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Mapping the Binding Sites for Chromatin Assembly Factor 1 on PCNA

Maintenance of normal cellular processes and prevention of diseased states, including cancer, requires proper gene silencing. Gene silencing is controlled in large part by the specific and precise packaging of DNA in the nucleus. This process is carried out by two proteins: proliferating cell nuclear antigen (PCNA) and chromatin assembly factor 1 (CAF-1).[1] Although the interaction between PCNA and CAF-1 is known to be critical in the proper packaging of DNA, their interaction is not currently well understood.

PCNA interacts with over 50 different proteins during DNA replication, repair, and recombination.[2] Previous studies have identified the specific region of PCNA to which almost all of these PCNA-interacting proteins bind.[2] However, our preliminary data suggests that CAF-1 may interact with PCNA at a novel, additional site.[3] To determine if this is in fact a secondary site of interaction, I created seven single amino acid PCNA mutants using site-directed mutagenesis. The mutated amino acids were selected based on their side chains and their location in and around the cavity that we hypothesize is a secondary site of interaction for CAF-1 on PCNA, as seen in Figure 1. Each of the amino acids were mutated to alanine residues.

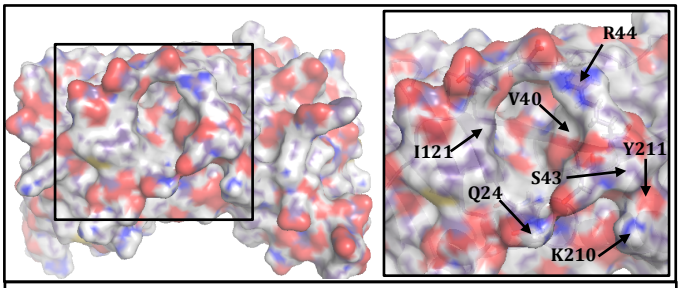

Figure 1. Appearance of the surface cavity on one monomer of PCNA. The box shows the locations of the amino acids in and around the cavity chosen for mutation.

After performing site-directed mutagenesis, I overexpressed and purified all seven mutant PCNA proteins. In order to determine if there were differences in binding between wild type and mutant PCNA proteins with CAF-1, I began using a fluorescence anisotropy (FA) binding assay.

A summary of FA can be seen in Figure 2. In our experiments, polarized light is used to excite fluorescently tagged CAF-1. When unbound, CAF-1 has a low anisotropy as it is free to rapidly rotate and reorient itself before emitting light, resulting in a depolarization of the light.

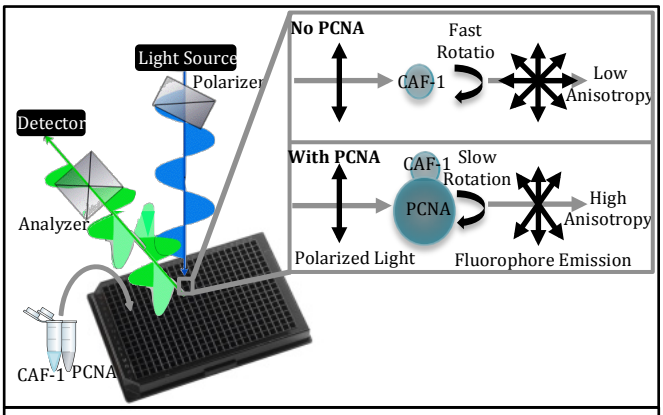

Figure 2. Principle of fluorescence anisotropy assay for characterizing protein-protein interactions.

As increasing amounts of PCNA are added, the anisotropy increases because CAF-1 is no longer able to rotate and reorient itself as rapidly and thus the light remains more polarized. This assay can be used to determine the binding constant (K_d) for each of the PCNA proteins and CAF-1. Throughout much of the fall semester and into the spring semester, I worked to optimize an FA assay. I optimized conditions for the fluorescent labeling reaction of CAF-1 as well as the concentrations and buffer conditions to use during the actual FA assay. However, after collecting lots of data, we determined that our system was not able to capture the interaction between PCNA and CAF-1 well because the reaction was occurring to quickly. Thus, although I collected data and calculated approximate K_d values for the interaction between PCNA and CAF-1, this data is likely not reliable.

I also began enzyme-linked immunosorbent assays (ELISAs) with the purified CAF-1 and both wild type and mutant PCNA proteins. For the ELISAs, I coated the plates with PCNA and added the CAF-1 as the second protein. To detect the CAF-1, I used an antibody for CAF-1. I ran this assay in duplicate for wild type PCNA and all seven PCNA mutants. In an ELISA, binding is quantified by the amount of absorbance, with increased absorbance corresponding to increased binding. As seen in Figure 3, the results from this assay suggest that R44A shows

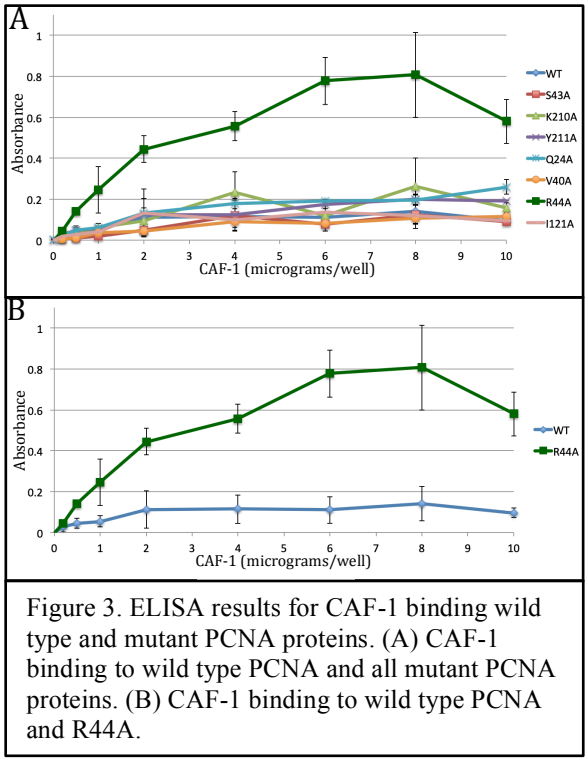

Figure 3. ELISA results for CAF-1 binding wild type and mutant PCNA proteins. (A) CAF-1 binding to wild type PCNA and all mutant PCNA proteins. (B) CAF-1 binding to wild type PCNA and R44A.

increased affinity for CAF-1 than wild type PCNA or any of the other mutants. This result is surprising as a mutant that affected binding would be expected to decrease the affinity rather than increase it. One potential explanation for this apparent increase in affinity is that R44A might function normally as a self-inhibitor to prevent PCNA from binding too tightly to CAF-1, which could result in global gene silencing.

In addition to the new ELISA, I also completed a preliminary EMSA assay. In an EMSA, the proteins of interest are allowed to react at different concentrations and then run on a native polyacrylamide gel. If the proteins interact, they will have a higher molecular weight and so will run more slowly on the gel in comparison to the individual proteins on their own. The darkness of bands (amount of protein in each band) can be quantified using an imaging program and approximate K_d values can be ascertained from this assay based on when the protein is split evenly between the two bands. In this assay, I used a constant concentration of PCNA (at 300 nM) and an increasing concentration of CAF-1 (from 0 nM to 300 nM). To visualize the bands, I silver-stained the gel, as seen in Figure 4. From this gel, it appears that there was a successful shift, as the amount of PCNA trimer in the lower band decreased

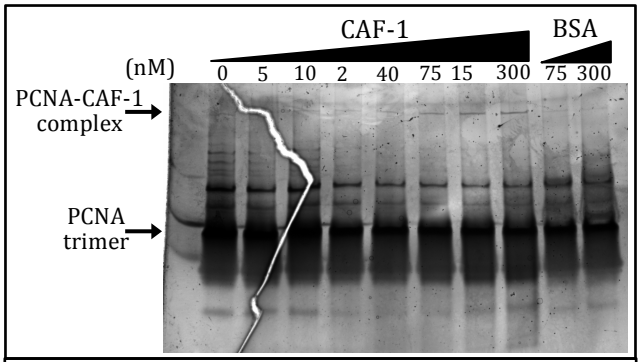

Figure 4. EMSA results for wild type PCNA and CAF-1. In the EMSA, increasing amounts of CAF-1 were added to a constant concentration of PCNA. BSA was run as a negative control.

with increasing amounts of CAF-1. However, the range of CAF-1 concentrations used were not sufficient to determine the K_d.

The results from the ELISAs suggest that this cavity is involved in a novel interaction with CAF-1. This alternate site of interaction could help explain how PCNA distinguishes CAF-1 from its many different binding partners, as this interaction between PCNA and CAF-1 is essential for proper gene silencing.

In future experiments, the EMSA assay can be further optimized and used to determine the K_d of wild type PCNA and CAF-1 as well as of the various PCNA mutants to determine if and how they affect binding to CAF-1. This assay can also be used to verify the results from the ELISA in which the R44A mutant showed higher affinity for CAF-1 than wild type PCNA.

References

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2. G. Maga, U. Hübscher, Proliferating cell nuclear antigen (PCNA): a dancer with many partners. *Journal of Cell Science*. 116:15, 3051-3060 (2003).
3. C. Kondratick, J. Litman, K. Shaffer, M. Washington, L. Dieckman, Crystal structures of PCNA mutant proteins defective in gene silencing suggest a novel interaction site on the front face of the PCNA ring. *PLoS ONE*. 13:3 (2018).