# Mechanism of Inhibition of Proliferating Cell Nuclear Antigen-Dependent DNA Synthesis by the Cyclin-Dependent Kinase Inhibitor p21†

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ABSTRACT: It is known that the direct binding of the cyclin-dependent kinase (Cdk) inhibitor p21, also called Cdk-interacting protein 1 (p21), to proliferating cell nuclear antigen (PCNA) results in the inhibition of PCNA-dependent DNA synthesis. We provide evidence that p21 first inhibits the replication factor C-catalyzed loading of PCNA onto DNA and second prevents the binding of DNA polymerase δ core to the PCNA clamp assembled on DNA. The second effect contributes most to the inhibition of pol δ holoenzyme activity. p21 primarily inhibited the DNA synthesis resulting from multiple reassembly of DNA polymerase δ holoenzyme. On the other hand, an ability of the PCNA clamp to translocate along double-stranded DNA was not affected by p21. These data were confirmed with a mutant of p21 that is unable to bind PCNA and therefore neither inhibited clamp assembly nor prevented the loading of DNA polymerase δ core onto DNA. Our data suggest that p21 does not discriminate *in vitro* "repair" and "replication" DNA synthesis based on template length but does act preferentially on polymerization which encounters obstacles to progress.

At least five different DNA polymerases (pols),[1] called α, β, γ, δ, and ε, are known in eukaryotic cells. Three of them (pols α, δ, and ε) are involved in DNA replication [reviewed in Hübscher and Spadari (1994)]. Studies of SV40 *in vitro* DNA replication indicated that several additional factors such as PCNA, RF-C, and RP-A are required for DNA replication [reviewed in Hübscher and Spadari (1994)]. Cooperative action of these auxiliary factors strongly inhibited the DNA synthesis by pol α (Tsurimoto & Stillman, 1991; Podust & Hübscher, 1993). On the other hand, PCNA/RF-C/RP-A efficiently stimulated the activity of pol δ and ε (Tsurimoto & Stillman, 1989; Lee et al., 1991a,b; Burgers, 1991; Podust et al., 1992a,b). RP-A and PCNA were later identified as essential factors for DNA repair reaction such as NER *in vitro* (Coverley et al., 1991; Shivji et al., 1992). This suggests that the NER gap-filling step might be performed by some of the same machinery as the chromosomal DNA synthesis. Since both DNA replication (Waga et al., 1994a) and NER (Aboussekhra et al., 1995) have been reconstituted with purified proteins, the minimal sets of components to perform these DNA transactions *in vitro* are now known. Besides events that are either unique to DNA replication, such as origin unwinding, the initiation reaction, and Okazaki fragment maturation, or unique to NER, such as incision and excision steps, both these DNA transactions also require an identical step, namely, assembly of the RF-C/PCNA clamp onto DNA, followed by the loading of pol core, resulting in the formation of pol δ or pol ε holoenzymes. Numerous studies on the role of pol δ and pol ε in DNA synthesis have not conclusively resolved the functions of these pols in DNA replication and/or DNA repair. Many enzymatic properties of pol δ and pol ε holoenzymes are rather similar (Burgers, 1991; Podust et al., 1992a,b). Furthermore, these two pols can substitute for one another in the reactions *in vivo* (Budd & Campbell, 1995).

Recent studies showed that p21, an inhibitor of G1 Cdks (Harper et al., 1993; Xiong et al., 1993) also directly binds to PCNA and thus inhibits PCNA-dependent DNA synthesis *in vitro* (Waga et al., 1994b; Flores-Rozas et al., 1994). This finding became even more intriguing when it could be demonstrated that p21 inhibits PCNA-dependent DNA replication but not PCNA-dependent NER (Li et al., 1994; Shivji et al., 1994). This indicated that p21 might play a significant role in the coordination of cell cycle progression, DNA replication, and DNA repair reactions in the cell.

In order to get insight into how p21 might discriminate between replication and repair reactions, one should understand first how the binding of p21 to PCNA influences the assembly and the action of pol δ or pol ε holoenzyme. This binding of p21 to PCNA might have consequences for three events, namely, (i) inhibition of the RF-C-catalyzed loading of PCNA onto DNA, (ii) prevention of the binding of pol δ (pol ε) core to the assembled PCNA clamp, and (iii)

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[1] *Abbreviations:* pol(s), DNA polymerase(s); PCNA, proliferating cell nuclear antigen; ph-PCNA, proliferating cell nuclear antigen modified so that it contains a site for *in vitro* phosphorylation; RP-A, replication protein A; RF-C, replication factor C; SSB, *Escherichia coli* single-strand DNA binding protein; p21, cyclin-dependent kinase inhibitor p21; ASM19, p21 mutant deficient in binding to PCNA; GST-p21 (or GST-ASM19), glutathione *S*-transferase N-terminal fusion p21 (or ASM19); Cdk, cyclin-dependent kinase; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; sccDNA, supercoiled circular DNA; SV40, simian virus 40; NER, nucleotide excision repair; nt, nucleotide.

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interference in the translocation of the PCNA clamp or the pol δ (pol ε) holoenzyme along the DNA template. In this paper we have analyzed the effect of p21 on each of these three reactions. Our results are discussed in the context of different effects of p21 in DNA replication and NER reactions.

## MATERIALS AND METHODS

### *Materials*

*Pol δ, Auxiliary Proteins, and Other Proteins.* Calf thymus pol δ (1 unit is defined as 1 nmol of dTMP incorporation into poly(dA)−oligo(dT) (base ratio 10:1) in the presence of 100 ng of PCNA in 60 min at 37 °C; Weiser et al., 1991), calf thymus RF-C (Mono Q fraction), and *Escherichia coli* SSB (Podust et al., 1992a) have been described. Human PCNA was expressed in *E. coli* and purified according to Fien and Stillman (1992). Restriction endonucleases, T4 polynucleotide kinase, and proteinase K were purchased from Boehringer Mannheim.

*DNA Substrates.* ssDNA and sccDNA from M13mp11 were prepared according to Sambrook et al. (1989). ssDNA was primed with 40-mer oligonucleotide complementary to nt 7041−7080 of the M13 genome. The primer was labeled with polynucleotide kinase and [$\gamma$-$^{32}$P]ATP. The labeled primer (2-fold molar excess over the template) was annealed to M13 ssDNA as described (Podust et al., 1992a). A gapped dsDNA substrate was prepared from replicative form I of M13mp11 DNA (Podust et al., 1994).

*Buffers.* Buffer A: 25 mM Tris-HCl (pH 8.0), 1 mM EDTA, 10 mM $NaHSO_3$, and 1 mM phenylmethanesulfonyl fluoride. Buffer B: 25 mM Tris-HCl (pH 7.5), 2 mM DTT, 0.15 M NaCl, and 1 mM EDTA. Buffer C: 40 mM Tris-HCl (pH 7.5), 0.2 mg/mL bovine serum albumin, 1 mM dithiothreitol, and 10 mM $MgCl_2$. Buffer D: same as buffer C but with triethanolamine hydrochloride (pH 7.5) instead of Tris-HCl.

### *Methods*

*Expression Constructs of p21 and Its Derivatives.* Human p21 cDNA cloned in the pBS vector was kindly provided by D. Beach. p21 cDNA was recloned as an *Nco*I−*Bam*HI cassette in the pT7-14b vector (Novagen), removing the polyhistidine stretch and the thrombin site. p21 mutants were generated by the alanine-scanning mutagenesis method (ASM). The selected mutant called ASM19 contained the four mutations H152A, K154A, R155A, and R156A and was unable to interact with PCNA (Goubin & Ducommun, 1995). GST-p21 and GST-ASM19 were constructed by cloning of the corresponding cDNAs as *Nco*I−*Hin*dIII cassettes in the pGEX-KG vector (Pharmacia) (Goubin & Ducommun, 1995).

*Purification of p21 Proteins.* The *E. coli* strain BL21-(DE3)pLysS was transformed with the corresponding expression construct and grown at 37 °C in 200 mL of Luria broth to an $A_{600}$ of 0.8. Isopropyl thiogalactoside was added to a final concentration of 0.4 mM and growth was continued for 3 h. The harvested cells were lyzed by freezing/thawing in 30 mL of buffer A containing 50 mM NaCl. The lysate was sonicated to decrease the viscosity and centrifuged for 20 min at 25000*g*. The pellet was washed three times in different buffers followed by centrifugation for 20 min at 25000*g*. They included first 40 mL of buffer A containing 0.5 M NaCl, second 40 mL of buffer A containing 0.5% (v/v) Triton X-100, and third 40 mL of buffer A containing 0.5 M urea. The inclusion bodies were solubilized with 30 mL of 25 mM Tris-HCl (pH 7.5) containing 8 M urea and 20 mM β-mercaptoethanol at 37 °C. The solution was clarified by centrifugation and the supernatant was dialyzed first against 500 mL of buffer B containing 4 M urea for 5 h, second against 500 mL of buffer B containing 2 M urea for 5 h, and third against 2 L of buffer B overnight. The dialyzed solution was centrifuged for 30 min at 25000*g*. The supernatant, containing solubilized p21, was loaded onto a 1-mL SP Sepharose Fast Flow (Pharmacia) column. p21 was eluted with a gradient of NaCl from 0.15 to 1 M in buffer B. GST-p21 and the mutant GST-ASM19 after solubilization were further purified with glutathione−sepharose (Pharmacia) chromatography. The solubilized proteins were bound to 1 mL of glutathione−sepharose for 30 min at 4 °C in batch, and the resin was then packed into the column and washed with 20 mL of 25 mM Tris-HCl (pH 8.0) containing 0.3 M NaCl and finally with 10 mL of 50 mM Tris-HCl (pH 8.0). Proteins were eluted with 50 mM Tris-HCl (pH 8.0) containing 10 mM glutathione. All three purified proteins (Figure 1) were frozen in liquid nitrogen and stored at −80 °C until use.

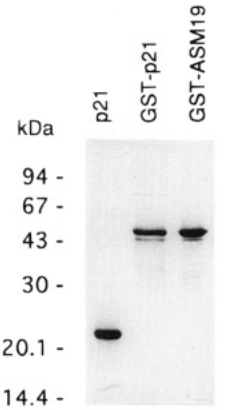


FIGURE 1: SDS−polyacrylamide gel electrophoresis of p21 and its derivatives used in this study. p21, GST-p21, and GST-ASM19 (1.5 μg each) were loaded onto a 12.5% polyacrylamide gel, electrophoresed, and stained with Coomassie blue.

*Production of Phosphorylatable PCNA and Its in Vitro Phosphorylation.* We recently constructed a recombinant PCNA that can be phosphorylated at the N-terminus with a cAMP-dependent protein kinase *in vitro*. This resulted in a radioactive protein that still contained full activity (Podust et al., 1995a). The phosphorylatable PCNA, called ph-PCNA, was expressed in *E. coli*, purified, and $^{32}$P-phosphorylated by cAMP-dependent protein kinase (catalytic subunit) using [$\gamma$-$^{32}$P]ATP as described (Podust et al., 1995a).

*Analysis of [$^{32}$P]-ph-PCNA/DNA Complexes.* A reaction mixture (final volume of 25 μL) contained buffer D, 1 mM ATP, 15 ng of [$^{32}$P]-ph-PCNA, 45 ng of RF-C, 50 ng of sccDNA (or gapped circular DNA), and p21 (or its derivative) as indicated. The samples were incubated for 3 min at 37 °C, and then glutaraldehyde was added to a final concentration of 0.1% (w/v) and the mixture was incubated further for 10 min at 37 °C. The samples were loaded and analyzed on a neutral 0.8% agarose gel in the presence of 0.1% SDS as described (Podust et al., 1995a). The gel was then fixed in 10% acetic acid containing 12% methanol, dried

under vacuum, and exposed to a X-ray film.

*DNA Synthesis on Gapped dsDNA Substrate.* A reaction mixture (final volume of 25 $\mu$L) contained buffer C, 1 mM ATP, 50 $\mu$M each dGTP, dCTP, and dTTP, 1.4 $\mu$Ci of [$\alpha$-$^{32}$P]dATP (3000 Ci/mmol), 50 ng of gapped DNA, 15 ng of PCNA, 45 ng of RF-C, 0.3 unit of pol $\delta$, and p21 (or its derivative) as indicated. After a 10-min incubation at 37 °C, DNA synthesis was stopped by the addition of SDS and EDTA to final concentrations of 1% and 20 mM, respectively. The products were analyzed by neutral 0.8% agarose gel electrophoresis. The gel was first stained with EtdBr and photographed and then fixed with acid and dried for autoradiography.

*DNA Synthesis on Primed ssDNA.* A reaction mixture (final volume of 25 $\mu$L) contained buffer C, 1 mM ATP, 50 $\mu$M each dATP, dGTP, dCTP, and dTTP, 50 ng of primed ssDNA (5′-$^{32}$P-phosphorylated primer), 15 ng of PCNA, 45 ng of RF-C, 175 ng of *E. coli* SSB, pol $\delta$, and p21 (or its derivative) as indicated. The samples were incubated for 15 min at 37 °C and the reactions were terminated by treating them with proteinase K (60 $\mu$g/mL) for 30 min at 37 °C in the presence of 1% (w/v) SDS and 20 mM EDTA (pH 8.0). The DNA was then precipitated with ethanol and the products were analyzed on an alkaline 1.5% agarose gel as described (Podust et al., 1995b).

## RESULTS

The experiments carried out in this study were performed with both p21 and GST-p21. The results obtained were essentially the same and therefore the data are shown only in Figure 2 (panels A and B) for both proteins, but not in Figures 3−6. The experiments were also simultaneously performed with GST-ASM19, a p21 mutant unable to bind PCNA but which fully interacts with Cdks (Goubin & Ducommun, 1995). This was done to assure that all the effects of p21 shown and discussed below were due to the p21−PCNA interaction exclusively.

*Effect of p21 on the RF-C-Catalyzed Loading of PCNA onto Circular dsDNA.* The RF-C-catalyzed loading of PCNA onto DNA has been analyzed using a recombinant PCNA containing phosphorylation site (Podust et al., 1995a). [$^{32}$P]-ph-PCNA was assembled on sccDNA, and the protein/DNA complex was fixed with glutaraldehyde and analyzed by agarose gel electrophoresis. The appearance of radioactivity comigrating with DNA was absolutely dependent on RF-C and ATP and was due to [$^{32}$P]-ph-PCNA exclusively (Podust et al., 1995a). Preincubation of [$^{32}$P]-ph-PCNA and p21 in the reaction mixture had a concentration-dependent effect. At amounts of GST-p21 up to 200 ng (Figure 2A,C) and of p21 up to 100 ng (Figure 2B), both proteins appeared to stimulate the loading of PCNA onto DNA, while further addition of GST-p21 and p21 inhibited RF-C/PCNA complex assembly. Agarose gel analysis also allowed us to estimate the availability of free PCNA in the presence of a defined amount of GST-p21 (Figure 2C). The migration of [$^{32}$P]-ph-PCNA, treated with glutaraldehyde, in the absence (lane 1) or presence of excess GST-p21 (lane 13) was noticeably different. The [$^{32}$P]-ph-PCNA and GST-p21 were also incubated in the absence of RF-C and DNA, followed by glutaraldehyde cross-linking and analysis on SDS−10% PAGE (data not shown). ph-PCNA in the presence of 600 ng of GST-p21 yielded cross-linked complexes of molecular

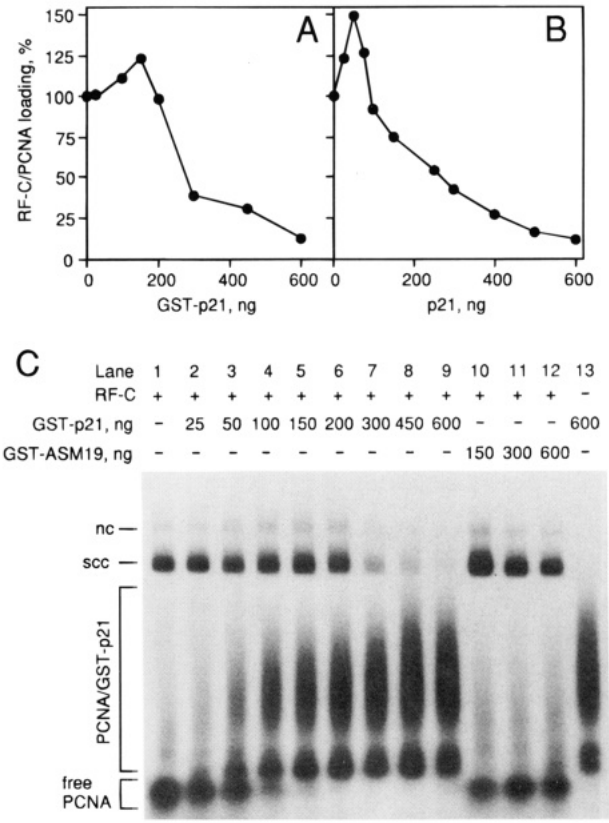


FIGURE 2: Effect of p21 on the RF-C-catalyzed loading of PCNA onto circular dsDNA. Fifteen nanograms of [$^{32}$P]-ph-PCNA, 45 ng of RF-C, 50 ng of sccDNA, and different amounts of p21 were incubated on ice for 10 min in buffer D. ATP (1 mM) was then added and samples incubated for 3 min at 37 °C followed by glutaraldehyde fixation and analysis on a neutral 0.8% agarose gel in the presence of 0.1% SDS. The radioactivity comigrating with sccDNA was quantified with a PhosphorImager. The p21 dependence of PCNA clamp loading onto sccDNA was determined either (A) with GST-p21 or (B) with p21. (C) Autoradiography of the agarose gel analysis of [$^{32}$P]-ph-PCNA loading onto DNA: lane 1, no GST-p21; lanes 2−9, different amounts of GST-p21; lanes 10−12, different amounts of GST-ASM19, a p21 mutant that does not bind PCNA (Goubin & Ducommun, 1995); lane 13, RF-C omitted, control of loading. scc, sccDNA; nc, nicked circular DNA; PCNA/GST-p21, cross-linked complexes of PCNA and GST-p21 of indefined composition (>200 kDa); free PCNA, cross-linked PCNA molecules (mixture of 110- and 70-kDa products).

mass >200 kDa. At lower concentrations of GST-p21, various proportions of PCNA could be cross-linked to GST-p21 as seen on Figure 2C. The cross-linking of ph-PCNA alone or in the presence of GST-ASM19 (150−600 ng) yielded two bands of about 110 kDa (apparently three cross-linked monomers of PCNA) and about 70 kDa (two cross-linked monomers) in a ratio of 70−80% to 20−30%, respectively, again confirming the inability of the mutant protein ASM19 to bind PCNA (Figure 2C, compare lanes 5, 7, and 9 to lanes 10−12). The data in Figure 2C demonstrate that even when essentially all PCNA is complexed with GST-p21, RF-C still loads the PCNA as efficiently as in the absence of p21 (compare lanes 1 and 6). In sum, RF-C displayed the ability to overcome the binding of p21 to PCNA and only a much higher amount of p21 inhibited loading of PCNA onto DNA.

*p21 Does Not Prevent the Dissociation of the RF-C/PCNA Complex from the Linearized Template.* We have recently shown that the RF-C/PCNA complex can be assembled on circular but not linear DNA. If the RF-C/PCNA complex

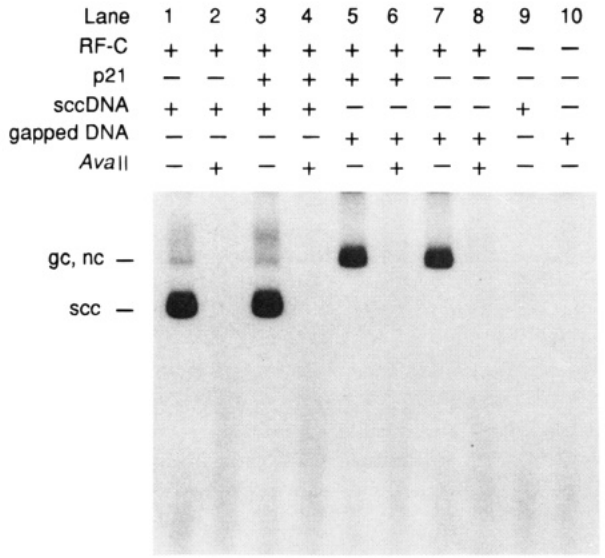


FIGURE 3: p21 does not prevent dissociation of the PCNA clamp from the linearized template. A reaction mixture (24 $\mu$L) containing buffer D, 1 mM ATP, 50 ng of supercoiled or gapped circular DNA, 15 ng of [$^{32}$P]-ph-PCNA, and 45 ng of RF-C was mixed on ice. For loading of PCNA onto DNA, the samples were incubated for 3 min at 37 °C. The tubes were then transferred on ice and 600 ng of p21 in a volume of 1 $\mu$L was added. After a 10-min incubation on ice, the DNA was linearized by *Ava*II (2 units) for 3 min at 37 °C, the proteins were fixed with glutaraldehyde for 10 min at 37 °C, and the samples were analyzed on a neutral 0.8% agarose gel containing 0.1% SDS. scc, supercoiled DNA; gc, gapped circular DNA; nc, nicked circular DNA.

was first loaded on circular DNA followed by linearization of the latter, the protein complex dissociated from the DNA. These data suggested that the RF-C/PCNA complex can track along dsDNA (Podust et al., 1995a). We therefore tested whether p21 can impede the sliding of the RF-C/PCNA complex along the template. [$^{32}$P]-ph-PCNA was first loaded by RF-C onto sccDNA or gapped circular DNA, and then p21 was added in the amount sufficient to bind all PCNA in the reaction mixture. The DNA was cut with *Ava*II at the unique site and the samples were finally fixed with glutaraldehyde. Three minutes of incubation in the presence of *Ava*II was enough for both RF-C/PCNA and RF-C/PCNA/p21 complexes to dissociate from the linear DNA yielding no protein/DNA cross-linked products (Figure 3). These data excluded in addition a hypothetical mechanism for the inhibitory effect of p21. This protein did not counteract RF-C by removing the assembled RF-C/PCNA complexes from the DNA (Figure 3, compare lane 1 to lane 3 and lane 5 to lane 7).

*Wild-Type but Not Mutant p21 Inhibits Gap-Filling DNA Synthesis by Pol δ Holoenzyme.* As a substrate for short-patch DNA synthesis we used gapped circular DNA (Podust et al., 1994). The ability of pol $\delta$ holoenzyme to fill the 45-nt gap in the presence of GST-p21 directly correlated with the ability of RF-C to load PCNA onto DNA: with 200 ng of GST-p21 (8 $\mu$g/mL) the gap-filling was quite efficient (Figure 4A, lane 5), while 600 ng of GST-p21 (24 $\mu$g/mL) strongly inhibited DNA synthesis (lane 6) as it might be expected from the experiment with loading of the PCNA onto DNA (Figure 2C, lane 9). The inhibiting effect of GST-p21 could be seen only at limiting amounts of PCNA. No inhibition was evident even with 600 ng of GST-p21, if 50 ng (2 $\mu$g/mL) instead of 15 ng (0.6 $\mu$g/mL) of PCNA was used per assay (data not shown).

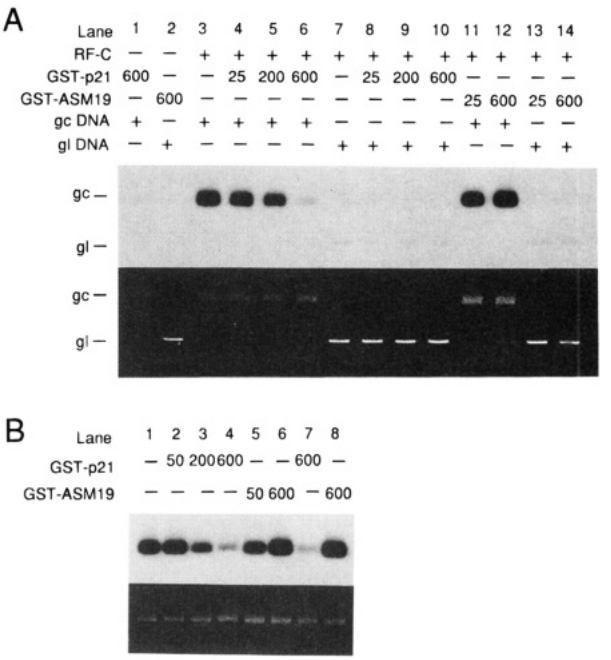


FIGURE 4: Wild-type but not mutant p21 inhibits gap-filling DNA synthesis by pol $\delta$ holoenzyme. Gap-filling DNA synthesis by pol $\delta$ holoenzyme was carried out in two ways: (i) all components were mixed together before DNA synthesis (panel A) or (ii) the clamp loading reaction was carried out first, then GST-p21 (GST-ASM19) was added, and finally DNA synthesis was started by addition of pol $\delta$ and dNTPs (panel B). Panel A: A reaction mixture (final volume of 25 $\mu$L) contained buffer C, 1 mM ATP, 50 ng of gapped (circular or linearized with *Bgl*I) DNA, dGTP, dCTP, and dTTP each at 50 $\mu$M, 1.4 $\mu$Ci of [$\alpha$-$^{32}$P]dATP, 15 ng of PCNA, 45 ng of RF-C, 0.3 unit of pol $\delta$, and varying amounts of GST-p21 (or GST-ASM19) and was mixed on ice. The samples were incubated for 10 min on ice and then transferred to 37 °C. After 10 min the reactions were terminated and the products were analyzed on a neutral 0.8% agarose gel followed by EtdBr staining and autoradiography. Panel B: A reaction mixture (23 $\mu$L) contained buffer C, 1 mM ATP, 50 ng of gapped circular DNA, 15 ng of PCNA, and 45 ng of RF-C and was incubated for 3 min at 37 °C to load the PCNA clamp onto DNA. Then the samples were cooled on ice and GST-p21 (or GST-ASM19) was added in a volume of 1 $\mu$L. The samples were incubated for 10 min on ice (lanes 1−6) or processed immediately without further incubation on ice (lanes 7 and 8), and then pol $\delta$, dGTP, dCTP, dTTP, and [$\alpha$-$^{32}$P]dATP were added in the same amounts as in panel A. After a 10-min incubation at 37 °C, the reactions were terminated and the products were analyzed on a neutral 0.8% agarose gel followed by EtdBr staining and autoradiography. gc, gapped circular DNA; gl, gapped linearized DNA.

The gap-filling experiments were also performed with a different protocol. The PCNA clamp was first assembled on gapped circular DNA, then GST-p21 was added, and finally DNA synthesis was started with pol $\delta$ and the four dNTPs. GST-p21 (24 $\mu$g/mL) strongly inhibited gap-filling by pol $\delta$, even though the PCNA clamp was already assembled equally for each sample. The interaction between p21 and PCNA was very fast. Either when GST-p21 was preincubated with PCNA for 10 min on ice or when the DNA synthesis was processed immediately after sample preparation, 600 ng of GST-p21 strongly inhibited the polymerization reaction (Figure 4B, compare lanes 4 and 7). The data indicated also that p21 binds both to free PCNA in solution and to PCNA clamps assembled on DNA.

We have earlier found that the linearization of the gapped circular DNA template completely abolished DNA synthesis by pol $\delta$ holoenzyme (Podust et al., 1994). The RF-C/PCNA

complex was suggested to be able to track along dsDNA and this was demonstrated in the experiments on RF-C/[$^{32}$P]-ph-PCNA clamp assembly (Podust et al., 1995a). Using the assay independent of DNA synthesis and in the absence of pol $\delta$, we have shown that p21 does not impede the sliding of RF-C/PCNA complex along DNA (Figure 3). To substantiate these data with another approach, we tested the ability of pol $\delta$ holoenzyme to replicate linear DNA in the presence of p21. If p21 does impede the tracking of the clamp or pol $\delta$ holoenzyme along DNA and, correspondingly, prevents their sliding off the linearized template, we might expect that the inhibitor would slow the replication rate but nevertheless allow some DNA synthesis on a 45-nt gap. No concentration of GST-p21 tested rescued the DNA synthesis on linearized template (Figure 4A, lanes 7−10).

*DNA Polymerase δ Competes with p21 for Binding to the PCNA Clamp.* The inhibitory effect of p21 on the PCNA-dependent DNA synthesis could be seen even if RF-C was allowed first to load PCNA onto DNA (Figure 4, compare panels A and B), but an interference with the PCNA clamp sliding along the DNA appeared not to be a reason for the inhibition (Figures 3 and 4). We therefore analyzed the question of whether p21 inhibits the loading of pol $\delta$ core onto the assembled PCNA clamp. As a template, M13 ssDNA primed with a 40-mer oligonucleotide was used. This primer was positioned close to several strong pause sites downstream (Podust et al., 1995b). When all components of the replication assay including GST-p21 were mixed on ice before incubation, increasing amounts of GST-p21 gradually inhibited DNA synthesis (Figure 5A, compare lanes 1−8). When the PCNA clamp was assembled first, followed by the addition of GST-p21, and DNA synthesis was started by pol $\delta$, the inhibitory effect of GST-p21 was stronger and was very marked with 300 ng of GST-p21 (Figure 5, compare lanes 4 and 17). Under these conditions GST-p21 prevented mainly the synthesis of long products (Figure 5). This indicated that inhibition of the PCNA clamp assembly was not the main cause of the overall inhibitory effect on DNA synthesis. Prevention of clamp sliding would have given the same effect in the experiments shown in panels A and B of Figure 5, but since sliding is not noticeably inhibited (Figure 3), the data suggested that p21 interfered with binding of pol $\delta$ to the PCNA clamp.

If the primary effect of p21 was to interfere with binding of pol $\delta$ core to the PCNA clamp, we could expect that increasing amounts of pol $\delta$ would restore the p21-inhibited DNA synthesis. To test this, the PCNA clamp was first assembled on the template, 400 ng of p21 was then added per sample, and finally increasing amounts of pol $\delta$ were supplied for DNA synthesis (Figure 6). Four hundred nanograms of p21 inhibited the activity of 0.3 unit of pol $\delta$ strongly (compare lanes 5 and 7). However, by addition of increasing amounts of pol $\delta$ (up to 6 units) the inhibitory effect of p21 was efficiently overcome (lanes 8−11), indicating that p21 and pol $\delta$ core directly compete for the PCNA clamp.

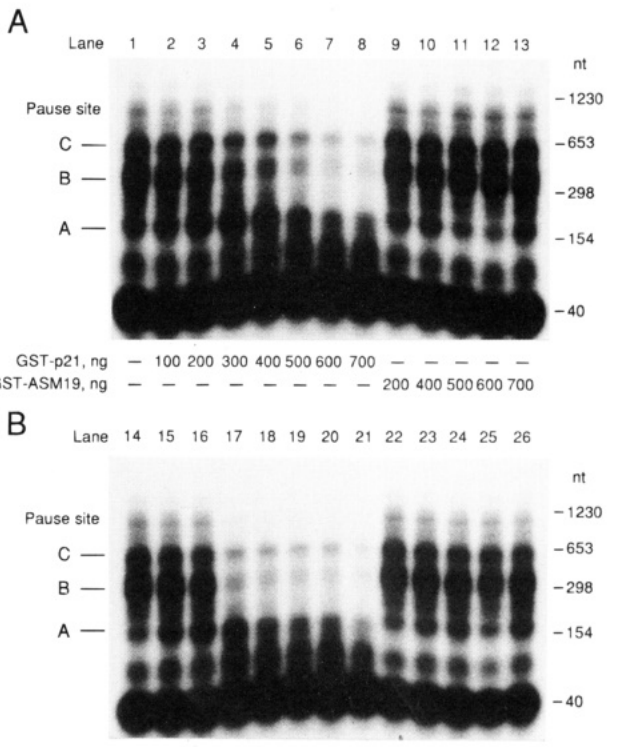


FIGURE 5: p21 inhibits DNA synthesis by pol $\delta$ holoenzyme on singly primed ssDNA. Primer extension DNA synthesis by pol $\delta$ holoenzyme was carried out in two ways: (i) all components were mixed together before DNA synthesis (panel A) or (ii) the clamp loading reaction was carried out first, then GST-p21 (GST-ASM19) was added, and finally DNA synthesis was started by addition of pol $\delta$ (panel B). Panel A (lanes 1−13): A reaction mixture (22.5 $\mu$L) contained buffer C, 1 mM ATP, 50 ng of primed M13 ssDNA (5′-$^{32}$P-phosphorylated primer), 15 ng of PCNA, 45 ng of RF-C, 175 ng of *E. coli* SSB, 0.3 unit of pol $\delta$, and varying amounts of GST-p21 (or GST-ASM19) and was mixed on ice. The samples were first incubated for 10 min on ice, and then all four dNTPs were added (2.5 $\mu$L) to a final concentration of 50 $\mu$M, followed by incubation at 37 °C. After 15 min the reaction was terminated and the products were analyzed on an alkaline 1.5% agarose gel. Panel B (lanes 14−26): A reaction mixture (23 $\mu$L) contained buffer C, dATP, dGTP, dCTP, and dTTP each at 50 $\mu$M, 1 mM ATP, 50 ng of primed M13 ssDNA (5′-$^{32}$P-phosphorylated primer), 15 ng of PCNA, 45 ng of RF-C, and 175 ng of *E. coli* SSB and was incubated 3 min at 37 °C to load the PCNA clamp onto DNA. Then the samples were transferred on ice and GST-p21 (or GST-ASM19) in the same amounts as in panel A were added in a volume of 1 $\mu$L. The samples were incubated for 10 min on ice, and then 0.3 unit of pol $\delta$ (1 $\mu$L) was added, followed by incubation at 37 °C. After 15 min the reaction was terminated and the products were analyzed on an alkaline 1.5% agarose gel.

## DISCUSSION

The experiments performed in this study indicated that inhibition of PCNA-dependent DNA synthesis *in vitro* by p21 occurs through a complex effect. The increasing concentrations of p21 in the reaction mixture first stimulated RF-C-catalyzed loading of PCNA onto DNA, but further addition of p21 inhibited this reaction (Figure 2). Interestingly, a similar observation has been made for the effect of p21 on kinase activity in the p21/cyclin A/Cdk2 complexes (Zhang et al., 1994). Our data suggest that p21 does not impede the sliding of the clamp along DNA. The RF-C/PCNA complex as well as the RF-C/PCNA/p21 complex dissociated from the linearized DNA within 3 min of incubation (Figure 3). Therefore, the impediment of clamp sliding could not contribute to inhibition of DNA synthesis occuring after tens of minutes.

The effect of p21 on PCNA loading onto DNA appeared not to be the main reason for inhibition of pol $\delta$ holoenzyme activity as a whole. Even stronger inhibition of DNA

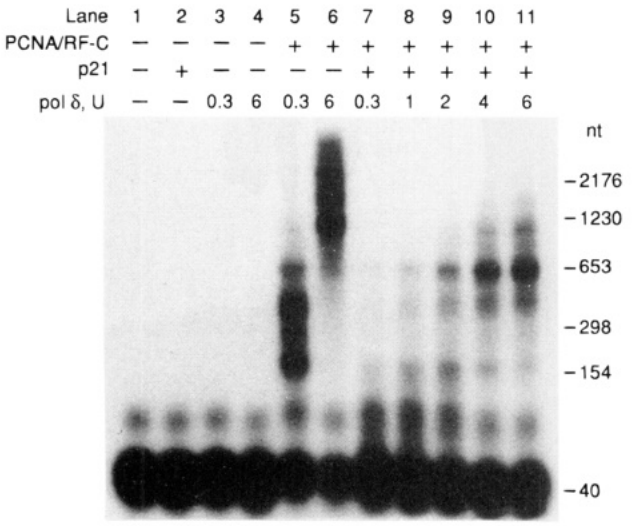


FIGURE 6: Pol $\delta$ can compete with p21 for binding to the PCNA clamp. A reaction mixture (23.5 $\mu$L) contained buffer C, dATP, dGTP, dCTP, and dTTP each at 50 $\mu$M, 1 mM ATP, 50 ng of primed M13 ssDNA (5′-$^{32}$P-phosphorylated primer), 15 ng of PCNA, 45 ng of RF-C, and 175 ng of *E. coli* SSB and was incubated for 3 min at 37 °C to load the PCNA clamp onto DNA. Then 400 ng of p21 was added in a volume of 0.5 $\mu$L. The samples were incubated for 5 min at 37 °C, and then varying amounts of pol $\delta$ in a volume of 1 $\mu$L were added, followed by the incubation at 37 °C. After 15 min the reaction was terminated and the products were analyzed on an alkaline 1.5% agarose gel.

synthesis was observed when PCNA was first assembled on DNA, then complexed with p21, and pol $\delta$ was added last (Figure 5). DNA synthesis was less sensitive to inhibition if pol $\delta$ holoenzyme was assembled simultaneously with the PCNA clamp (Figure 5). As was expected from earlier studies (Flores-Rozas et al., 1994; Li et al., 1994), p21 affected primarily the synthesis of long DNA products. The template−primer used for this study was chosen because it contains three pause sites downstream from the primer on the template. These pause sites caused the dissociation of pol $\delta$ core from the holoenzyme while the PCNA clamp remained stably bound to DNA (Podust et al., 1995b). Pol $\delta$ holoenzyme was concluded earlier to be only moderately processive and this was based on several observations. First, under single-turnover conditions bovine pol $\delta$ could not synthesize products longer than those corresponding to the first pause site (Podust et al., 1995b). Human pol $\delta$ holoenzyme also was not able to synthesize the long DNA products upon a single binding event (Tsurimoto & Stillman, 1989). Second, bovine pol $\delta$ holoenzyme and a replicative DNA helicase (SV40 T antigen or protein E1 from bovine papilloma virus 1) could not cooperate in a "head-to-tail" synthesis mode on dsDNA template as occurs in *E. coli* or T4 systems (Mok & Marians, 1987; Cha & Alberts, 1989) and RP-A was required for coordinated action of pol and helicase (Podust et al., 1995b). Pol $\delta$ holoenzyme replicates faster than the SV40 T antigen unwinds DNA duplexes (Murakami & Hurwitz, 1993), and it might therefore be expected that the pol $\delta$ holoenzyme periodically collides with the DNA helicase, thus resulting in the dissociation of pol $\delta$ core from DNA. This would explain the rather moderate processivity of bovine pol $\delta$ holoenzyme (Podust et al., 1995b), and it appears this might be also the case for the human enzyme in the SV40 *in vitro* system (Eki et al., 1992).

Analysis of inhibition of the PCNA-dependent DNA synthesis by p21 showed that the inhibition does not occur by an "all-or-none" mechanism (Flores-Rozas et al., 1994). Our data agree well with this finding. The replication products synthesized by pol $\delta$ holoenzyme in the presence of inhibiting amounts of p21 corresponded mostly to the first pause site (Figure 5). Very similar products synthesized by pol $\delta$ holoenzyme were obtained on the same template under single-turnover conditions (Podust et al., 1995b). On the basis of our previous studies (Podust et al., 1995b) and the results presented in this paper, we propose the following mechanism of differential inhibition of short "repair" and long "replication" DNA synthesis by p21. The continuous assembly of pol $\delta$ holoenzyme and the first turnover DNA synthesis seem to be relatively resistant to p21 inhibition. This would be enough to complete, for instance, a short patch "repair" mission, such as NER. Inhibition of long tract "replication" DNA synthesis might result from a direct competition of p21 and pol $\delta$ core for the PCNA clamp during multiple reloading of pol $\delta$ onto DNA (Figure 6).

*In vivo* the cell might even have some mechanism to protect PCNA-dependent DNA synthesis from direct interference of p21. Crude fractions required for NER supplemented with a controlled amount of purified PCNA could perform the repair DNA synthesis even in the presence of 40 $\mu$g/mL GST-p21 (Shivji et al., 1994). In our experiments with purified proteins, however, 24 $\mu$g/mL of GST-p21 inhibited the loading of PCNA onto DNA and any DNA synthesis nearly completely. Other PCNA binding proteins, such as Gadd45 (Smith et al., 1994), might interfere with binding of p21 to PCNA. DNA replication in *Xenopus* extract could be strongly inhibited by p21, but this was due to the inhibition of early stages of DNA replication and could be rescued by addition of cyclins A or E but not PCNA. No inhibition of the elongation stage synthesis by p21 was evident (Strausfeld et al., 1994). Recent studies revealed two separated domains on the p21 molecule. The Cdk2 binding domain was shown to be located in the N-terminal part of the protein, between amino acid residues 45 and 60, while the PCNA binding region was localized to the C-terminus, between residues 142 and 163 (Goubin & Ducommun, 1995; Nakanishi et al., 1995). The C-terminal fragments of p21 interacting with PCNA but not with Cdk2 were unable to inhibit DNA synthesis *in vivo* and to suppress cell growth (Chen et al., 1995; Nakanishi et al., 1995).

We speculate that the inhibitory effect of p21 on *in vivo* DNA replication might be selective rather than global. Replication of chromosomal DNA proceeds simultaneously at several thousand forks throughout the nucleus. If one particular replication fork encounters damage in the DNA, it would be unnecessary and inefficient to block DNA synthesis at all other forks. When DNA synthesis at some definite locus is stopped by the DNA damage, this might cause the partial disassembly of the replication complex. Therefore pol $\delta$ core could dissociate, while the PCNA clamp remains stably bound to DNA (Podust et al., 1995b). The remaining PCNA clamp might reload the pol core onto DNA, and pol $\delta$ (or pol $\epsilon$) would bypass DNA damage (O'Day et al., 1992) with potentially mutagenic consequences. Interrupted DNA synthesis might be a target for direct p21 inhibition, to prevent such bypass synthesis by pol $\delta$ or pol $\epsilon$.

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