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UNG2 and RPA Activity on ssDNA-dsDNA Junctions

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Abstract

Uracil DNA glycosylase, or UNG2, is an enzyme that is involved in DNA repair. Its primary job is to eliminate harmful uracil bases from DNA strands. To do this, the enzyme is assisted by replication protein A (RPA). RPA helps UNG2 in the identification of uracil bases by targeting UNG2 activity near ssDNA-dsDNA junctions (1-3). The results from assays presented here agree with published findings that showed UNG2 is heavily targeted by RPA to uracil bases that are close to ssDNA-dsDNA junctions (for example, uracil located 9 bps from the junction as opposed to 33 bps) (1,2). However, these previous experiments were performed in the absence of a macromolecular crowding agent. Inert compounds such as PEG8K can be used experimentally to better represent the physiologic environment inside a cell, which is very crowded with proteins and other small molecules and differs from dilute conditions often used in enzyme assays. In the presence of a crowding agent (PEG8K), we found that RPA balances UNG2's selectivity for uracil sites near ssDNA-dsDNA junctions that contain a 5' ssDNA section. In other words, UNG2 becomes less targeted to uracils that are very close to the junction (i.e., U9). Interestingly, this effect was not seen when we examined RPA effects on UNG2 activity using ssDNA-dsDNA junctions substrates that contain a 3' ssDNA section.

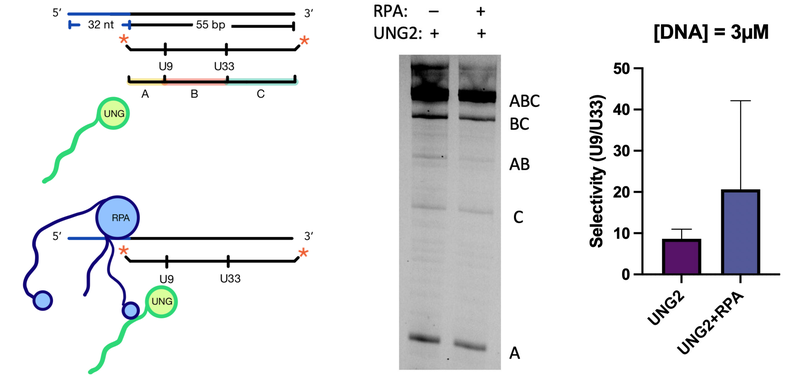
Methods

During enzyme assays, the concentration of uracilated DNA was kept constant at 3μM. Initially, RPA was excluded and variable UNG2 concentrations were tested to identify optimal steady-state conditions for the enzyme. Assay time points were 5 minutes and UNG2 concentrations ranged from 0.1-100 nM. Enzyme assays were then run with a stoichiometrically-equivalent amount of RPA and DNA (3μM) and the determined concentration of UNG2. The assay buffer was 10mM Tris-Cl, 100mM NaCl, 0.1mM EDTA, and the reaction volume was 15μL. The reactions were quenched with NaOH. A solution of formamide-5mM EDTA was added to the samples as a loading buffer, then the samples were run under denaturing conditions using UREA-PAGE. DNA gel fragments that reflect UNG2 activity were visualized by nature of fluorescein end-labels present on the uracilated DNA strand. Gel band intensity was analyzed and quantified using FIJI. This progression was also repeated with 10% PEG8K added to the buffer. The gel band intensities were used to calculate kobs values for UNG2 under the various conditions using the following equation:

$$Kobs = \frac{\frac{[substrate\ processed]\ (\mu M)}{rxn\ time}}{[enzyme]\ (\mu M)}$$

UNG2 and RPA activity on ssDNA-dsDNA substrates containing a 5' ssDNA section

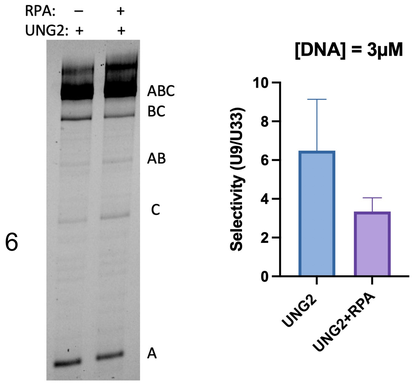
Figure 1



Effects of PEG8K on UNG2 activity for 5' ssDNA substrate

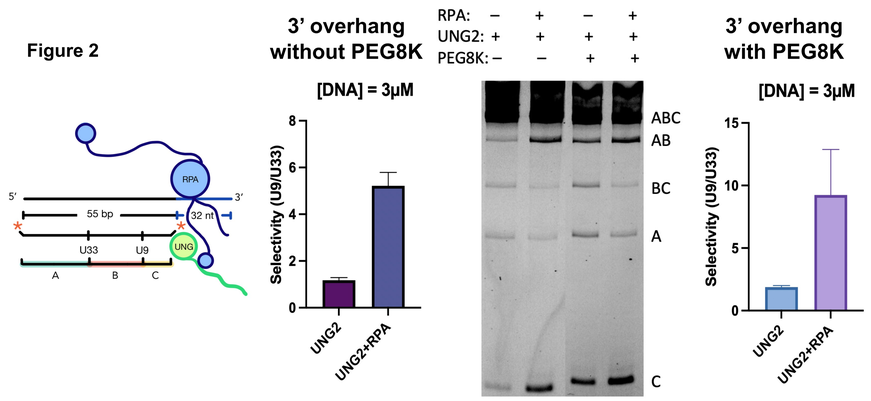
With a 5' overhang, UNG activity becomes somewhat balanced at U33 in the presence of PEG8K.

The selectivity ratio differs from about 9 and 20 to about 6 and 3 with the addition of PEG8K.



UNG2 and RPA Activity on ssDNA-dsDNA substrates containing a 3' ssDNA section

Figure 2



The balancing effect is not seen with the 3' DNA.

Summary of UNG2 Kobs values determined from all assays with RPA and PEG8K

3 μM 5'U9U33 DNA, 1nM UNG2 and 3μM RPA

UNG		U9/U33: 8.667
Ptrans	Kobs U33	Kobs U9
0	7.783	65.413

UNG+ RPA		U9/U33: 20.611
Ptrans	Kobs U33	Kobs U9
0.111	7.307	57.934

3 μM 5'U9U33 DNA, 2nM UNG2 and 3μM RPA with PEG8K

UNG		U9/U33: 5.563
Ptrans	Kobs U33	Kobs U9
0.161	10.493	50.508

UNG+ RPA		U9/U33: 3.346
Ptrans	Kobs U33	Kobs U9
0.256	10.751	36.839

3 μM 3'U9U33 DNA, 3nM UNG2 and 3μM RPA

UNG		U9/U33: 1.177
Ptrans	Kobs U33	Kobs U9
0	4.431	5.119

UNG+ RPA		U9/U33: 5.220
Ptrans	Kobs U33	Kobs U9
0	4.662	24.013

3 μM 3'U9U33 DNA, 3nM UNG2 and 3μM RPA with PEG8K

UNG		U9/U33: 1.884
Ptrans	Kobs U33	Kobs U9
0.165	12.715	23.591

UNG+ RPA		U9/U33: 9.249
Ptrans	Kobs U33	Kobs U9
0.279	3.879	35.703

Conclusions

We now believe that the polarity of ssDNA-dsDNA junctions affects the role of RPA in targeting UNG2 activity. Our experiments with PEG8K more accurately reflect nuclear conditions then experiments in dilute buffer. These experiments indicate that consistent uracil base excision activity is important when a 5' ssDNA section is adjacent to a DNA duplex. In contrast, the ability of RPA to target UNG2 very close to a junction appears to be important when the junction contains a 3' ssDNA section.

Acknowledgements

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References

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