The Influence of the Proliferating Cell Nuclear Antigen-interacting Domain of p21\textsuperscript{CIP1} on DNA Synthesis Catalyzed by the Human and \textit{Saccharomyces cerevisiae} Polymerase \(\delta\) Holoenzymes*

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In eukaryotes, processive DNA synthesis catalyzed by DNA polymerases \(\delta\) and \(\epsilon\) (pol \(\delta\) and \(\epsilon\)) requires the proliferating cell nuclear antigen (PCNA). It has recently been shown that in humans (h), the PCNA function, required for both DNA replication and nucleotide excision repair, can be inactivated by p21\textsuperscript{CIP1} due to a specific interaction between hPCNA and the carboxyl terminus of p21\textsuperscript{CIP1}. In this report, we show that \textit{Saccharomyces cerevisiae} (\textit{S. cerevisiae}) PCNA-dependent pol \(\delta\)-catalyzed DNA synthesis was inhibited less efficiently than the human system by the intact p21\textsuperscript{CIP1} protein and was unaffected by the p21\textsuperscript{CIP1} carboxyl-terminal peptide (codons 139–160). This species-specific response of PCNA to p21\textsuperscript{CIP1}-mediated inhibition of DNA synthesis results from a marked difference in the ability of h and \textit{S. cerevisiae} PCNA to interact with p21\textsuperscript{CIP1}. As shown by binding studies using the surface plasmon resonance technique, hPCNA binds both full-length p21\textsuperscript{CIP1} and the p21\textsuperscript{CIP1} peptide (139–160) stoichiometrically with a similar affinity \((K_D \sim 2.5\text{ nM})\) while \textit{S. cerevisiae} PCNA binds p21\textsuperscript{CIP1} with 10-fold less affinity and does not interact with the p21\textsuperscript{CIP1} peptide (139–160).

The DNA replication machinery is functionally conserved from bacteria to mammals. The mechanism underlying DNA synthesis of primed templates in T4 bacteriophage (1), \textit{Escherichia coli} (2), \textit{Saccharomyces cerevisiae} (3), and humans (4, 5) depends on a protein complex (the “clamp-loader”) that binds to a primer-template junction. In each case, the multisubunit clamp loader complex loads a toroidal shaped protein (the “clamp”) (6–8) onto a primer end in an ATP-dependent reaction. The DNA polymerases that catalyze deoxynucleotide incorporation are tethered to DNA by a direct interaction with the clamp. This interaction, which occurs independently of the clamp loader, converts DNA polymerases from dispersive to highly processive enzymes capable of catalyzing extensive DNA synthesis (9–12). Furthermore, chromosomal replicates of \textit{E. coli} (pol\textsuperscript{1} III core, \(\gamma\) complex, and \(\beta\)), phage T4 (T4gp43, T4gp44/62 complex, and T4gp45) and eukaryotes (pol \(\delta\) or \(\epsilon\), RF-C, and PCNA) not only possess functional similarities but, in many cases, show marked sequence conservation (13, 14).

In eukaryotes, insight into chromosomal DNA synthesis has been derived from \textit{in vitro} studies of simian virus 40 replication. With the exception of one viral encoded protein, the SV40 T antigen, this replication pathway is dependent on the cellular replication machinery (15–17). The essential host proteins have been isolated, and in most cases their detailed mechanisms have been elucidated (9, 10, 18, 19). The DNA polymerase \(\alpha\)-DNA primase complex is responsible for the initiation of replication. DNA primase generates oligoribonucleotide primers that are elongated by pol \(\alpha\) for about 30 nucleotides resulting in the accumulation of pre-Okazaki fragments. In the absence of proteins that bind to 3'-hydroxyl primer ends, pol \(\alpha\) is capable of rebinding and elongating chains to mature Okazaki fragments on the lagging strand and generating DNA chains that are approximately one-half the length of the circular duplex DNA template on the leading strand (20). This non-physiological mechanism, referred to as the monopolysis system, requires relatively high levels of the pol \(\alpha\)-primase complex. When RFC and PCNA are present, they interfere with pol \(\alpha\) rebinding by binding to the 3'-hydroxyl primer ends where they facilitate the tethering of pol \(\delta\) (or pol \(\epsilon\)) (3, 5, 21). As a result, both leading strand synthesis and the completion of lagging strand pre-Okazaki fragments depend on pol \(\delta\) (or pol \(\epsilon\) and pol \(\epsilon\)).

In eukaryotes, PCNA has been shown to be the target of a number of factors that control cell growth. One such factor, p21\textsuperscript{CIP1}, is a checkpoint protein that acts as an antimitogenic signal by binding to and inhibiting cyclin-dependent kinases as well as by binding to PCNA and inhibiting \textit{in vitro} PCNA-dependent DNA replication (22–29). These two disparate inhibitory functions have been shown to reside in separate domains of p21\textsuperscript{CIP1}, a 164-amino acid protein. The cyclin-dependent kinase inhibitory activity of p21\textsuperscript{CIP1} is located within the amino-terminal domain while the PCNA binding region resides in the carboxy-terminal domain. These distinct inhibitory activities have been demonstrated both \textit{in vitro} and \textit{in vivo} using the overexpressed amino- or carboxy-terminal domains of p21\textsuperscript{CIP1} (30, 31).

A peptide derived from amino acids 141–160 within the carboxy-terminal domain of p21\textsuperscript{CIP1} that binds PCNA and inhibits PCNA-dependent DNA synthesis \textit{in vitro} has previ-
ously been described (32). In this report, we have examined the species specificity involved in the pol δ holoenzyme system using enzymes isolated from S. cerevisiae and HeLa cells and have shown that a chemically synthesized peptide that spans amino acid residues 139–160 of p21CIP1 specifically inhibits reactions dependent on human PCNA. This p21CIP1 peptide (139–160) had no effect on elongation reactions dependent on S. cerevisiae PCNA, although both the full-length p21CIP1 protein and a truncated p21CIP1 protein (truncated at amino acid residue 70–164) inhibited the S. cerevisiae pol δ holoenzyme system albeit to a lesser extent than that observed with the human pol δ holoenzyme system.

Binding studies using surface plasmon resonance demonstrated that hPCNA binds p21CIP1 and the p21CIP1 peptide (139–160) stoichiometrically (~three molecules bound per hPCNA trimer) with a similar affinity (K_D ~2.5 nM). The affinity between S. cerevisiae PCNA and p21CIP1 peptide (139–160) was 10-fold less than the affinity of hPCNA for p21CIP1, and there was no detectable interaction between the p21CIP1 peptide (139–160) and S. cerevisiae PCNA. Recently the crystal structure of the p21CIP1 peptide (139–160) complexed with hPCNA has been elucidated (33). Based on this structure and knowledge of the specific amino acid differences between S. cerevisiae and hPCNA, we propose a model explaining the differential effects of p21CIP1 and its derivatives on S. cerevisiae and hPCNA-dependent reactions.

MATERIALS AND METHODS

Preparation of Proteins—The following proteins were prepared as described previously: hpol δ, hRFC, hPCNA, HSSB, S. cerevisiae pol δ, S. cerevisiae RFC, S. cerevisiae PCNA, p21CIP1, p21CIP1 peptide (139–160), and hPCNA with an amino-terminal-cAMP-dependent protein kinase site (34–30).

The following proteins were commercially obtained: cAMP-dependent protein kinase (Sigma), T4gp32 protein, and E. coli SSB (Pharmacia Biotech, Inc.). The peptide acetyl-Gly-Arg-Lys-Arg-Arg-Gln-Thr-Arg-<i>d</i>Val containing 300 pmol, 65 pmol of amino-terminal tagged PCNA, and 6.66 units of hRFC in the presence of 1.2% alkaline agarose gel electrophoresis indicated the presence of an equal mixture of single-stranded DNA molecules and linear DNA fragments, reflecting the presence of a single nick in the duplex circular DNA. Consistent with this, agarose gel electrophoresis in TAE buffer (0.04 M Tris acetate + 1 mM EDTA (pH 8.0)) containing ethidium bromide indicated that the DNA had been quantitatively converted from a RF I structure to a RF II structure.

Results

Species Specificity of the Pol δ Holoenzyme—The elongation of a [32P]dCMP-labeled oligonucleotide primer (35 nucleotides) hybridized to circular single-stranded M13 mp7 (7.2 kilobases) DNA by both the h and S. cerevisiae pol δ holoenzymes was compared (Fig. 1). Three different DNA binding proteins, HSSB, E. coli SSB, and the T4 gp 32, were compared in each system. HSSB was more effective than the other SSBs in supporting elongation of the primed template by the human pol δ holoenzyme (Fig. 1, lanes 1–3), whereas all three SSBs supported elongation of the labeled primer by the yeast pol δ holoenzyme to a similar extent (lanes 4–6). For this reason all subsequent experiments were carried out using HSSB as the DNA binding protein.

The six different permutations possible for reconstituting the pol δ holoenzyme from combinations of h and S. cerevisiae PCNA, RFC and pol δ proteins were examined (Fig. 1, lanes 9–14). In all cases, the efficiency of full-length product formation with interspecies mixtures of these proteins was reduced compared with the full-length products formed in reactions containing proteins from a single species. The most pronounced species specificity was observed with mixtures containing hpol δ, hRFC, and S. cerevisiae PCNA (lane 10) and reactions carried out with S. cerevisiae pol δ, hRFC, and S. cerevisiae PCNA (lane 14). The finding that hRFC substituted poorly for S. cerevisiae RFC in the presence of S. cerevisiae pol δ is consistent with the data of Fien and Stillman (37). The experiments described by Fien and Stillman (37) were carried out with S. cerevisiae SSB, whereas the experiments described in Fig. 1 contained HSSB. Thus, the marked specificity noted is most likely independent of the SSB used. Limited elongation of primer chains was observed in reactions containing hPCNA, S. cerevisiae RFC, and S. cerevisiae pol δ (lane 9). The other combinations of human and S. cerevisiae proteins supported elongation (lanes 11–13), although a substantial number of products accumulated that had not been maximally extended. The formation of discrete shorter products in reactions containing heterologous mixtures of proteins indicated that the processive action of pol δ was reduced compared with reactions carried out with proteins from homologous species.

Primer extension assays were repeated with an unlabeled primed template in the presence of [α-32P]dNTPs (Table I).
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was isolated from human or S. cerevisiae three holoenzyme components (polatha
tm mixtures(10
hPCNAinhibits polnot presented).

Elongation reaction of a32P-labeled primed M13 single-stranded DNA was

The influence of the p21CIP1 peptide-(139–160) on the elongation of a 32P-labeled primed M13 single-stranded DNA was examined (Fig. 2A). As previously observed, high levels of full-length p21CIP1 blocked the elongation reaction catalyzed by the hpol δ holoenzyme (Fig. 2A, lane 3). Elongation catalyzed by the S. cerevisiae pol δ holoenzyme was also inhibited but, in contrast to the human system, the labeled primer was elongated without the accumulation of full-length material (Fig. 2A, lane 4). Interestingly, high levels of the p21CIP1 peptide-(139–160) had no effect on DNA elongation by the S. cerevisiae pol δ holoenzyme (lane 6). As previously noted, this p21CIP1 peptide-(139–160) quantitatively inhibited DNA synthesis by the hpol δ holoenzyme (Fig. 2A, lane 5). Elongation catalyzed by any combination of human and S. cerevisiae proteins that included hPCNA was also completely inhibited by the peptide (lanes 8, 10, and 12). The combination of hpol δ, S. cerevisiae RFC, and S. cerevisiae PCNA was the only heterologous pol δ holoenzyme complex that included S. cerevisiae PCNA and supported elongation (lane 13). This reaction was not altered by addition of the p21CIP1 peptide-(139–160) (lane 14).

In the presence of the hpol δ holoenzyme, the extent of inhibition of DNA elongation by p21CIP1 and its carboxyl-terminal derivatives was influenced by the ratio of PCNA to the inhibitor (27, 28, 38). For this reason, the influence of the p21CIP1 peptide-(139–160) on the S. cerevisiae pol δ holoenzyme was examined at various PCNA concentrations (Fig. 2B). DNA elongation became more efficient with increasing levels of S. cerevisiae PCNA (Fig. 2B, lanes 1, 4, and 7). Addition of the p21CIP1 peptide-(139–160) did not affect the elongation of the labeled primer irrespective of the level of S. cerevisiae PCNA present (Fig. 2B, compare lanes 1 and 3; lanes 4 and 6; lanes 7 and 9). In contrast, the influence of the p21CIP1 peptide-(139–160) on hpol δ holoenzyme-catalyzed nucleotide incorporation was dependent on the level of PCNA added (Fig. 2C). In the presence of 170 and 68 nM hPCNA, 120 and 45 nM of the p21CIP1 peptide-(139–160) were required to inhibit DNA elongation by 50%.

Using overlapping 20-amino acid peptides Warbrick et al. (32) showed that the critical region of p21CIP1 required for its interaction with PCNA spans amino acids 144–151 (see Table II). Consistent with this finding high levels of a 12-mer peptide derived from amino acids 139–160 of p21CIP1 containing a deletion of amino acids 146–156 (see Table II) did not affect DNA synthesis catalyzed by the hpol δ holoenzyme (Fig. 2C).

Full-length p21CIP1 and Truncated p21CIP1 Proteins-(70–164) Inhibit Human and, Less Efficiently, Yeast PCNA-dependent DNA Synthesis—Full-length p21CIP1 inhibited the S. cerevisiae pol δ holoenzyme reaction and the length of DNA formed was dependent upon the ratio between the inhibitor and PCNA. Thus, reducing the level of PCNA in the presence of a fixed amount of p21CIP1 resulted in the production of less full-length material (Fig. 2B, compare lanes 2, 5, and 8).

The influence of p21CIP1 on DNA synthesis carried out in the presence of higher levels of singly-primed M13 DNA substrate

Qualitatively, the level of nucleotide incorporation mirrored the results shown in Fig. 1, i.e. reactions containing S. cerevisiae PCNA, hRFC, and hpol δ were virtually inactive. The combination of S. cerevisiae pol δ, hRFC, and hPCNA resulted in substantial nucleotide incorporation (Table I), but the resulting DNA chains were highly heterogeneous in length (data not presented).

Elongation reactions catalyzed by either the h or S. cerevisiae pol δ holoenzyme were absolutely dependent on addition of all three holoenzyme components (pol δ, hRFC, and PCNA) as well as an SSB protein. Thus, in the absence of PCNA no detectable elongation of labeled primer was observed with either the human proteins (Fig. 1, lane 7) or the S. cerevisiae proteins (Fig. 1, lane 8). Likewise, omission of S. cerevisiae pol δ or S. cerevisiae RFC (Fig. 2A, lanes 15 and 16, respectively) prevented DNA synthesis. Similar results were obtained following omission of any one of the pol δ holoenzyme components or SSB protein (data not presented).

The p21CIP1 Peptide-(139–160) Inhibits Human but Not Yeast PCNA-dependent DNA Synthesis—The interaction between p21CIP1 and the carboxyl-terminal domain of p21CIP1 and hPCNA inhibits in vitro SV40 DNA replication and hPCNA-dependent elongation of primed DNA templates by the pol δ holoenzyme (27, 28, 32, 38).

The influence of the p21CIP1 peptide-(139–160) on the elongation of a 32P-labeled primed M13 single-stranded DNA was examined (Fig. 2A). As previously observed, high levels of full-length p21CIP1 blocked the elongation reaction catalyzed by the hpol δ holoenzyme (Fig. 2A, lane 3). Elongation catalyzed by the

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\text{Table I: Species specificity measured by direct nucleotide incorporation}\\
\begin{array}{|c|c|}
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\text{Additions} & \text{Nucleotide incorporation} \\
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\text{hpol} & 20.3 \\
\text{scpol} & 20.6 \\
\text{scpol} & 4.85 \\
\text{scpol} & 1.93 \\
\text{hpol} & 15.3 \\
\text{hpol} & 8.72 \\
\text{hpol} & 5.21 \\
\text{hpol} & 3.43 \\
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was examined in the presence of a low concentration of *S. cerevisiae* PCNA (Fig. 3A). Addition of a high level of p21CIP1 to reactions containing a limiting amount of *S. cerevisiae* PCNA resulted in marked inhibition of DNA synthesis (lane 4). Addition of a lower level of p21CIP1 in the presence of the same concentration of *S. cerevisiae* PCNA resulted in more extensive DNA synthesis (lane 6).

Further experiments investigating the influence of p21CIP1 on the incorporation of labeled nucleotides confirmed that the extent of inhibition of hpold holoenzyme-catalyzed elongation by p21CIP1 was dependent upon the level of hPCNA present (Fig. 3B). Nucleotide incorporation was reduced by 50% in the presence of 0.37, 0.18, and 0.10 mM p21CIP1 in reactions containing 170, 68, and 34 nM hPCNA (monomer), respectively. In the presence of the *S. cerevisiae* pol δ holoenzyme, 50% inhibition was observed with 1.2 μM p21CIP1 in the presence of either 170 or 17 nM *S. cerevisiae* PCNA, whereas reactions containing a low level of *S. cerevisiae* PCNA (1.7 nM) required the addition of 0.8 mM p21CIP1 to inhibit DNA synthesis by 50%. Altering the amount of *S. cerevisiae* RFC or *S. cerevisiae* pol δ added to reactions containing a limiting concentration of *S. cerevisiae* PCNA did not significantly alter the level of p21CIP1 required to inhibit DNA synthesis by 50% (data not shown).

The inhibition of the *S. cerevisiae* pol δ holoenzyme by p21CIP1 and the lack of inhibition by the p21CIP1 peptide-(139–160) prompted us to examine the effects of the addition of a

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**Table II**

| Sequences of peptides derived from the carboxyl terminal of p21CIP1 |  
|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|
|                        | 139  | 140  | 141  | 142  | 143  | 144  | 145  | 146  | 147  | 148  | 149  | 150  | 151  | 152  | 153  | 154  | 155  | 156  | 157  | 158  | 159  | 160  |
|                        | GRK  | RQR  | TSMT | DYP  | H    | SKR  | RRL  | IFS  |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| p21CIP1 peptide-(139–160) | 139  | 140  | 141  | 142  | 143  | 144  | 145  | 146  | 147  | 148  | 149  | 150  | 151  | 152  | 153  | 154  | 155  | 156  | 157  | 158  | 159  | 160  |
| p21CIP1 peptide-(139–145 + 156–160) | 139  | 140  | 141  | 142  | 143  | 144  | 145  | 146  | 147  | 148  | 149  | 150  | 151  | 152  | 153  | 154  | 155  | 156  | 157  | 158  | 159  | 160  |
| p21CIP1 peptide-(141–152) | 141  | 152  |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| p21CIP1 peptide-(144–151) | 144  | 151  |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| p21CIP1 peptide-(139–145 + 156–160) | 139  | 140  | 141  | 142  | 143  | 144  | 145  | 146  | 147  | 148  | 149  | 150  | 151  | 152  | 153  | 154  | 155  | 156  | 157  | 158  | 159  | 160  |
| p21CIP1 peptide-(144–151) | 144  | 151  |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| p21CIP1 peptide-(139–160) | 139  | 140  | 141  | 142  | 143  | 144  | 145  | 146  | 147  | 148  | 149  | 150  | 151  | 152  | 153  | 154  | 155  | 156  | 157  | 158  | 159  | 160  |
peptide that spans an additional 70 amino acids within the carboxyl terminus of p21CIP1 (Fig. 3C). Previous studies have shown that a truncated p21CIP1 protein derived from amino acids 70–164 within the carboxyl-terminal of p21CIP1 was capable of binding to hPCNA as well as inhibiting hpol δ holoenzyme-catalyzed elongation of primed templates (31). In con-
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FIG. 4. Analysis of interactions between h or S. cerevisiae PCNA and p21\(^{\text{CIP1}}\) or the p21\(^{\text{CIP1}}\) peptide-(139–160). A–C, during a 7-min time interval, a solution of 100 pmol of p21\(^{\text{CIP1}}\) in 35 \(\mu\)l of buffer containing 10 mM Hepes-HCl (pH 7.4), 150 mM NaCl, and 3.4 mM EDTA was passed over each of three sensor chips to which either 110 fmol of hPCNA (3320 RU) (A), 92 fmol of S. cerevisiae (sc) PCNA (2748 RU) (B), or buffer (C) had been coupled in the presence of carbodiimide. Following completion of this 7-min injection step, buffer was passed over each chip at the same flow rate (5 \(\mu\)l/min). D–F, during a 7-min time interval, 12 pmol of the p21\(^{\text{CIP1}}\) peptide-(139–160) in 35 \(\mu\)l of buffer containing 10 mM Hepes-HCl (pH 7.4), 150 mM NaCl, and 3.4 mM EDTA was passed over each of three sensor chips that were carbodiimide-coupled to either 110 fmol of hPCNA (3320 RU) (D), 92 fmol of S. cerevisiae PCNA (2748 RU) (E), or buffer (F) was then passed over each chip.

In this experiment, 100 fmol (281.4 RU) and 1 fmol (30 RU) of the full-length p21\(^{\text{CIP1}}\) protein-(70–164) inhibited the elongation reaction catalyzed by the S. cerevisiae pol \(\delta\) holoenzyme (Fig. 3C, lanes 7–9). These results suggest that amino acids within codons 70–139 are capable of interaction with S. cerevisiae PCNA. Like the full-length p21\(^{\text{CIP1}}\) protein, the effects of the truncated p21\(^{\text{CIP1}}\) protein-(70–164) were more pronounced in the presence of low levels of PCNA than at higher PCNA concentrations (compare lanes 9 and 7).

The Binding of p21\(^{\text{CIP1}}\) and the p21\(^{\text{CIP1}}\) Peptide-(139–160) to Human and S. cerevisiae PCNA—In order to explain the different effects of the p21\(^{\text{CIP1}}\) and p21\(^{\text{CIP1}}\) peptide-(139–160) on S. cerevisiae and hPCNA-dependent reactions, we determined whether these proteins exhibit differential binding affinities for PCNA. p21\(^{\text{CIP1}}\) was shown previously to directly interact with hPCNA by coimmunoprecipitation, gel filtration, and surface plasmon resonance binding (27, 28). The interaction detected using the latter procedure indicated that ~2.3 molecules of p21\(^{\text{CIP1}}\) monomer bound to each PCNA monomer. Consistent with this, passage of solutions of p21\(^{\text{CIP1}}\) over a sensor surface on which 110 fmol of hPCNA (3320 RU) was immobilized resulted in an immediate increase in mass (Fig. 4A). In this experiment, 140 fmol of p21\(^{\text{CIP1}}\) (3009 RU) was retained on the hPCNA sensor chip, corresponding to a stoichiometry of ~1 molecule of p21\(^{\text{CIP1}}\) monomer bound per molecule of PCNA monomer. Fourteen-fold less p21\(^{\text{CIP1}}\) (10 fmol, 222 RU) bound to a sensor surface to which 92 fmol (2748 RU) of S. cerevisiae PCNA had been covalently coupled (Fig. 4B). The background values have been subtracted from these data and were calculated following passage of p21\(^{\text{CIP1}}\) solutions over a blank sensor chip surface (Fig. 4C). These data indicate that the affinity of S. cerevisiae PCNA for p21\(^{\text{CIP1}}\) is 14-fold less than the affinity of hPCNA for p21\(^{\text{CIP1}}\). These results were confirmed following passage of S. cerevisiae or hPCNA solutions over a sensor surface chip to which p21\(^{\text{CIP1}}\) was immobilized (data not shown).

A 100-fold difference in the association between S. cerevisiae and hPCNA with the p21\(^{\text{CIP1}}\) peptide-(139–160) was observed.

In this experiment, 100 fmol (281.4 RU) and 1 fmol (30 RU) of the p21\(^{\text{CIP1}}\) peptide-(139–160) were retained by sensor chips to which hPCNA (110 fmol, 3320 RU) or S. cerevisiae PCNA (92 fmol, 2748 RU) had been covalently coupled, respectively (Fig. 4, D and E). As described above, background values have been subtracted and were calculated following passage of the p21\(^{\text{CIP1}}\) peptide-(139–160) over a blank sensor chip surface (Fig. 4F). These data indicate that approximately 1 molecule of the p21\(^{\text{CIP1}}\) peptide-(139–160) binds to each monomer molecule of hPCNA, similar to the stoichiometry observed with the full-length p21\(^{\text{CIP1}}\) protein. In contrast, no significant interaction was observed between the p21\(^{\text{CIP1}}\) peptide-(139–160) and S. cerevisiae PCNA. Identical results were obtained following p21\(^{\text{CIP1}}\) peptide-(139–160) immobilization on a sensor chip over which solutions of h or S. cerevisiae PCNA were continuously flowed (data not shown).

Sensorgrams recorded using different hPCNA concentrations passing over the surface of chips to which p21\(^{\text{CIP1}}\) or the biotinylated p21\(^{\text{CIP1}}\) peptide-(139–160) had been coupled are shown in Fig. 5, A and B, respectively. The apparent dissociation constants (\(K_D\)) for hPCNA binding to both p21 and the p21\(^{\text{CIP1}}\) peptide-(139–160) are very similar (p21 \(K_D = 2.55 \times 10^{-9}\) M ± 1.05 \(\times 10^{-9}\) M and p21\(^{\text{CIP1}}\) peptide-(139–160) \(K_D = 2.3 \times 10^{-9}\) M ± 1.5 \(\times 10^{-9}\) M) as calculated using the...
The experiments presented here investigated the interactions between p21^{CIP1} and a p21^{CIP1} carboxyl-terminal derived peptide with PCNA and the subsequent effect on pol δ-catalyzed DNA elongation. Full-length p21^{CIP1} inhibited DNA synthesis catalyzed by h or S. cerevisiae pol δ holoenzymes to an extent dependent upon the ratio between p21^{CIP1} and PCNA. In contrast, addition of the p21^{CIP1}-derived peptide-(139–160) only inhibited reactions dependent upon hPCNA, not S. cerevisiae PCNA. Thus, reactions containing S. cerevisiae pol δ and S. cerevisiae RFC with hPCNA were inhibited by the p21^{CIP1} peptide-(139–160), whereas reactions containing h or S. cerevisiae pol δ, S. cerevisiae RFC, and S. cerevisiae PCNA were unaffected by the peptide. The species origin of the pol δ and RFC did not influence DNA synthesis inhibition by p21^{CIP1} and its derivatives.

Real time interaction analysis presented here demonstrated that the affinity of the p21^{CIP1} for hPCNA was ~15-fold greater than its affinity for S. cerevisiae PCNA. This is consistent with the biochemical data presented indicating that 12 times more p21^{CIP1} was required to inhibit S. cerevisiae PCNA-dependent nucleotide incorporation by 50% than was required to inhibit hPCNA-dependent incorporation by 50%. Furthermore, the affinity of the p21^{CIP1} peptide-(139–160) for hPCNA was 100-fold greater than its affinity for S. cerevisiae PCNA. These data are also consistent with the singly-primed M13 elongation assays presented which indicated that addition of the p21^{CIP1} peptide-(139–160) in amounts stoichiometric with hPCNA inhibited nucleotide incorporation by 50%, whereas the addition of excess peptide did not affect reactions dependent upon S. cerevisiae PCNA.

The crystal structure of the p21^{CIP1} peptide-(139–160) complexed with hPCNA has recently been determined (33). This analysis demonstrated that the p21^{CIP1} peptide-(139–160) is bound to the inter-domain connector loop that links the amino- and carboxyl-terminal domains of each PCNA monomer (residues 119–133), Additional interactions are formed with both domains of PCNA. A schematic model of the hPCNA-p21^{CIP1} peptide-(139–160) complex derived from crystal structure analyses is presented in Fig. 7. Although the α-carbon backbone traces of h and S. cerevisiae PCNA are very similar (8, 41), significant differences in the backbone conformation are located in the regions where the p21^{CIP1} peptide-(139–160) interacts with hPCNA. These differences may account for the distinct effects of both p21^{CIP1} and the p21^{CIP1} peptide-(139–160) in these two systems.

First, and of particular interest, the configuration of the hPCNA interconnector loop that forms part of the p21^{CIP1} peptide-(139–160) binding surface differs in S. cerevisiae PCNA. A number of residues in this interconnector loop contribute to the formation of two hydrophobic pockets in hPCNA that bind the Met-147, Phe-150, Tyr-151, and Ile-158 residues of the p21^{CIP1} peptide-(139–160). There is also an extensive array of hydrogen bonding-mediated contacts between hPCNA and the p21^{CIP1} peptide-(139–160) in this region. Thus both the conformation and sequence of the interconnector loop of hPCNA are important in forming the interface with the p21^{CIP1} peptide-(139–160). Specific differences in the amino acid sequence between the h and S. cerevisiae PCNA are likely to exclude some favorable contacts with the p21^{CIP1} peptide-(139–160) and S. cerevisiae PCNA. For example, Ile-158 of the p21^{CIP1} peptide-(139–160) fits snugly into a small hydrophobic pocket adjacent to the hPCNA connector loop, Substitution of a cysteine residue (Cys-27) for asparagine at this position in S. cerevisiae PCNA would significantly alter the nature of the binding site. Additionally, Arg-156 of the p21^{CIP1} peptide-
The amino acids of the p21\textsuperscript{CIP1} peptide-(139–160) complex derived from crystal structure analysis. The amino acids of the p21\textsuperscript{CIP1} peptide-(139–160) are indicated in 
white letters on a blue background. The critical pocket domains of hPCNA involved in its interaction with the p21\textsuperscript{CIP1} peptide-(139–160) are schematically drawn as red circles, and the hPCNA interconnector domain is indicated in green. The amino acids within each domain that are not conserved between h and \textit{S. cerevisiae} PCNA are indicated with the hPCNA amino acid indicated to the left followed by an arrow and the \textit{S. cerevisiae} PCNA amino acid at that position. The amino acid sequences of the h and \textit{S. cerevisiae} PCNA interconnector regions (119–133) are shown at the bottom of the figure.

The connector loop of hPCNA also appears to be critical for its interaction with pol δ (42). A monoclonal antibody against hPCNA that blocks the interaction between hpol δ and hPCNA was competed by a peptide derived from amino acids 121–135 within the connector loop of PCNA. Further evidence for the importance of this region of PCNA comes from cold-sensitive mutations of the \textit{S. cerevisiae} PCNA gene (POL30) that are clustered in the interconnector domain region and exhibit a cell division cycle phenotype at the restrictive temperature arresting with a 2C DNA content (43).

The effects of p21\textsuperscript{CIP1} and the p21\textsuperscript{CIP1} peptide-(139–160) on hPCNA-dependent DNA synthesis, the complex stoichiometry, and dissociation constants between each of these agents and hPCNA are all quantitatively similar. This suggests that the biological consequence of the p21\textsuperscript{CIP1} interaction with hPCNA is quantitatively due to the region contained within the 22-amino acid p21\textsuperscript{CIP1} peptide-(139–160). In contrast, the effects of full-length p21\textsuperscript{CIP1} and the p21\textsuperscript{CIP1} peptide-(139–160) on \textit{S. cerevisiae} PCNA are different. Furthermore, p21\textsuperscript{CIP1} was much less effective in blocking \textit{S. cerevisiae} PCNA-dependent reactions than hPCNA-dependent reactions. These findings indicate that p21\textsuperscript{CIP1} may affect the \textit{S. cerevisiae} pol δ-holoenzyme by a mechanism distinct from that observed with the hpol δ-holoenzyme. Since \textit{S. cerevisiae} PCNA has a low affinity for p21\textsuperscript{CIP1} and no detectable affinity for the p21\textsuperscript{CIP1} peptide-(139–160), other sites within p21\textsuperscript{CIP1} (present in the p21\textsuperscript{CIP1} peptide-(70–164)) may contribute to its interaction with \textit{S. cerevisiae} PCNA and subsequent effects on the \textit{S. cerevisiae} pol δ-holoenzyme activity.

The loading of PCNA onto DNA by RFC was maximally inhibited by 50% following addition of concentrations of p21\textsuperscript{CIP1} that completely blocked the elongation of primed DNA. Hubacher’s laboratory (44) has shown that PCNA loaded onto circular DNA can still slide off the DNA in the presence of p21\textsuperscript{CIP1} after linearization of the DNA. These results suggest that p21\textsuperscript{CIP1} may act to inhibit the interaction between the polymerase and PCNA through its ability to bind to the interconnector domain of PCNA. This would suggest that all reactions dependent upon PCNA and pol δ (or pol ε) would be affected by p21\textsuperscript{CIP1} including DNA repair reactions in which PCNA plays the same role as in replication, acting to tether the polymerase to DNA (45, 46).

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