Computational protein design suggests that human PCNA-partner interactions are not optimized for affinity

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INTRODUCTION

Proliferating cell nuclear antigen (PCNA) mediates DNA replication and repair in eukaryotes through the recruitment of numerous DNA-modifying enzymes to the replication fork.1 PCNA forms a sliding platform to enhance the processivity and catalytic activity of its many different partners by tethering them to the DNA template.2,3 Many PCNA partners compete for the same binding site on PCNA through a conserved binding motif.2,3 Such multispecific binding is widespread in biological signaling and regulation, posing special challenges for their analysis.4,5 The mechanism by which PCNA switches between its different partners during DNA replication and repair is unknown and is the subject of on-going and intense research.1–3 For example, post-translational modification of PCNA partners was suggested as one of the mechanisms that enable PCNA to switch from processive DNA replication to repair of damaged DNA through the switching between partners.1 Previous examination of the importance of these interactions for DNA replication and repair utilized mutational approaches to abolish PCNA-partner interactions.6–8 However, due to the functional redundancy exhibited by PCNA partners,9 abolishing these interactions often led to minor phenotypic defects in yeast or human cells. To investigate the importance of accurate regulation of PCNA-partner interactions for DNA replication and repair, we previously utilized a directed evolution approach to generate yeast PCNA mutants with enhanced affinities.

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for different partners.\textsuperscript{10} In vivo analysis of these mutants in yeast revealed the susceptibility of DNA replication and repair for tighter PCNA-partner binding, highlighting the importance of a balance between the different PCNA-partner binding affinities for the progression of DNA replication and repair in vivo.\textsuperscript{10} To generate such mutants we used saturation mutagenesis of four residues at the interdomain connecting loop (IDCL) of PCNA that is the main surface mediating interaction with PCNA’s different partners.\textsuperscript{2,3}

Relative to the yeast PCNA, the human PCNA interacts with many more partners and is involved in regulating diverse biological processes including cell cycle and apoptosis.\textsuperscript{1} Thus, the generation and examination of PCNA mutants with altered affinity and specificity can be highly useful for examining the mechanism of PCNA switching between partners during different biological processes. In addition, PCNA plays an important role in uncontrolled cell proliferation in different cancers\textsuperscript{1–3} and potent inhibitors for PCNA-partner interactions can arrest cancer cell proliferation. Thus, engineering of human PCNA for enhanced affinity for different partners will deepen our understanding of the molecular basis for human PCNA-partner interactions and can significantly aid the design of new potent inhibitors of these interactions. With the initial intent of generating human PCNA mutants with enhanced affinities to the different PCNA partners, we used a similar directed evolution strategy focusing on the IDCL region of the human PCNA. Surprisingly, despite the high conservation of the structure and function of PCNA between yeast and human,\textsuperscript{11} our directed evolution experiments failed to identify any significantly improved human PCNA mutants (data not shown). Seeking sites outside the IDCL where the PCNA affinity toward its partners could be modulated, we used a computational approach to design human PCNA mutants.

In recent years, computational design has emerged as a powerful approach for \textit{de novo} design of proteins with new catalytic and binding properties,\textsuperscript{12,13} and for engineering novel specificities.\textsuperscript{14–17} Computational design methodology suffers from inaccuracies in the representation of protein energetics, and although computed and measured binding affinities are correlated,\textsuperscript{18} the small binding energy increases (~0.5 kcal/mol per mutation) associated with the affinity-maturation process are not accurately captured by the energy function,\textsuperscript{12,19} requiring testing of a variety of alternative mutants suggested by design.\textsuperscript{20} On the other hand, computational design offers valuable clues for the affinity maturation process by focusing mutations on a few positions and amino acid residue identities that could increase affinity. This approach alleviates the need to construct and screen large mutant libraries, which can often be much larger than the capacity of \textit{in vitro} evolution strategies (up to $10^{11}$ variants), and thus can uncover new sources of affinity enhancements that are missed by \textit{in vitro} evolution strategies.

Here we used computational design to generate PCNA mutants with increased binding affinity for four different PCNA partners including p66, a subunit of polymerase delta, Flap endonuclease 1 (FEN1), p15, a PCNA-associated factor with increased expression in tumor cells,\textsuperscript{21} and polymerase eta (polη).

**MATERIAL AND METHODS**

**Computational design of PCNA**

Prior to design, the natural complexes were relaxed by full side chain repacking followed by RosettaDock\textsuperscript{22} local-refinement with the default all-atom Rosetta energy function followed by rigid-body and side chain minimization. The resultant complexes were designed using RosettaDesign,\textsuperscript{23} as used in the design of \textit{de novo} interfaces\textsuperscript{19} restricting designed residues to a sphere of PCNA residues within 10 Å of the peptides, and allowing all amino acid identity substitutions at these sites, except for Pro, Gly, and Cys, which could destabilize the PCNA. All residues on the peptides were allowed to repack (side chain conformational sampling, without amino acid substitutions). RosettaDesign conducts combinatorial packing and design optimization, allowing it to identify optimal combinations of residue substitutions, each of which on its own could be destabilizing. Following RosettaDesign, we modeled the effects of other residue variants of similar physicochemical properties as those suggested by RosettaDesign and computed these mutants’ binding affinities. Binding energy was evaluated as the difference in energy of the repacked bound and repacked unbound configurations. In addition to the default rotamers used by RosettaDesign, we added extra rotamers for chi1 and chi2 within one standard deviation of the standard rotamers (using the Rosetta command line flags: -ex1 –ex2) in all calculations.

**Expression and purification of human PCNA**

For \textit{E. coli} expression, WT PCNA and the different mutants were cloned into plasmid pET28 (Novagen) fused to 6xhistidine tag. Expression and purification of the WT and mutant PCNA were performed as previously described.\textsuperscript{10}

**ELISA assay for the detection of PCNA-PIP peptide interactions**

ELISA plates (Griener Microlon 96W) were coated with 0.2 µg streptavidin (Pierce) and 1 µM of biotinylated PIP peptides (sequences are shown in Supporting Information, Fig. S1), as described.\textsuperscript{10,24} Following peptide coating, the plates were incubated with 0.01 mg/ml of the purified WT or mutant PCNA and shaken at 25°C for 1 h. Plates were then washed with PBS supplemented with 0.05% Tween-80 (PBST) and each well was incubated with mouse α-6xHis-tag antibodies (Santa-Cruz Biotechnology, 1:2000) and then with secondary
Fluorescence anisotropy assays

Fluorescence anisotropy of fluorescein-labeled p66 peptide and polη peptide was measured at increasing concentrations of PCNA variants. In each binding assay, fluorescein-labeled peptide (50 nM) was incubated with increasing concentrations of WT or mutant PCNA (monomer concentration of 0-80 μM) in a reaction buffer containing 150 mM NaCl, 1 mM EDTA, 30 mM Hepes, pH 7.5. Samples (50 μL) were loaded onto a 384-well plate and fluorescence anisotropy was recorded by a Synergy-2 (BioTek) plate reader using a 485 nm excitation filter and a 535 nm emission filter. The increases in anisotropy were plotted against the PCNA concentrations and the dissociation constant (K_D) values were determined by fitting the data to a single site binding model:

$$ r = r_{\text{max}} \cdot \left( \frac{[P]}{K_D + [P]} \right) $$

where r is the level of fluorescence anisotropy at a given PCNA (P) concentration.

Inhibition constants (K_i) of FEN1 peptide were determined by competition experiments between the fluorescently labeled p66 peptide and unlabeled FEN1 peptide in binding to PCNA. In these assays, 50 nM of fluorescently labeled p66 peptide was incubated with fixed concentrations of WT or mutant PCNA variants at a concentration that is equal to the calculated K_D. Fluorescence anisotropy was then measured at increasing concentrations of unlabeled FEN1 peptide (0-8 μM) (Supporting Information, Fig. S5). The K_i values were determined by fitting the data to a competitive inhibition model:

$$ r = \frac{r_{\text{max}}[P]}{K_D \cdot (1 + [I]/K_i) + [P]} $$

where r is the level of fluorescence anisotropy at a given PCNA (P) and inhibitor (I) concentrations. K_D values were fixed according to the data obtained with the fluorescently labeled p66 peptide (see Table II). All measurements were carried out in 384-well plates, using the Synergy-2 (BioTek) plate reader with a 485 nm excitation filter and a 535 nm emission filter. All experiments were performed in triplicates and the mean is presented ± standard deviation. For data fitting to the different equations we used the sigmaplot software.

Yeast two hybrid (Y2H) analysis of PCNA

The Y2H analysis was performed as previously described. Briefly, the pJ69a host strain was cotransformed with pAD-PCNA WT/mutant and pBD-partner plasmids in all possible combinations using the LiAC method. Single transformants were grown overnight in SC-Leu-Trp, washed twice with DDW and diluted by a factor of 1:50 into 10 ml of pre-warmed SC-Leu-Trp-His. OD_600 measurements of the cultures were taken at the indicated time. The generation time of the indicated mutants and their respective partners were calculated from their growth curves in liquid SC-Leu-Trp-His media. The generation time (τ) was calculated from the growth curves, according to the equation OD_t = OD_0 e^{-\tau t}. The generation time calculated for each culture is an average of at least three independent experiments.

### RESULTS

**Computational design of PCNA affinity-enhancing mutants**

To identify mutations outside of the IDCL region that may enhance PCNA affinity to its partners we used RosettaDesign, which uses combinatorial repacking of amino acid side chains and has been used design de novo binding interactions to design the entire surface of PCNA that interacts with its partners for enhanced intermolecular binding energy (Fig. 1). As expected from the initial experimental screen, affinity-enhancing substitutions in the IDCL region were not identified by this step. However, we found two positions (131 and 233) where the mutant pair V233W/Q131M was predicted to modestly increase affinity (Table 1). To better characterize the basis for the predicted increase in affinity and to identify other variants that may provide similar enhancements, we manually modeled and evaluated each substitution using the Rosetta energy function. Modeling showed the

<table>
<thead>
<tr>
<th>PCNA</th>
<th>PIP peptide</th>
<th>Binding energy (Rosetta energy units)</th>
<th>Buried surface area (Å²)</th>
<th>Hydrophobic buried surface area (Å²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>1a7b (Fen1)</td>
<td>-23.2</td>
<td>1436</td>
<td>1108</td>
</tr>
<tr>
<td></td>
<td>p15</td>
<td>-20.3</td>
<td>1476</td>
<td>1157</td>
</tr>
<tr>
<td></td>
<td>p66</td>
<td>-21.8</td>
<td>1469</td>
<td>1183</td>
</tr>
<tr>
<td></td>
<td>polη</td>
<td>-22.8</td>
<td>1409</td>
<td>1182</td>
</tr>
<tr>
<td>Q131M</td>
<td>1a7b (Fen1)</td>
<td>-25.4</td>
<td>1479</td>
<td>1159</td>
</tr>
<tr>
<td>V233W</td>
<td>p15</td>
<td>-22.1</td>
<td>1422</td>
<td>1116</td>
</tr>
<tr>
<td></td>
<td>p66</td>
<td>-24.8</td>
<td>1531</td>
<td>1199</td>
</tr>
<tr>
<td></td>
<td>polη</td>
<td>-28.4</td>
<td>1507</td>
<td>1243</td>
</tr>
</tbody>
</table>

The double mutant shows a decrease in binding free energies, and in all cases but p15, a small increase in buried surface area. The Rosetta binding energy calculations suggest that the free energy changes are mostly due to additional hydrophobic contacts, reduced desolvation penalties at the interface (due to the elimination of Gln131), and reductions in side chain conformational strain.
V233W substitution to be highly destabilizing on its own due to steric overlaps with Gln131. Substitution of Gln131 to any of the hydrophobic amino acids Ile, Leu, Met, or Val together with the V233W was predicted to enhance PCNA-partner interaction affinities, with the double mutant V233W/Q131M being most favored.

The majority of PCNA partners contain a conserved binding motif termed the PCNA-interacting protein (PIP) region, located in the N- or C-terminal region of the partner distinct from its active site. Our calculations show clear improvements in binding energy of the designed mutants, relative to the WT, for the four different PIP peptides derived from the different partners (Table I). Structural analysis shows that the V233W substitution increases the van der Waals contacts between the peptides and PCNA and eliminates a small cavity at the interface, providing a potential rationale for the affinity enhancement (Fig. 1). Rosetta energy calculations suggest that contributions to binding free energy decreases were also made by reductions in the desolvation penalty and residue-conformational strain at the interface (Table I). The predicted role of the substitutions at the Gln131 position is to restrict the side chain conformation of the substituted Trp233 to the designed conformation (Fig. 1B). As these substitutions interact solely with the conserved phenylalanine residues of the PIP peptides, the PCNA substitutions were predicted to improve affinities across the board to PIP-containing PCNA partners (see below). Computational analysis showed that other aromatic identities at position 233 would not interact as favorably with the peptide as the Trp even in the presence of other residue identities at position 131.

Experimental characterization of mutant PCNA binding to its partners

To experimentally examine the effect of the mutations suggested by computational modeling at positions 131 and 233 on the binding of PCNA to different partners, we generated the single V233W mutant and four double mutants containing the V233W and the Q131I/L/M/V substitutions. The WT and mutant PCNA genes were cloned into a bacterial vector and overexpressed in E. coli following growth and induction. We found that the single V233W mutant is expressed to a low level in the crude cell extracts (Supporting Information, Fig. S2), in agreement with the expectation from modeling that the pair Trp233/Gln131 would form destabilizing steric overlaps. By contrast, all the double mutants expressed to a high and similar level as the WT protein, suggesting that these mutations did not impair PCNA folding (Supporting Information Fig. S2). Next, the four double mutants were purified to homogeneity using Ni-NTA affinity and gel filtration chromatography, revealing PCNA trimerization levels similar to that of the WT protein, and allowing the separation and purification of these mutant trimers from aggregated forms of the protein (for...
example, see Supporting Information Fig. S3). We then examined the binding of these trimeric mutants to the four different PIP peptides including p66, FEN1, polh and p15 (Supporting Information, Fig. S1) using ELISA. The ELISA analysis showed a substantial increase of up to 4.6 fold of the mutants’ binding signal relative to the WT protein, in qualitative agreement with computational modeling (Fig. 2).

As a more sensitive measure of affinity, we characterized the affinity of the WT and mutant PCNA to the PIP peptides using a fluorescence anisotropy binding assay. To this end, all four peptides were synthesized with an addition of an N-terminal cysteine. 5-iodacetamidofluorescein (5-IAF) was then used to label each peptide and the resulting fluorescent peptides were purified by reverse-phase high-performance liquid chromatography (HPLC). For each peptide, successful labeling and purification were confirmed by mass spectrometry. All peptides except FEN1 were efficiently labeled, and of those, binding of p66 and polh to our PCNA variants could be easily detected by fluorescence anisotropy measurements. Thus, titration experiments indicated that the double mutants bound peptides with affinities up to 14-fold higher than the WT protein (Fig. 3, Table II, and Supporting Information Fig. S4), in agreement with the ELISA results and computational modeling (Fig. 2).

To verify that the PCNA mutants showing increased affinity for the PIP peptides also exhibit increased affinity for the full-length partner, a Y2H analysis was performed. To facilitate the Y2H analysis, we cloned the four full length PCNA partners fused to the DNA binding domain (BD) and the WT and designed PCNA mutants to the activating domain (AD). Strains containing different combinations of plasmids were grown on selective media to examine the relative level of the PCNA-partner interactions. To obtain a quantitative analysis of the difference in growth rates, the different strains were grown in liquid selective media and their growth rate was monitored. The Y2H strains containing the different PCNA mutants exhibited an up to 2.2-fold increase in the yeast growth rate in selective media (Fig. 4 and Table S1), indicating increased binding affinity. The increase in growth rate was further supported by a spotting assay in which different concentrations of cells were plated on agar (Supporting Information Fig. S6). Overall, the Y2H results further support the ELISA and fluorescence polarization (FP) analysis and show that the increase in binding affinity of the PCNA to the PIP pep-
tide leads to increased affinity to the full-length PCNA partner (Supporting Information Table S2). Since the human PCNA has many different partners it is possible that the designed mutations increased the binding affinity to all peptides examined here but reduced the binding affinity to other partners. However, this possibility is made unlikely by the very high conservation of the two PIP-domain Phe residues (Fig. 1) among all PCNA partners.

**DISCUSSION**

In this study, we used a computational approach to design human PCNA mutants with increased binding affinity for four different partners. This approach allowed the direct generation of mutants with enhanced interaction affinity for the different partners revealing an unexpected region that can modulate PCNA-partner interaction affinity, while alleviating the need to construct and screen large PCNA random mutant libraries. Based on structural information and modeling, we suggest that the V233W Q133V/I/L/M double mutant can lead to increased PCNA-partner binding affinities by forming additional hydrophobic interactions with conserved phenylalanine residues of the PIP region (Fig. 1). Our experimental analysis demonstrates that these interactions increase the affinity of PCNA to four representative client proteins.

Our target for the protein design was PCNA, an essential protein mediating DNA replication and repair in all eukaryotes. PCNA is a classical example of a hub protein that binds and releases many different DNA modifying enzymes during the replication process. Our current designed human PCNA mutants show that the affinity of PIP containing partners to PCNA was not fully optimized during the evolution of PCNA-partner interactions. It is possible that the specific mutations leading to increased binding affinities were suppressed to maintain the transient nature of these interactions and to allow rapid switching of partners on PCNA during replication (Supporting Information Fig. S7). This hypothesis is supported by our previous in vivo examination of yeast PCNA mutants with increased affinity for different partners highlighting the importance of maintaining transient PCNA-partner interactions during DNA replication. Our designed human PCNA mutants can be further examined both in vitro and in vivo using different DNA replication assays to shed new light on the regulation of PCNA-partner interactions during PCNA-mediated DNA replication and repair in humans. In addition, these mutants allow us to more deeply understand the molecular basis of PCNA-partner interactions and can enable the design of potent inhibitors for PCNA-partner interactions that can lead to the inhibition of uncontrolled cell proliferation for cancer therapy.

**Figure 3**
Fluorescence anisotropy assays for binding of human PCNA to p66 or polη peptides. The Q131M/V/I/L mutants also include the V233W mutation. Fluorescence anisotropy of fluorescein-labeled p66 peptide (A) and polη peptide (B) was measured at increasing concentrations of PCNA variants, as indicated. In each binding assay, fluorescein-labeled peptide (50 nM) was incubated with increasing concentrations of WT or mutant PCNA.

**Table II**
Dissociation Constants (K_D) and Inhibition Constants (K_I) Between WT and Designed Mutant PCNA and PIP Peptides Determined by FP Analysis

<table>
<thead>
<tr>
<th>PCNA</th>
<th>K_D (µM)</th>
<th>K_I (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p66</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>34.4 ± 7.5</td>
<td>47.5 ± 2.8</td>
</tr>
<tr>
<td>Q131I</td>
<td>2.4 ± 0.4 (14.3)</td>
<td>3.6 ± 0.4 (13.1)</td>
</tr>
<tr>
<td>Q131L</td>
<td>5.5 ± 0.8 (5.3)</td>
<td>6.6 ± 0.3 (7.2)</td>
</tr>
<tr>
<td>Q131M</td>
<td>3.2 ± 0.3 (10.8)</td>
<td>5.1 ± 0.5 (8.3)</td>
</tr>
<tr>
<td>Q131V</td>
<td>5.4 ± 0.7 (6.4)</td>
<td>6.9 ± 0.3 (6.9)</td>
</tr>
</tbody>
</table>

*In parenthesis is the fold increase relative to the WT protein.
*The indicated mutations also include the V233W mutation. The indicated K_D values were determined by fitting the data of fluorescence anisotropy assays. Inhibition constants (K_I) were determined by competition experiments between the fluorescently labeled p66 peptide and unlabeled FEN1 peptide in binding to PCNA.
REFERENCES


