Cdk-interacting protein 1 directly binds with proliferating cell nuclear antigen and inhibits DNA replication catalyzed by the DNA polymerase δ holoenzyme

(cell cycle regulation/processivity/protein-protein interaction)

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ABSTRACT

Cdk-interacting protein 1 (Cipl) is a p53-regulated 21-kDa protein that inhibits several members of the cyclin-dependent kinase (CDK) family. It was initially observed in complexes containing CDK4, cyclin D, and proliferating cell nuclear antigen (PCNA). PCNA, in conjunction with activator 1, acts as a processivity factor for eukaryotic DNA polymerase (pol) δ, and those three proteins constitute the pol δ holoenzyme. In this report, we demonstrate that Cipl can also directly inhibit DNA synthesis in vitro by binding to PCNA. Cipl efficiently inhibits simian virus 40 replication dependent upon pol α, activator 1, PCNA, and pol δ, and this inhibition can be overcome by additional PCNA. Simian virus 40 DNA replication, catalyzed solely by high levels of pol α-primase complex, is unaffected by Cipl. Using the surface plasmon resonance technique, a direct physical interaction of PCNA and Cipl was detected. We have observed that Cipl efficiently inhibits synthesis of long (7.2 kb) but not short (10 nt) templates, suggesting that its association with PCNA is likely to impair the processive movement of pol δ during DNA chain elongation, as opposed to blocking assembly of the pol δ holoenzyme. The implications of the Cipl-PCNA interaction with respect to regulation of DNA synthesis, cell cycle checkpoint control, and DNA repair are discussed.

The eukaryotic cell cycle is regulated by a family of cyclin-dependent kinases (CDKs). Several small proteins have been detected that bind and inhibit CDKs (reviewed in ref. 1); one such protein, cdk-interacting protein 1 (Cipl; also known as wild-type p53-activated fragment 1, or WAF-1), was initially observed in a quaternary complex that also included cyclin D, CDK4, and proliferating cell nuclear antigen (PCNA) (2). Cipl can directly interact with CDKs (3–5), indicating that PCNA is not required for the interactions between Cipl and CDKs.

Cipl is regulated by the tumor suppressor gene p53, a transcription factor that controls the G1 cell cycle arrest checkpoint in response to DNA damage (6). Cipl is transcriptionally induced by DNA damage in a p53-dependent fashion and may mediate cell cycle arrest (7). The G1 checkpoint is thought to arrest the cell cycle to prevent DNA replication of damaged templates while allowing repair.

Although kinases are thought to regulate DNA synthesis, the appearance of Cipl and PCNA in a complex suggested the possibility of a more direct link to replication. PCNA, in conjunction with activator 1 (A1; also known as RF-C), acts as a processivity factor for DNA polymerase (pol) δ (8–10). Recent studies have shown that PCNA (as a trimer) and the β subunit (a dimer) of DNA pol III holoenzyme of Escherichia coli both form a tight clamp that tethers their cognate DNA polymerase to the template and translocates along the DNA with the polymerase during replication (11–13). Thus any agent that can interact with the sliding clamp might impede the movement of the replication complex.

Here we demonstrate that Cipl directly binds PCNA to form a complex that inhibits the activity of the PCNA-dependent pol δ holoenzyme, which reduces the replication of simian virus 40 (SV40) DNA and the elongation of primed DNA templates. However, SV40 DNA replication carried out with the pol δ-independent monomeric polymerase system was unaffected by Cipl. The inhibitory effects of Cipl were independent of the cdk2-cyclin A complex.

MATERIALS AND METHODS

Preparation of DNAs and Proteins. (dT)12-18 and (dA)5500 were obtained from Life Sciences Inc.; poly(dA) was annealed to oligo(dT) (20:1) as described (14). Singly primed DNA was prepared by hybridizing a 2-fold molar excess of a 34-nt oligonucleotide to circular single-stranded M13 mp7 (7.2 kb) DNA (containing 10–15% linear molecules) at nucleotide positions 6300–6633. The annealed product was labeled by the incorporation of a single dCMP residue (corresponding to position 6299) using the Klenow fragment of DNA pol I. The resulting product (~2000 cpm/mol), after phenol extraction, was isolated by filtration through a G-50 Sephadex column. Cytosolic extracts of HeLa cells, SV40 origin-containing DNA (pSV01AEP), human single-stranded DNA-binding protein (HSSB; 600 units/mg of protein; also called RP-A), pol α-primase complex (3.7 × 106 and 4.9 × 106 units/mg, respectively), SV40 large tumor antigen (T antigen), and PCNA (2000 units/mg of protein) were prepared as described (15–17). pol δ, isolated from HeLa cells, was purified as described by Lee et al. (14). The preparation used here contained 1500 units/mg of protein. The purification of A1, based on its ability to stimulate pol δ activity, was carried through procedure 2, as described (14). In all experiments reported here, the single-stranded DNA-cellulose fraction (1200 units/mg of protein) was used. Cipl was expressed in E. coli and isolated as described (3). Cipl (at concentrations between 0.5 and 1 mg/ml) was stored at a salt concentration of 0.2–0.4 M NaCl to avoid aggregation and was diluted 5- to 10-fold prior to use. Human cyclin A and cdk2 (kindly

Abbreviations: HSSB, human single-stranded DNA binding protein; SSB, single-stranded DNA binding protein; A1, activator 1; PCNA, proliferating cell nuclear antigen; pol, DNA polymerase; SV40, simian virus 40; T antigen, SV40 large tumor antigen; Cipl, p21 cdk-interacting protein 1; CDK, cyclin-dependent kinase; RU, response units.

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provided by E. Gibbs, Memorial Sloan–Kettering Cancer Center, overexpressed in E. coli, were prepared as described (18). The β and δ subunits of E. coli pol III holoenzyme were both overexpressed and cloned in E. coli and prepared as described (19, 20).

Binding and Replication Assays. The immobilization of PCNA and the E. coli β subunit to sensor chips was carried out using the carbodiimide covalent linkage procedure following the manufacturer’s (Pharmacia Biosensor) instructions. The response units (RU) indicated the concentration of PCNA and β subunit covalently linked to the chip and are reported in the figure legend.

SV40 DNA replication, the elongation of poly(dA)-oligo(dT), and singly primed M13 DNA were carried out as described (14). Where indicated, elongation reactions were analyzed by alkaline agarose gel electrophoresis as detailed in the figure legends.

RESULTS

Influence of Cipl on SV40 DNA Replication by Crude Extracts and the Monopolymerase System. The influence of Cipl on the T antigen-dependent replication of SV40 DNA using crude extracts of HeLa cells was examined. In this reaction, DNA synthesis is dependent on the combined action of the pol α–primase complex for the initiation of DNA chains and the pol δ holoenzyme for their elongation. As shown in Fig. 1, DNA synthesis, measured by incorporation of labeled deoxyribonucleotides, was inhibited by increasing levels of Cipl. In contrast, SV40 DNA synthesis carried out by the monopolymerase system was not affected by Cipl. In the monopolymerase system, relatively high levels of pol α carry out both leading- and lagging-strand synthesis, obviating the requirement for the PCNA-dependent pol δ holoenzyme (16). Since SV40 DNA replication with crude extracts and the monopolymerase system depend upon SV40 T antigen, HSSB, and the pol α–primase complex for activity, these observations suggest that Cipl blocked SV40 replication in crude extracts by inhibiting the pol δ holoenzyme system.

The inhibition of replication with crude extracts by Cipl could be partially reversed by supplementing reactions with purified PCNA. Thus, in the presence of 50 and 100 ng (120 nM) of Cipl, which inhibited replication 38% and 65%, respectively, the addition of 100 ng (86 nM monomer) of PCNA reduced the inhibition to 14% and 45%, respectively (data not presented). The addition of this level of PCNA had virtually no effect on DNA synthesis in the absence of Cipl. This suggests that the inhibition by Cipl in this system is specifically due to an effect on the action of PCNA.

Influence of Cipl on the pol δ Holoenzyme-Catalyzed Elongation of Primed DNA Templates. A direct measurement of the activity of the pol δ holoenzyme, which consists of PCNA, A1, and pol δ, and of the influence of Cipl was carried out using primed DNA templates. For this purpose, (dA)_{200} (dT)_{28} was used as the primer template. The synthesis of poly(dT) with this substrate is dependent on ATP, a SSB, PCNA, A1, pol δ, and dTTP. As shown in Fig. 2, elongation of oligo(dT) was markedly inhibited by Cipl, and the extent of inhibition depended on the level of PCNA added. In the presence of 10 ng (11.4 nM monomer) of PCNA, 50% inhibition of poly(dT) synthesis was observed in the presence of 50 ng (80 nM) of Cipl. Higher levels of PCNA require higher Cipl levels to inhibit the reaction. Under the conditions of the experiment, the inhibitory effects of Cipl were unaffected by increasing the levels of A1, pol δ, HSSB, or addition of cdk2 in the presence or absence of cyclin A (data not presented). These observations suggest that Cipl inhibits the activity of PCNA independent of the presence of cdk2–cyclin A.

![Fig. 1. Influence of Cipl on SV40 DNA replication by crude extracts of HeLa cells and the monopolymerase system. Replication of SV40 DNA by crude extracts was carried out in reaction mixtures (40 μl) containing 0.3 μg of pSV01AEP, 0.83 μg of SV40 T antigen, 0.3 μg of HSSB, 186 μg of crude extract of HeLa cells, Cipl as indicated, and other reagents as described (14, 17). Reaction mixtures were incubated for 90 min at 37 °C, and the amount of acid-insoluble radioactivity formed (using [α-32P]dCTP) was measured. In the absence of Cipl, 74.4 pmol of dCMP was incorporated. In the monopolymerase reactions, reaction mixtures (40 μl) contained the same amount of DNA, SV40 T antigen, and HSSB as above, but in place of the crude extract of HeLa cells, purified pol α–primase complex (0.4 and 1.1 units, respectively), 12 ng of E. coli cloned vaccinia topoisomerase I (a gift from S. Shuman, Memorial Sloan–Kettering Cancer Center), and 3 μg of bovine serum albumin were added. Cipl was added as indicated and other reagents were added as described (14). In the absence of Cipl, 28.2 pmol of dCMP was incorporated into DNA after 90 min at 37 °C.

![Fig. 2. Influence of Cipl on the synthesis of poly(dT) by the pol δ holoenzyme. Reaction mixtures (30 μl) contained 10 mM Tris- HCl (pH 7.8), 10 mM creatine phosphate (di-Tris salt, pH 7.7), 1 μM of creatine phosphokinase, 2 mM dithiothreitol, bovine serum albumin (150 μg/ml), 30 μM [α-32P]dTTP (1200 cpm/pmol), 2 mM ATP, 7 mM MgCl2, 300 pmol of (dA)_{200}(dT)_{28} (20:1), 0.6 μg of HSSB, 0.2 unit of A1, 0.1 unit of pol δ, PCNA, and Cipl as indicated. After 20 min at 37 °C, the amount of acid-insoluble poly(dT) formed was measured. In these reactions, A1 and pol δ were added after the addition of all other reagents, which were assembled at 0 °C. In the absence of Cipl, dTMP incorporation with 100 and 10 ng of PCNA was 132 and 55.2 pmol, respectively. In the absence of PCNA, A1, or pol δ, dTMP incorporation was 1.80, 1.46, and <0.1 pmol, respectively.](image-url)
If the loading of PCNA onto primer ends was completely blocked by Cipl, the pol δ-catalyzed elongation reaction would be totally inhibited. Such a mechanism would result in an all-or-none synthesis of DNA chains. In reactions partially inhibited by Cipl, the size of extension products would be unaffected, but their yield would be reduced. To determine whether Cipl blocked the elongation of primed templates in an all-or-none manner, the elongation of a single labeled primer annealed to single-stranded circular M13 DNA was carried out. In these experiments, two different amounts of PCNA, 50 ng (84 nM monomer) and 100 ng were used, each in the presence of 50 ng (120 nM) and 100 ng of Cipl. Under these conditions, nucleotide incorporation was inhibited between 30% and 80% (data not presented). Analysis of the replication products by alkaline agarose gel electrophoresis indicated that Cipl caused a marked reduction in the formation of longer products, concomitant with an increased accumulation of shorter chains (Fig. 3, lanes 5–8). Again, the addition of cdk2 (450 nM) and cyclin A (260 nM) did not alter the quantitative distribution of products formed in the presence (lanes 7–10) or absence (lanes 3, 4, 11, and 12) of Cipl.

These results indicate that Cipl inhibition does not lead to an all-or-none effect on the pol δ-catalyzed elongation reaction and most likely interferes with the sliding of PCNA along the DNA or its association with pol δ.

Further support for this notion was obtained by examining the influence of Cipl on short-chain elongation of the singly primed M13 DNA containing a labeled primer. In these experiments, chains were elongated for only 10 nt by the inclusion of ddCTP as a chain terminator (Fig. 4). In the presence of 0.25 nM template and 2 ng of PCNA (3.3 nM monomer), short-chain elongation was reduced <10% by concentrations of Cipl up to 50 ng (120 nM). At higher levels of Cipl (480 nM), this reaction was inhibited up to 70%. This inhibition was probably due to impaired loading of PCNA on the primer end; when PCNA was first loaded onto the primer end in the presence of A1 and the complex was then treated with 200 ng of Cipl (480 nM), the short-chain elongation reaction was reduced by only 10% (data not shown).

We interpret the short-chain elongation results as follows. In the presence of levels of Cipl that inhibit extensive elongation, there is no inhibition of short-chain elongation because the enzyme translocates over a distance that is not long enough to reflect its impaired movement. The likely mechanism by which Cipl interferes, in this short chain reaction, is by blocking the loading of the PCNA clamp on the primer end.

**Direct Interaction Between Cipl and PCNA.** Direct evidence that Cipl physically interacts with PCNA was obtained using the surface plasmon resonance technique (21). For the experiment shown in Fig. 5, human PCNA (expressed in E. coli) was coupled to the sensor surface over which a solution containing Cipl was passed. An immediate burst in accumulation of mass was observed upon exposure to Cipl, plateauing at a stoichiometry of ~2.3 molecules of Cipl (as monomer) per molecule of PCNA trimer (Fig. 5A). After 7 min, equilibration buffer lacking Cipl was passed over the surface,

**Fig. 4.** Influence of Cipl on the synthesis of a 10-nt DNA fragment by the pol δ holoenzyme. Reaction mixtures (20 μl) containing 60 mM Tris-HCl (pH 8.0), 60 mM creatine phosphate, 10 mM MgCl₂, 1 mM dithiothreitol, 5 μg of bovine serum albumin, 3 mM ATP, 100 mM each of dATP, dGTP, dTTP, and ddCTP, 5 fmol of labeled, singly primed M13 mp7 single-stranded circular DNA, and the indicated amounts of Cipl were preincubated at 37°C for 10 min. HSSB (210 ng), pol δ (0.025 unit), A1 (0.05 unit), and PCNA (2 ng, 22 fmol of trimer) were added, and primer extension was carried out at 37°C for 8 min. Reactions were terminated by the addition of 3 μl of 0.1 M EDTA/2% SDS, boiled for 5 min to dissociate the labeled oligonucleotide products from the M13 circular templates, and electrophoresed through a 12% polyacrylamide gel containing TBE buffer. (A) Autoradiograph of the dried gel. Lane 1, enzymes omitted; lane 2, no PCNA; lane 3, complete reaction but lacking Cipl. (B) Quantitation of the Cipl inhibition of primer extension. Extension of 2.7 fmol of substrate corresponded to 100% activity.

**Fig. 3.** Influence of Cipl on the elongation of labeled singly primed DNA by pol δ holoenzyme. Reaction mixtures (20 μl) contained 30 mM Tris-HCl (pH 7.8), 2 mM dithiothreitol, bovine serum albumin at 150 μg/ml, 100 μM dNTPs, 2 mM ATP, 7 mM MgCl₂, 16.2 fmol of singly primed M13 DNA (2600 cpm/fmol), 0.6 μg of HSSB, 0.15 unit of A1, 0.1 unit of pol δ, and where indicated 0.3 μg of cdk2 plus 0.3 μg of cyclin A, PCNA, and Cipl (in amounts indicated). Reaction mixtures lacking A1 and pol δ were incubated for 5 min at 37°C and cooled to 0°C, and then A1 and pol δ were added. After 40 min at 37°C, reaction mixtures were adjusted to 10 mM EDTA, loading dye containing SDS was added, and the mixtures were subjected to alkaline agarose gel (1.8%) electrophoresis. The gel was dried and autoradiographed. In lane 13, pol δ was omitted from the reaction; the lane marked M indicates the position of polynucleotide markers (in kb).
The results presented here demonstrate that Cipl directly interacts with PCNA, forming a complex that can be readily detected by real-time interaction analysis using the BIAcore (Pharmacia Biosensor). The strong RU observed revealed that each mol of PCNA trimer complexed 2.3 mol of Cipl, almost equivalent to the binding of one Cipl molecule to one PCNA molecule. The lower observed value may represent the unavailability of portions of the PCNA trimer covalently linked to the sensor chip. Analysis of Cipl interaction with *E. coli* pol β subunit revealed specificity for PCNA. Further direct evidence for the interaction between Cipl and PCNA was obtained by gel-filtration analysis.

The complex formed between PCNA and Cipl reduced the ability of PCNA to support pol δ-catalyzed elongation reactions. At molar ratios of Cipl to PCNA trimer between 2 and 5, the elongation of singly primed M13 templates was inhibited, resulting in a marked decrease in the synthesis of full-length products and the accumulation of DNA chains 0.1–1 kb in length. At higher molar ratios, the accumulation of short DNA chains was reduced.

The steps involved in formation of the PCNA clamp and its loading by A1 on primed DNA templates in the eukaryotic system (pol δ and pol ε) have been elucidated (8–11). The initial step in this process involves the selective binding of A1 to a primer terminus on SSB-coated template DNA. The primed template–A1 complex, in the presence of ATP, then interacts with PCNA, forming a PCNA adduct that is stable to gel filtration. This DNA–A1–PCNA complex can then interact with pol δ, or pol ε, which, in the presence of dNTPs, catalyzes processive DNA synthesis. The interaction of Cipl with PCNA could interfere with the binding of PCNA to A1, to pol δ, or to both. Cipl (present at low levels) could impair the movement of the replication complex along the DNA. The data presented above suggest that probably each of these reactions can be affected by Cipl. Results supporting this suggestion include the following. (i) The most pronounced effect of relatively low levels of Cipl (preincubated with PCNA prior to the addition of A1 and pol δ) was on the synthesis of long DNA products. (ii) Inhibition of the elongation of chains over a short 10-nt stretch required high molar ratios of Cipl to PCNA (>40-fold). (iii) When PCNA was first loaded on to primed DNA by A1, subsequent incubation with Cipl only slightly inhibited the 10-nt elongation reaction. This indicates that Cipl can block the clamp-loading step when present at high molar ratios to PCNA. (iv) Preliminary experiments indicated that the loading of PCNA on A1-primed DNA was the same in the presence and absence of 2-fold molar excess of Cipl (data not presented). These observations suggest that Cipl, when present at molar ratios to PCNA of 4:1 or lower, interferes with the rate of pol δ-catalyzed DNA synthesis. It could do this by interfering with only the sliding of the complex along DNA or by decreasing the association of pol δ with PCNA. The latter possibility, however, seems less likely since the inhibition by Cipl was unaffected by supplementation of reactions with more pol δ, and preincubation of A1 and PCNA with the singly primed substrate prevented inhibition by Cipl of the short-stretch elongation reaction.

The finding that Cipl can alter rates of DNA synthesis may have significant implications for understanding cell cycle checkpoints. Previously checkpoints were thought to block major cell cycle transitions in order to prevent replication and segregation of damaged templates. Available data are consistent with a direct role for Cipl in this p53-dependent G1 checkpoint induced by DNA damage via its ability to associate with and inactivate cyclin E–Cdk2 complexes, which function at the G1/S transition (7). The data presented here indicate that Cipl may provide a second checkpoint function by directly inhibiting PCNA-dependent chain elongation catalyzed by pol δ. While PCNA has been shown to participate in repair reactions (23), it is likely that only very high levels of Cipl complexed to PCNA would inhibit repair, because it involves the synthesis of relatively short lengths of DNA. The polymerase that participates in the repair of damaged DNA has been shown in some instances to be pol ε (24, 25), whose activity can utilize the PCNA sliding clamp (10, 26). We suspect that, as for pol δ, the elongation of primed templates by pol ε will also prove to be sensitive to Cipl. D-type cyclins have been shown to physically associate with PCNA in the absence of a Cdk (27). Thus, it is possible that
the affinity of Cip1 for PCNA could be substantially enhanced in the presence of Cdk-cyclin D complexes through the formation of multiple protein–protein interactions.

p53 has also been implicated in DNA damage-dependent apoptosis, and Cip1 is induced during this process (7). The decision to remain arrested or undergo apoptosis may depend in part on the extent of damage or the presence of conflicting growth control signals. Cip1, through its association with PCNA, could play a role in transducing signals leading to apoptosis, perhaps by permanently inhibiting DNA synthesis.

Several DNA tumor viruses express proteins that bind and inactivate p53. If replication of these agents were sensed as DNA damage by the cell, Cip1 could prevent their replication by sequestering PCNA. Thus the necessity to remove p53 may not only facilitate exit from G1 to S but could also optimize cells for high-level viral DNA synthesis by blocking Cip1 formation. Whether Cip1 is involved in checkpoints, apoptosis, or both, it is likely that the alteration of the rate and extent of DNA synthesis by Cip1 is an important aspect of the cell's ability to regulate S phase in response to signals elicited by DNA damage.

Note Added in Proof. Recently, a cyclin-dependent kinase inhibitor implicated in G1 phase arrest, p27Kip1, has been isolated (28). This protein possesses some regions of sequence similarity to Cip1. However, recombinant p27Kip1 did not inhibit the elongation of pre-existing DNA templates in transcription mixtures as described in Fig. 3, nor did it interact with PCNA, measured by SPR as described in Fig. 5A (K. Poljak, J. Massagué, A. Koff, Z.K., M.O., and J.H., unpublished results).

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