RPA is to bind the DNA repair enzyme uracil DNA glycosylase (UNG2) and direct its activity towards ssDNA-dsDNA junctions[1].



Model of RPA binding to ssDNA and UNG2 during replication

UNG2 removes uracil bases from DNA which can appear through dUMP misincorporation or through cytosine deamination. If uracil is present instead of a cytosine, then the original GC pair becomes a GU pair. The uracil will then base pair to adenine in the replicated daughter strand. This results in a GC → AT mutation that could contribute to cancer formation.



Cytosine deamination to uracil, and a thymine base is shown for comparison.

RPA is known to target UNG2 towards individual uracil bases. We hypothesize that RPA will target UNG2 to uracil bases in DNA regardless of the number of uracils in the DNA strand.

Materials and Methods

DNA oligonucleotides were purchased from Integrated DNA Technologies . Oligos were annealed by mixing complementary strands, heating to 95˚C, then cooling to room temperature. The DNA sequences contained ~50% GC content and the uracil bases were flanked by an identical palindromic sequence. An adenine base was placed opposite to the uracil on the complementary strand to mimic a dUTP misincorporation during replication. 5' and 3' fluorescein end-labels were conjugated to the phosphate of the terminal nucleotide.

Activity assays were performed at room temperature by mixing DNA, RPA, and UNG2 in a buffer solution containing 10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM DTT, and 0.1 mM EDTA. Assays contained 0.4 µM DNA, 1 nM UNG2, and varying concentrations of RPA. Reactions were run for 4 minutes to capture the initial rate of reaction. The assay was quenched by adding NaOH to a concentration of 300 mM then heating for 15 min at 70˚C. The NaOH treatment also cleaves the abasic sites generated by UNG2 removing uracil. The samples were then diluted 5-fold with formamide containing 5 mM EDTA and the DNA fragments were resolved on 15% polyacrylamide-8M urea TBE gels. The fluorescein-labeled fragments were imaged and the gel bands were quantified using Fiji/ImageJ.

Calculations for k_{obs} used the equation:

$$k_{obs}\ (\text{min}^{-1}) = \frac{(\%\ \text{uracil excised}) * [\text{Substrate}]}{(\text{Reaction Time}) * [\text{Enzyme}]}$$

Calculations for P_{trans}, or the percent of DNA strands contained excision at both sites,[2] used the equation:

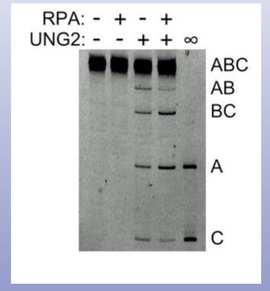
$$P_{trans} = \frac{(A+C)-AB-BC}{A+C+AB+BC}$$

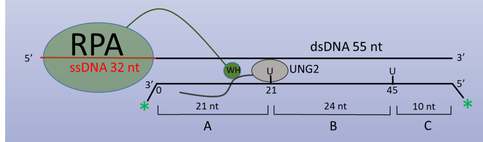
The kinetic data are reported as means with standard errors derived from four independent trials for each condition. Data was plotted using GraphPad Prism 6.

Electrophoretic mobility shift assays (EMSA) used 0.9% agarose—0.1x TAE gels and contained 0.4 µM DNA and varying amounts of RPA. High concentrations of UNG2 was added to fully excise all uracils to produce abasic sites. The buffer solution used during binding was identical to the assay buffer but contained 10% glycerol. After electrophoresis, the free and bound DNA bands were imaged with a Typhoon 9500 imager.

UNG2 Site Selectivity Assay

RPA Directs UNG2 Activity

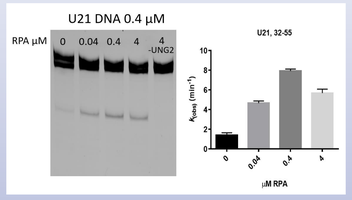


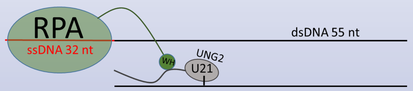


RPA binds to the ssDNA and tethers UNG2 to the closer uracil

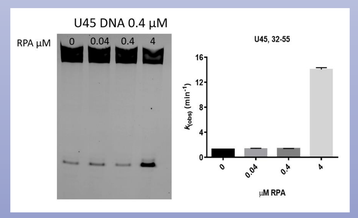
UNG2 Site Selectivity: One Uracil in the Duplex

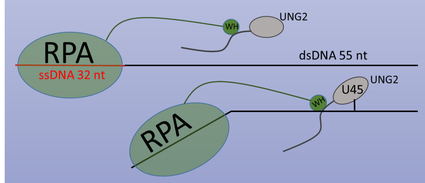
RPA Targets UNG2 to U21





RPA Targets to U45 only with High RPA

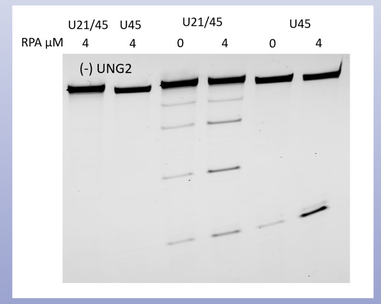


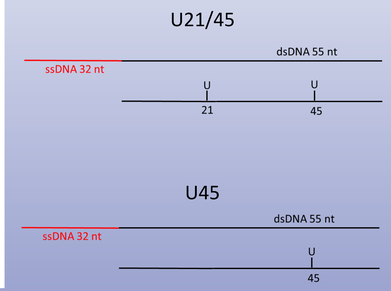


Additional RPA binding possibly unwinds dsDNA and extends the reach of UNG2 to U45

Site Selectivity Changes with Two Uracils in the Duplex

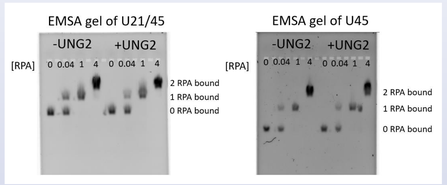
High RPA **Does Not** increase U45 activity with U21 present

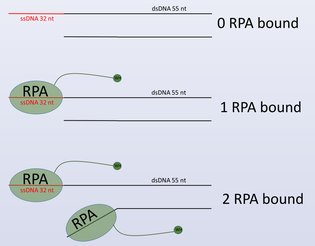




Possible Mechanism 1

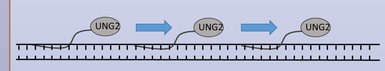
Does the Uracil or Abasic Site Prevent RPA Binding?



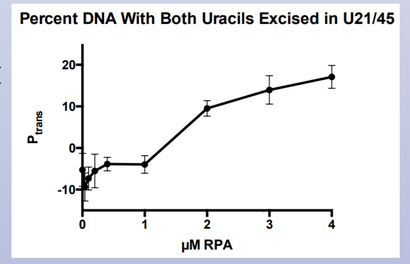


Possible Mechanism 2

Can UNG2 Only Cut Once Before Dissociating?

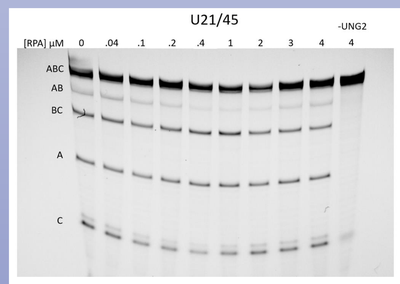


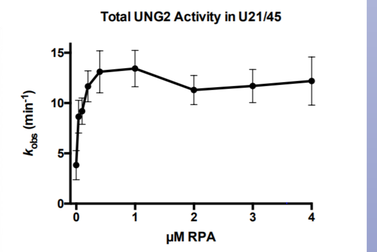
UNG2 alone can translocate along a strand of DNA



Ongoing Hypothesis

Is UNG2 Being Limited by its Reaction Speed?





Future Work: Adding an Additional Uracil Between U21 and U45

Acknowledgements

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References

1. Weiser, Brian P, et al. “N-Terminal Domain of Human Uracil DNA Glycosylase (hUNG2) Promotes Targeting to Uracil Sites Adjacent to ssDNA–DsDNA Junctions.” *Nucleic Acids Research*

2. Rodriguez, Gaddiel, et al. “Disordered N-Terminal Domain of Human Uracil DNA Glycosylase (hUNG2) Enhances DNA Translocation.” *ACS Chemical Biology*