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Mediation of Proliferating Cell Nuclear Antigen (PCNA)-dependent DNA Replication through a Conserved p21^{Cip1}-like PCNA-binding Motif Present in the Third Subunit of Human DNA Polymerase δ *

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The subunit that mediates binding of proliferating cell nuclear antigen (PCNA) to human DNA polymerase δ has not been clearly defined. We show that the third subunit of human DNA polymerase δ , p66, interacts with PCNA through a canonical PCNA-binding sequence located in its C terminus. Conversely, p66 interacts with the domain-interconnecting loop of PCNA, a region previously shown to be important for DNA polymerase δ activity and for binding of the cell cycle inhibitor p21^{Cip1}. In accordance with this, a peptide containing the PCNA-binding domain of p21^{Cip1} inhibited p66 binding to PCNA and the activity of native three-subunit DNA polymerase δ . Furthermore, pull-down assays showed that DNA polymerase δ requires p66 for interaction with PCNA. More importantly, only reconstituted three-subunit DNA polymerase δ displayed PCNA-dependent DNA replication that could be inhibited by the PCNA-binding domain of p21^{Cip1}. Direct participation of p66 in PCNA-dependent DNA replication *in vivo* is demonstrated by co-localization of p66 with PCNA and DNA polymerase δ within DNA replication foci. Finally, *in vitro* phosphorylation of p66 by cyclin-dependent kinases suggests that p66 activity may be subject to cell cycle-dependent regulation. These results suggest that p66 is the chief mediator of PCNA-dependent DNA synthesis by DNA polymerase δ .

DNA replication requires the recruitment of multiple components during the S phase of the cell cycle. Of chief importance are DNA polymerases, of which three, α , δ , and ϵ , have been shown by genetic studies in the yeast *Saccharomyces cerevisiae* to be essential for the replication of the eucaryotic genome (1, 2). Among these, DNA polymerase α -primase is the only enzyme that can start DNA synthesis *de novo*. It is thus ideally suited for the synthesis of short primers that serve to initiate leading strand synthesis at the replication origin and

Okazaki fragments on the lagging strand (3, 4). Both DNA polymerases δ and ϵ can elongate primers synthesized by DNA polymerase α and appear to have overlapping or complementary functions (5). Although DNA polymerase δ alone can replicate both leading and lagging strands *in vitro* (5), mounting evidence suggests that DNA polymerase ϵ may be implicated in DNA replication in ways that may not necessarily involve its DNA polymerase activity (6–10).

We have been interested in how DNA polymerase δ interacts with other factors to accomplish replication of the mammalian genome. Extensive research over a number of years has shown that DNA polymerase δ requires interactions with at least two factors, a toroidal-shaped homotrimeric protein known as proliferating cell nuclear antigen (PCNA)¹ and a heteropentameric complex termed replication factor C. In the presence of ATP, replication factor C interacts with the 3' primer-template terminus and recruits PCNA onto the DNA, creating a mobile platform that tethers DNA polymerase δ to the primer terminus. Once bound to PCNA, DNA polymerase δ acquires new dynamic properties, including high processivity, low nonproductive binding to single-stranded DNA, and propinquity to factors engaged in ancillary activities at the replication fork (5).

Replication complex formation involving PCNA, DNA polymerase δ , and replication factor C is inhibited by p21^{Cip1} (11, 12), a Cdk inhibitor whose increased synthesis following DNA damage results in mitotic arrest in the G₁ phase of the cell cycle (13, 14). In contrast, the effect of p21^{Cip1} on nucleotide excision repair, which requires PCNA-dependent DNA synthesis by DNA polymerase δ , remains controversial (15, 16). The C-terminal region of p21^{Cip1} contains the canonical PCNA-binding domain (17). A peptide containing this domain inhibits DNA synthesis by DNA polymerase δ *in vitro* (17–19). Crystallographic studies have shown that the peptide interacts with the domain-interconnecting loop of PCNA, a region that has also been shown to mediate DNA polymerase δ binding to PCNA (20). These data strongly suggest that p21^{Cip1} inhibition of DNA polymerase δ involves competition between their respective PCNA-binding domains for binding to PCNA. However, despite the fundamental importance of this interaction, the identity of the subunit of mammalian DNA polymerase δ that mediates PCNA binding remains controversial.

All three DNA polymerases mentioned have complicated

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¹ The abbreviations used are: PCNA, proliferating cell nuclear antigen; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; PBA, phosphate-buffered saline; Ni-NTA, nickel-nitrilotriacetic acid; RBV, recombinant baculovirus; PAGE, polyacrylamide gel electrophoresis; Cdk, cyclin-dependent kinase.

subunit compositions consisting of a large catalytic subunit and smaller subunits whose functions in many cases remain to be elucidated. DNA polymerase δ from the yeast *Schizosaccharomyces pombe* consists of at least four subunits: Pol3, Cdc1, Cdc27, and Cdm1 (21). The Cdc27 subunit was recognized early on to contain a C-terminal sequence motif that shares significant homology with the consensus PCNA-binding motif found in a variety of PCNA-binding proteins such as p21^{Cip1}, Fen1, 5' cytosine methyltransferase, and DNA ligase I (22). Since then, the region has been shown to interact directly with PCNA and to be essential for cell viability, indicating that Cdc27 plays an essential function by bridging the catalytic subunit of DNA polymerase δ to PCNA (23). Likewise, purified DNA polymerase δ complexes from *S. cerevisiae* contain three subunits (24), one of which, Pol32, has been shown to be the functional homologue of Cdc27 (25). In mammals, however, DNA polymerase δ has up until recently been consistently purified as a heterodimeric complex of only two subunits (26), a catalytic subunit of 125 kDa and a small subunit of 48–50 kDa that display homology respectively to Pol3 and Cdc1 of *S. pombe*. PCNA stimulation of the two-subunit complex and the absence of a visible Cdc27 homologue led to the identification of binding sites for PCNA on the large subunit of human DNA polymerase δ (27). However, none of these binding sites display significant homology to the canonical PCNA-binding site found in other PCNA-binding proteins such as p21^{Cip1}, although one of the sites resembles the novel PCNA-binding site recently identified by screening a random peptide library for PCNA-binding proteins (28). The necessity of the small 48–50-kDa subunit for the reconstitution of active two-subunit DNA polymerase δ led to the alternative suggestion that the PCNA-binding site was located on the small subunit or was formed at the interface between both subunits (29).

Recently, new purification procedures for DNA polymerase δ from mouse (30), calf thymus (31), and human cells (32) yielded a novel subunit of 66–68 kDa, termed p66 or subunit C according to the newly proposed nomenclature for DNA polymerase δ subunits (34). Here, we present results that strongly suggest that this subunit mediates PCNA binding to DNA polymerase δ and corresponds to the subunit whose interaction with PCNA is targeted by p21^{Cip1}.

MATERIALS AND METHODS

Reagents, Peptides, Buffers, and Enzymes—Isopropyl- β -D-thiogalactopyranoside and dithiothreitol (DTT) were from ICN Biomedicals Inc. Antibiotics were from Eurobio. ELISA plates (Maxisorb) were from Nalgen-Nunc International. Restriction enzymes and bovine serum albumin (BSA) (Fraction V) were from Roche Molecular Biochemicals. Phosphate-buffered saline (PBS) tablets (pH 7.2) were from Life Technologies, Inc. Where indicated, protease inhibitors (ICN Biomedicals Inc.) were added at the following concentrations: 5 μ g/ml antipain, 5 μ g/ml leupeptin, 5 μ g/ml aprotinin, and 5 μ g/ml pepstatin. Streptavidin was from Promega. Streptavidin-agarose beads were from Sigma-Aldrich. Nitrocellulose membranes (BioTrace NT 0.45 μ) were from Gelman Sciences. Ni-NTA agarose was from Qiagen. The synthetic peptides had the following sequences: N-terminal biotinylated p66 peptide, Btn-KGTAALGKANRQVSITGFFQRK; p21^{Cip1} peptide, GRKRRQTSMTDFYHSKRRLIFS; and mutated p21^{Cip1} peptide, GRKRRQTSATDFYHSKRRLIFSKRKP. The methionine to alanine substitution in the mutated peptide is indicated in bold type. All peptides were greater than 95% pure and were obtained from Sigma-Genosys. Cyclin-Cdk complexes were a kind gift of Dr. Heinz-Peter Nasheuer. Buffer PBS-T contained PBS plus 0.1% Tween 20, and buffer PBS-TM was buffer PBS-T plus 5% skimmed milk. Mouse DNA polymerase δ complex was purified as described previously (30).

Antibodies—Monoclonal anti-p66 antibody was prepared by Q-BIO-gene using recombinant p66 protein expressed in *Escherichia coli*. One hybridoma clone (2C11) was selected for its ability to recognize p66 on Western blots and immunoprecipitate p66 from mouse cell extracts. Rabbit anti-serum directed against recombinant p50 (I₄ 527) was kindly provided by Dr. Antero So.

Unless indicated otherwise, the antibodies were diluted as follows for Western blot analyses: goat anti-p125 antibody (C-20, Santa Cruz Biotechnology) at 1:1000; rabbit anti-p50 antibody at 1:1,000; rabbit anti-phosphoserine antibodies (Zymed Laboratories Inc.) at 1:1000; and mouse anti-phosphothreonine antibodies (Zymed Laboratories Inc.) at 1:1000.

Purification of His-tagged PCNA Protein—His-tagged PCNA protein was purified as described previously (35) except that proteins eluted from the Ni-NTA column were dialyzed at 4 °C against buffer containing 20 mM HEPES-KOH, pH 7.5, 1 mM DTT, 100 mM NaCl, and 10% glycerol and charged onto a 1-ml Hi-Trap ion exchange column (Amersham Pharmacia Biotech). PCNA was eluted at about 0.4 M NaCl after applying a gradient of 0.1 M NaCl to 1 M NaCl in the same buffer to the column at 4 °C. Biotinylation of PCNA and recombinant human RP-A (replication protein A) was achieved by using the biotin labeling kit of Roche Molecular Biochemicals according to the manufacturer's instructions.

Induction and Purification of Recombinant p66—The coding sequence of human-p66 was cloned into pET19b as described previously (30), and the recombinant plasmid was transferred into *E. coli* BLR DE3. Transformed cells were incubated at 37 °C with continual shaking in 1 liter of LB medium containing 100 μ g/ml ampicillin and 40 mM glucose. When the cells reached an A_{600} of about 0.3, the cells were resuspended in 1 liter of LB medium containing 100 μ g/ml of ampicillin and 1 mM isopropyl- β -D-thiogalactopyranoside. The culture was incubated for 6 h at 37 °C. After centrifugation, the pellet was resuspended in 50 ml of 50 mM Na₂HPO₄, 100 mM KCl, 0.1% Tween 20, 10 mM β -mercaptoethanol, 10 mM EDTA, 10 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 200 μ g/ml lysozyme, and protease inhibitors. After 20 min on ice, the suspension was sonicated and centrifuged at 19,000 \times g for 10 min at 4 °C, and the pellet was resuspended in 50 ml of buffer A, pH 8.0 (100 mM Na₂HPO₄, 10 mM Tris-HCl, pH 8.0, 1% Triton X-100, 1 mM β -mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitors). The resuspension was agitated gently overnight at 4 °C. After another sonication, the extract was centrifuged at 19,000 \times g for 10 min at 4 °C. The pellet was resuspended in 10 ml of buffer B (100 mM Na₂HPO₄, 10 mM Tris-HCl, pH 8.0, 6 M guanidine HCl, 1 mM β -mercaptoethanol, and protease inhibitors), and the suspension was shaken at 4 °C for at least 6 h. After centrifugation at 19,000 \times g for 10 min at 4 °C, the supernatant containing p66 was collected. Renaturation of p66 was achieved by diluting the p66 suspension dropwise into a 100-fold excess of renaturation buffer with continual stirring (20 mM Tris-HCl, pH 7.5, 200 mM ammonium sulfate, 10 mM magnesium acetate, 1 mM DTT, and 10% glycerol). The protein was further purified by Ni-NTA column chromatography. The column (500 μ l) was equilibrated with 5 ml of renaturation buffer and charged three times with 500 μ g of renaturated p66 protein. After washing the column with 5 ml of column buffer (20 mM Tris-HCl, pH 7.5, 200 mM ammonium sulfate, 10 mM magnesium acetate, 1 mM DTT, 10% glycerol, and 50 mM imidazole, pH 7.5), six fractions of 250 μ l were collected from the column with column buffer containing 450 mM imidazole, pH 7.5, and protease inhibitors.

Preparation of the C-terminal Deleted p66 Protein Derivative—pBlue-script SK+ containing the full-length KIAA0039 cDNA was digested with *Nco*I and *Apa*I, which liberated a fragment of 1324 base pairs whose sequence started just upstream of the first ATG codon of the p66 gene and stopped 80 base pairs before the stop codon. The truncation resulted in the loss of the last 22 amino acids from the C terminus of p66, which comprises the putative PCNA-binding domain. The fragment was subjected to the 3'-5' exonuclease and DNA polymerase activities of T4 DNA polymerase, resulting in a blunt-ended fragment. The fragment was then inserted by blunt end ligation into the blunt-ended *Xho*I site of pET19b, which placed it downstream of the T7 lac promoter and in frame with the His tag coding region. The synthesis of products longer than that encoded by the inserted fragment is prevented in this construct by the presence of a stop codon in each open reading frame on the 3' side of the insertion. *E. coli* BLR DE3 was transformed with the recombinant plasmid by electroporation, and the truncated protein was expressed and purified as described for full-length p66 protein.

Construction of Recombinant Baculovirus Vectors Expressing p125, p66, and p50—The open reading frames of the three subunits of human DNA polymerase δ (GenBankTM) were amplified by polymerase chain reaction from 50 ng of HeLa cDNA using the Klenow polymerase mix (CLONTECH, Palo Alto, CA) and the following primer sets designed from sequences deposited in GenBankTM: p125, 5'-GGGATGGATGGC-AAGCGGCGGCCAGG-3' and 5'-CCATGGGATGCTTGC AAGGTCAC-CAGG-3'; p66, 5'-ACCATGGCGGACCAGCTTTATCTGG-3' and 5'-CC-AAGTCTCTGATCTACCAGAGATGG-3'; and p50, 5'-GCCATGTTTC-

TGAGCAGGCTGCCC-3' and 5'-CAGCCTCCATCTGGGCCTCTC-TGGTC-3'.

The cycling parameters were: 94 °C for 60 s; 36 cycles at 94 °C for 30 s, and 67 °C for 3–5 min; and finally 10 min at 67 °C. The polymerase chain reaction products were cloned into pCR2.1 topo (Invitrogen), the sequences were verified by dideoxy sequencing, excised by *EcoRI* digestion, and subcloned into *EcoRI* sites of the baculovirus transfer vectors pVL1393 or pAcHLT-A (PharMingen) for production of untagged or histidine-tagged recombinant products, respectively. Recombinant baculoviruses were generated by co-transfecting the appropriate baculovirus transfer vector containing the desired gene and *Bsu36I* linearized BapK6 baculovirus DNA into insect *Sf9* cells.

Insect Cell Culture and Purification of Recombinant DNA Polymerase δ —The *Sf9* cell line was maintained as a monolayer culture at 28 °C in TC-100 medium (Life Technologies, Inc.) supplemented with 0.37 g/liter α -ketoglutaric acid, 0.4 g/liter β -D-fructose, 0.055 g/liter fumaric acid, 0.67 g/liter malic acid, 0.06 g/liter succinic acid, 2.7 g/liter sucrose, 0.2 g/liter choline chloride, 0.2 g/liter β -alanine, 0.35 g/liter NaHCO₃, 3.33 g/liter lactalbumin hydrolysate, 100,000 units/liter penicillin, 0.1 g/liter streptomycin, 0.25 mg/liter amphotericin B, and 10% fetal calf serum (Life Technologies, Inc.). The medium was buffered to pH 6.2 using NaOH. The cells were split every 4 days to maintain a density of 10⁵ cells/ml. Recombinant baculoviruses (RBVs) were amplified to 10⁸ plaque-forming units/ml and used for protein expression. *Sf9* cells in exponential phase were layered at a density of 5 × 10⁵ cells/ml and infected simultaneously with different RBVs. For a 175-cm² cell culture, dish cells were infected using 3 ml of RBV-p125, 2 ml of RBV-p66, and/or 1 ml of RBV-p50.

After 2 days of incubation at 28 °C, the cells were harvested and centrifuged at 400 × *g* for 10 min. The pellets were washed twice with ice-cold PBS. The cells were centrifuged and resuspended in 10 volumes of hypotonic buffer (25 mM HEPES-KOH, pH 7.7, 5 mM KCl, and 1.5 mM MgCl₂) and immediately centrifuged at 800 × *g* for 3 min. The cells were resuspended in 5 volumes of hypotonic buffer containing protease inhibitors. The suspension was left on ice for 10 min and lysed with 16 strokes of a Dounce homogenizer. After incubating on ice again for 20 min, the ionic strength was adjusted to 0.4 M NaCl followed by constant mixing for 30 min. The cell extracts were cleared by centrifugation (20,000 × *g* for 30 min). The supernatant was adjusted to 5 mM imidazole, pH 7.7, and applied to a Ni-NTA column (1 ml for 10 175-cm² cell culture dishes) equilibrated beforehand in buffer A (25 mM HEPES-KOH, pH 7.7, 0.4 M NaCl, 1.5 mM MgCl₂, 5 mM imidazole, pH 7.7, and 10% glycerol). After washing with 20 volumes of buffer A, the column was equilibrated with 10 volumes of buffer A containing 75 mM NaCl. The proteins were eluted using buffer A containing 100 mM imidazole, pH 7.7.

ELISAs—Incubations were done at room temperature, and the wells were washed three times between each incubation step with PBS-T unless otherwise indicated. To measure p66 binding to PCNA, a 96-well ELISA plate was coated with 1 μ g of streptavidin in 50 μ l of PBS/well for 1 h. The wells were then saturated by incubating them with 1% BSA in PBS for 1 h. Various concentrations of biotinylated PCNA or biotinylated, recombinant human RP-A were added to 50 ng of p66 in 50 μ l of PBS containing 1% BSA, 0.1% Triton X-100, and 150 mM NaCl, and the mixtures were incubated in the streptavidin-coated wells for 1 h. After washing, mouse monoclonal antibody against p66 diluted 100-fold in PBS-TM was then added to the wells and incubated for 1 h followed by goat anti-mouse IgG peroxidase-conjugated secondary antibody, diluted 2000-fold in PBS-TM for 1 h. After three final washes in PBS-T, the interactions were revealed by adding to each well 100 μ l of 0.4 mg/ml of *o*-phenylenediamine in a buffer containing 0.012% hydrogen peroxide, 0.1 M citric acid, and 0.1 M Na₂HPO₄, pH 5.0. The reaction was stopped with 0.1 M H₂SO₄, and the intensity of the yellow color was measured at A₄₅₀. The values were used as a measure of the interaction between both proteins after correcting for the background values of PCNA- or RP-A-uncoated wells. To demonstrate p21^{Cip1} inhibition of the binding of the C-terminal domain of p66 to PCNA, the wells were coated with a streptavidin-biotinylated p66 peptide complex by adding 50 μ l of 20 μ g/ml streptavidin plus 100 ng of biotinylated p66 peptide in PBS to each well for 1 h. After rinsing the wells once with PBS, the remaining binding capacity of the wells was saturated by filling them with 1% BSA in PBS for 1 h. PCNA (30 ng in 50 μ l of PBS, 150 mM NaCl, 1% BSA, and 0.1% Triton X-100) along with various concentrations of p21 peptide or mutated p21 peptide, were then added to the wells and incubated for another hour. Anti-PCNA monoclonal antibody diluted 1000-fold in PBS-TM was then added to the wells and incubated for 2 h followed by secondary antibody (goat peroxidase-conjugated anti-mouse

IgG) diluted 1000-fold in 50 μ l of PBS-TM for 1 h. The wells were rinsed and processed as described above.

Pull-down Assays—The reaction mixtures contained 200 ng of wild-type p66 along with the indicated amounts of biotinylated PCNA or biotinylated human RP-A in reaction buffer (PBS, 350 mM NaCl, 1% Triton X-100, and protease inhibitors). The reactions were incubated for 2 h at 30 °C. Then 20 μ l of a 50% suspension of streptavidin-agarose beads was added to each tube followed by incubation on a shaker for another 2 h at 30 °C. The tubes were centrifuged at 3,000 × *g* for 2 min, and the pellets were washed three times for 15 min at 37 °C by resuspending and centrifuging them in reaction buffer. After a fourth wash, the beads were resuspended in 20 μ l of loading buffer. The samples were processed for Western blotting with 100-fold diluted anti-p66 monoclonal antibody as described below. For the pull-down assays with the PCNA mutant derivatives, the same amount of each derivative (0.5 μ g) was added to reactions containing 10 μ g of biotinylated p66 peptide in reaction buffer (PBS, 0.1% Triton X-100 and 1 mg/ml BSA). The reactions were processed as described above except that 1000-fold diluted monoclonal anti-PCNA antibody was employed for Western blotting (see below). For pull-down assays of recombinant three- and two-subunit DNA polymerase δ complexes, 20 μ l of a 50% suspension of streptavidin-agarose beads in PBS was added to purified two- and three-subunit preparations of DNA polymerase δ in PBS-T (500 μ l) containing 200 ng of biotinylated PCNA. After 1 h of incubation at room temperature with agitation, the beads were recuperated by centrifugation and washed five times with PBS-T as described above, and the samples were processed for Western blot analysis with anti-p125 antibody as described below.

Kinase Assays—The reactions were performed in 10 μ l of reaction mixture containing kinase buffer (25 mM Tris-HCl, pH 7.5, and 6 mM MgCl₂), 10 μ Ci of [γ -³²P]ATP (3000 Ci/mmol; PerkinElmer Life Sciences), 1 μ M ATP, the indicated amounts of histone H1 or p66 protein, and 1 μ l of cyclin-Cdk complex. The reactions were incubated for 20 min at 37 °C and stopped by adding 3 μ l of 200 mM Tris-HCl, pH 6.8, 8 mM EDTA, 4% (w/v) β -mercaptoethanol, 32% (v/v) glycerol, and 0.1% (w/v) bromophenol blue. The samples were boiled and then subjected to 12.5% SDS-PAGE. After drying, radioactive bands on the gel were revealed by autoradiography of the gel with Fuji medical x-ray film.

Immunofluorescence Microscopy—All operations were carried out at room temperature unless indicated otherwise. HeLa cells were grown on the surfaces of coverslips in 6-well culture plates containing Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 μ g/ml penicillin, 100 units/ml streptomycin, and 1% nonessential amino acids (Life Technologies, Inc.) at 37 °C under a humidified atmosphere containing 5% CO₂. Exponentially growing cells were washed twice in PBS and fixed for 10 min in PBS containing 2% formaldehyde. The cells were then permeabilized in PBS containing 0.2% Triton X-100 for 5 min at 4 °C, washed twice in PBS, and fixed for 20 min in methanol at -20 °C. After two washes in PBS, the cells were incubated with 100 μ l of appropriate dilutions of the one or both of the following antibodies diluted in PBS containing 5% BSA for 1 h: PCNA-fluorescein isothiocyanate-conjugated monoclonal antibody (Ab-1, Oncogene) diluted 100-fold, p66 monoclonal antibody (2C11) diluted 100-fold, monoclonal PCNA antibody (PC10, Roche Molecular Biochemicals) diluted 600-fold, and goat polyclonal antibody against the large subunit of DNA polymerase δ diluted 100-fold. The coverslips were washed three times in PBS-T. Depending on the first antibody, the cells were then incubated as for the first antibody with either one or both of the following secondary antibodies; fluorescein isothiocyanate- or rhodamine-conjugated Affinipure goat anti-mouse IgG diluted 100-fold (Jackson ImmunoResearch) or rhodamine-conjugated donkey anti-goat IgG (Santa Cruz Biotechnology). After three washes in PBS, the coverslips were incubated in 1 μ g/ml of 4,6-diamidino-2-phenylindole in PBS for 5 min. Finally, after two washes in PBS, coverslips were placed face down on a drop (2 μ l) of 100 mg/ml of DABCO in PBS and 50% glycerol and sealed with nail polish. Fluorescent images were captured through a three-dimensional image capture system on a DMRXA LEICA microscope using Metamorph software (Universal Imaging). The images were deconvoluted using software developed by Sibarita and DeMey.²

Co-immunoprecipitation Assays—200 μ l (700 μ g of protein) of partially purified mouse FM3A cell extract (phosphocellulose 0.4 M fraction) (30) was added to 160 μ l of PBS containing 40 μ l of the supernatant of the hybridoma clone 2C11 expressing p66 monoclonal

² J. B. Sibarita and J. R. DeMey, manuscript in preparation.

antibodies. The reactions were incubated on ice for 30 min, and immunocomplexes were precipitated by adding 20 μ l of a 50% suspension of protein G-Sepharose beads (Amersham Pharmacia Biotech) and centrifuging at $12,000 \times g$ for 2 min. After four washes with PBS, the samples were processed for Western blotting with monoclonal anti-p66 and anti-p125 antibodies as described below.

Western Blot Analysis—Samples along with prestained protein standards (Bio-Rad) were boiled in loading buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol, 100 mM DTT) and subjected to 12.5% SDS-PAGE. After transfer to 0.45- μ m nitrocellulose membranes, the membranes were blocked with PBS-TM for 30 min. The blots were then incubated with the appropriate antibody diluted in PBS-TM at 4 $^{\circ}$ C overnight. The blots were washed four times with PBS-T for 5 min each, followed by incubation for 1 h with secondary antibody, which was either horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (Jackson Immunoresearch Laboratories) or donkey anti-goat IgG (Santa Cruz) diluted 1:2000 in PBS-TM. For anti-phosphoserine, anti-phosphothreonine, and anti-phosphotyrosine antibodies, PBS-T containing 3% BSA was employed for blocking and antibody dilution. The blots were washed four times in PBS-T for 5 min each, and the membranes were incubated with the chemiluminescence reagent of PerkinElmer Life Sciences and exposed to Fuji medical x-ray film.

Far Western Blot Analysis—Proteins were transferred to 0.45- μ m nitrocellulose membranes from a SDS-12.5% polyacrylamide gel. The membrane was incubated in BLOTTO (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM KCl, 5% fat-free milk, 1% BSA, 0.05% Tween 20) for 1 h at room temperature. The membrane was then incubated overnight at 4 $^{\circ}$ C in BLOTTO containing 1 μ g/ml of PCNA with constant agitation. After three rinses with BLOTTO, the membrane was incubated with monoclonal PCNA antibody (diluted 1:600) with or without the p21 or mutated p21 peptides in BLOTTO for 3 h at room temperature. The membrane was washed three times with BLOTTO and incubated for 1 h with horseradish peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, diluted 1:2000) and further processed as described above under Western blotting.

In Vitro DNA Synthesis—DNA replication assays were performed as described previously (35). The template (0.25 μ g of DNA or 0.83 nmol of nucleotide/reaction) was poly(dA)_{–300} annealed to oligo(dT)_{12–18} (Amersham Pharmacia Biotech) at a template-primer base ratio of \sim 10:1. Nucleotide was [*methyl*-³H]thymidine 5' triphosphate (Amersham Pharmacia Biotech) at 0.5 μ Ci/reaction (0.38 mmol/Ci, 200 cpm/pmol thymidine 5' triphosphate), corresponding to a final concentration of 10 μ M. The reactions were incubated at 37 $^{\circ}$ C for 60 min and processed for counting as described previously (35).

RESULTS

Recombinant p66 Protein Binds to PCNA—Previously, we showed that mammalian DNA polymerase δ complexes containing at least four subunits, a large catalytic subunit of 125 kDa, a small subunit of 48–50 kDa, and two novel components of 66 and 38 kDa, can be isolated by PCNA affinity chromatography (30). By immunoblot analyses, the 38-kDa component was shown to be either the 38- or the 40-kDa subunit of replication factor C or both. The 66-kDa component was identified as the product of the Kazusa cDNA clone KIAA0039. RNA 5'-extension analysis showed that the cDNA was complete, although potentially it would code for a protein of 51.5 kDa. Retarded mobility on the SDS-polyacrylamide gel, caused by the high charge/mass ratio of p66, was suggested to explain the discrepancy between the observed and theoretical molecular masses. The translated KIAA0039 cDNA sequence contains a very good match to the extreme N-terminal and C-terminal PCNA-binding sites found in a wide range of other PCNA-binding proteins, including the third subunits of DNA polymerase δ from yeast, Cdc27 and Pol32 (30) (Fig. 1A). This observation as well as the association of this protein with active DNA polymerase δ complexes from mouse (30), calf thymus (31), and human (32) has led to the suggestion that p66 may be the third subunit of DNA polymerase δ . To test this hypothesis, we first of all purified recombinant human p66 protein from *E. coli* (Fig. 1B) and tested its ability to bind to purified PCNA (Fig. 1, C and D). His-tagged p66 protein was expressed as an

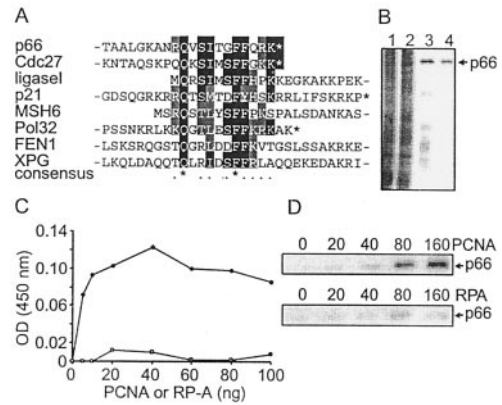


FIG. 1. Recombinant p66 protein binds to PCNA. A, alignment of canonical PCNA-binding domains of known PCNA-binding proteins, including that of p66 (18). B, silver-stained SDS-polyacrylamide gel of different purified fractions of p66 overexpressed in *E. coli*. Lanes 1 and 2, noninduced and induced *E. coli* extracts (3 μ g); lane 3, insoluble pellet (0.5 μ g) resuspended in 6 M guanidine HCl; lane 4, eluted nickel column fraction (0.5 μ g). C, ELISA of p66 binding to PCNA. A constant amount of recombinant p66 protein (50 ng) was added to streptavidin-coated wells containing increasing amounts (ng) of bound, biotinylated PCNA (closed circles) or biotinylated human RP-A (open squares) (purified as described in Ref. 36). The reactions were processed as described under "Materials and Methods." The absorbance at 450 nm indicates the level of p66 binding to the wells. D, pull-down assays of p66 binding to biotinylated PCNA or biotinylated human RP-A. The assays were performed by incubating constant amounts of recombinant p66 (200 ng) to reactions containing increasing amounts (in ng) of either biotinylated PCNA (upper panel) or biotinylated human RP-A (lower panel) bound to streptavidin beads. Immunoblots of the streptavidin-bound fractions were probed with anti-p66 monoclonal antibodies.

insoluble aggregate after 6 h of induction in *E. coli*. The insoluble pellet was resolubilized in 6 M guanidine HCl, rapidly diluted into guanidine HCl free buffer to achieve renaturation, and then further purified by Ni-NTA chromatography (see "Materials and Methods") (Fig. 1B). The binding between PCNA and p66 was tested using modified ELISAs. Basically, increasing amounts of biotinylated human PCNA or biotinylated human RP-A as a control were bound to the bottom of a streptavidin-coated 96-well ELISA plate, and constant amounts of recombinant p66 protein were added to the wells. Fig. 1C shows that p66 binding to the well was dependent on the amount of prebound PCNA but not on the amount of prebound RP-A. Likewise, in pull-down assays with streptavidin-coated beads, the amount of p66 pulled down by the streptavidin-coated beads depended on the amount of biotinylated-PCNA protein added to the reactions. Only very little p66 was pulled down in the absence of PCNA or in the presence of RP-A (Fig. 1D). These results indicate that p66 binds specifically to PCNA.

The Extreme C-terminal Region of p66 Mediates PCNA Binding—The extreme C-terminal region of p66 contains a consensus PCNA-binding domain (Fig. 1A). To determine whether this domain contributes to PCNA binding by p66, we deleted the entire C-terminal region starting at amino acid 444, effectively removing the 22-amino acid region containing the putative PCNA-binding domain. In Fig. 2A, increasing amounts of p66 and the deleted p66 derivative were transferred to a nitrocellulose membrane for blotting with anti-p66 monoclonal antibody (upper panel) or anti-PCNA monoclonal antibody (lower panel) after incubation of the blot with PCNA (far Western blotting). The results demonstrate that PCNA bound only very weakly to the p66 deletion derivative lacking the PCNA-binding domain. Similarly, using the same ELISAs as described above, the deleted p66 derivative showed markedly reduced affinity for PCNA (Fig. 2B). These results indicate that the C-terminal 22-amino acid region containing the PCNA-binding

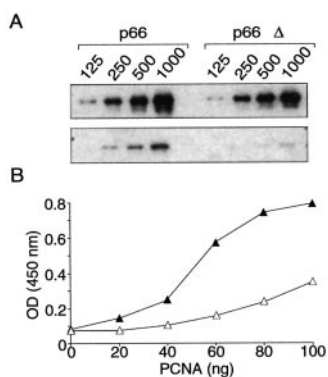


FIG. 2. p66 binding to PCNA depends on its C-terminal region. A, Western blots. Increasing amounts (in ng) of recombinant wild-type or C-terminal deleted p66 protein (p66 Δ) were transferred to nitrocellulose membranes and probed either with anti-p66 monoclonal antibody (upper panel) or anti-PCNA monoclonal antibody after incubation of the membrane with PCNA (1 μ g/ml). B, ELISAs of the binding of wild-type (closed triangles) and C-terminal deleted p66 (open triangles) (50 ng) to increasing amounts of biotinylated PCNA immobilized on the bottoms of 96-well ELISA plates. The absorbance at 450 nm indicates the level of p66 binding to the wells.

domain is critical for p66 binding to PCNA.

Affinity of the C-terminal Region of p66 for PCNA Derivatives with Mutations in Exposed Loop Regions—Having established that binding to PCNA requires the C-terminal region of p66, we analyzed the regions on PCNA that are important for p66 binding. Pull-down assays were used to measure the affinity of a biotinylated peptide, containing the extreme C-terminal 22-amino acid region of p66, for purified PCNA derivatives containing alanine substitutions within the loop regions on the surface of the PCNA trimer (for a detailed description of these mutants see Ref. 18). The QLGI125 mutant showed severely reduced binding affinity for the p66 peptide in the pull-down assays (Fig. 3A). The mutant contains alanine substitutions in residues Gln¹²⁵, Leu¹²⁶, Gly¹²⁷, and Ile¹²⁸ located within the middle of the domain-interconnecting loop. The region has previously been suggested to form part of the binding site for DNA polymerase δ on the basis of experiments showing that PCNA stimulation of DNA polymerase δ is inhibited by monoclonal antibodies that recognize residues 121–135 (37). This region is also the site of the pol30–79 mutation (I126A,I128A) in *S. cerevisiae* and that of other mutations (Val¹²³, Leu¹²⁶, Gly¹²⁷, and Ile¹²⁸) affecting DNA polymerase δ stimulation by PCNA (38, 39). The remaining three mutants showed nearly normal affinity for the p66 peptide, with the exception of LAPK251, which contains a triple mutation in the C-terminal region. However, this mutant has been suggested to aggregate (18), indicating that the weak interaction with the p66 peptide may reflect the inaccessibility of PCNA within the aggregate rather than any direct block to p66 binding. The results of the binding assays agree with the relative activities of the three PCNA mutants in the DNA replication assay shown in Fig. 3B and with the results of previous replication assays using the same mutants (18). Nearly normal binding of the p66 peptide to SHV43 and VDK188, which contain mutations within the hydrophobic pocket located on the C-side of the trimer and on the prominent loop located on the rear side of the trimer, respectively, suggests that these regions are not critical for p66 binding to PCNA.

The amino acids Gln¹²⁵ and Gly¹²⁷ that were targeted in the QLGI125 mutant are also involved in p21^{Cip1} binding to PCNA (20). Moreover, only the QLGI125 mutant showed a marked loss of binding for p21^{Cip1} in band shift assays on native polyacrylamide gels (18). Thus, p66 binding to PCNA mimics that of native DNA polymerase δ and the region involved is also im-

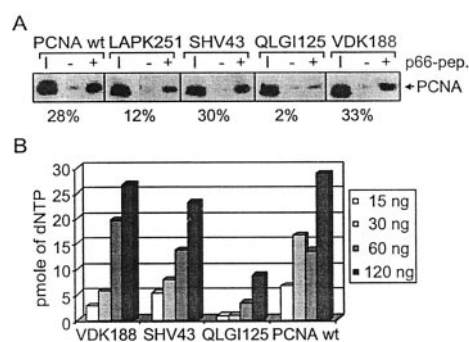


FIG. 3. Replication and binding activities of wild-type and mutant PCNA for the PCNA-binding domain of p66. A, pull-down assays of the binding of wild-type and mutant PCNA to the 22-amino acid C-terminal region of p66. A PCNA immunoblot of the streptavidin-bound fractions from pull-down assays of biotinylated p66 peptide (10 μ g) binding to wild-type and mutant PCNA (0.5 μ g each) (see text for details). The numbers below the lanes indicate the percentages of bound PCNA to total input PCNA (|). Lane |, one fifth (100 ng) of the input amount of PCNA; lane -, without the p66 peptide; lane +, with the p66 peptide. B, DNA replication activity of the PCNA mutants used in the pull-down assays.

portant for p21^{Cip1} binding. This suggests that the PCNA-binding domain of p66 must be important for PCNA-dependent DNA synthesis by DNA polymerase δ and that competition between p21^{Cip1} and p66 for the same binding site on PCNA underlies p21^{Cip1} inhibition of DNA polymerase δ .

p21^{Cip1} Inhibits p66 Binding to PCNA and DNA Synthesis by Native DNA Polymerase δ —The extreme C terminus of p21^{Cip1} contains the PCNA-binding domain that has been shown to compete for DNA polymerase δ binding to PCNA and inhibit DNA synthesis by DNA polymerase δ *in vitro* (18). In Fig. 4, we show that a peptide of 22 amino acids containing the PCNA-binding domain of p21^{Cip1} inhibits both p66 peptide binding to PCNA and DNA synthesis by a mouse DNA polymerase δ fraction containing p66 (30) (Fig. 4B, inset). A similar peptide (mutant p21 peptide) containing the entire C-terminal region of p21^{Cip1} but with a substitution of isoleucine for alanine within the consensus PCNA-binding domain was ineffective, confirming previous results that this amino acid is critical for p21^{Cip1} function (40). Competition between the PCNA-binding sites of p21^{Cip1} and p66 for binding to PCNA comforts the conjecture that p21^{Cip1} and p66 recognize the same binding site on PCNA, the middle of the domain interconnecting loop, and that this competition causes inhibition of PCNA-dependent DNA polymerase δ activity.

PCNA-dependent DNA Synthesis by Reconstituted Human DNA Polymerase δ Requires p66—Human DNA polymerase δ was reconstituted by co-expressing recombinant p125, p66, and p48–50 subunits in the baculovirus system (see “Materials and Methods”). The p48–50 subunit was tagged with six histidine residues on its N-terminal side to facilitate purification of the reconstituted DNA polymerase δ complex by nickel-agarose chromatography. Immunoblot analysis of the eluted fractions from the Ni-NTA column showed that all three subunits were eluted together, suggesting that the three subunits formed a complex when co-expressed in Sf9 insect cells (Fig. 5A, inset). Far Western analysis of the reconstituted complexes (Fig. 5A) showed that only p66 interacted with PCNA. As with the C-terminal p66 peptide described above, the interaction between recombinant p66 and PCNA was inhibited by the wild-type p21^{Cip1} peptide but not by the p21^{Cip1} peptide containing a mutation within the PCNA-binding domain. The failure to observe an interaction between PCNA and p125 subunit could be attributed to either the disproportional low amount of p125 in the complex (data not shown) or to poor renaturation of p125

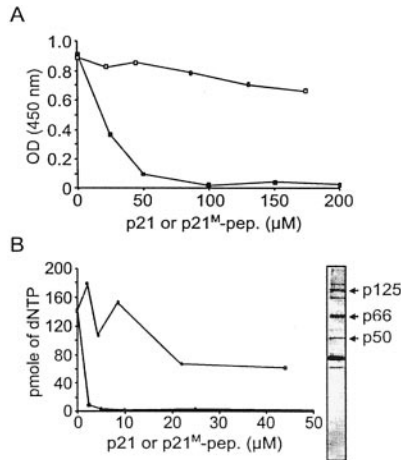


FIG. 4. The C-terminal region of p21^{Cip1} inhibits p66 peptide binding to PCNA and PCNA-dependent DNA synthesis by DNA polymerase δ *in vitro*. *A*, ELISAs of the effect of p21 (closed squares) and mutant p21 (open squares) (p21^M) peptides on p66 peptide binding to PCNA. Each assay contained 0.1 μ g of immobilized biotinylated p66 peptide and 30 ng of PCNA. The absorbance at 450 nm indicates the level of PCNA binding to the wells. *B*, *in vitro* replication assays of DNA synthesis by native three-subunit DNA polymerase δ in the presence of the p21 (closed squares) or mutant p21 (open squares) peptide. Replication assays were done as described under "Materials and Methods." *Inset*, silver-stained lane of an SDS-polyacrylamide gel of the PCNA-purified DNA polymerase δ complex used in the replication assays.

on the membrane filter. To circumvent these problems, we did pull-down assays by adding biotinylated PCNA-coated streptavidin beads to fractions containing either the three subunit complex or the two subunit complex lacking p66 (Fig. 5B). The p125 subunit was only pulled down when fractions containing the three-subunit complex were employed. We failed to detect the p125 band in pull-down assays with the two-subunit complex. These results suggest that the p66 mainly mediates the interaction between p125 and PCNA.

In Fig. 5C we confirm these findings by showing that PCNA-dependent DNA synthesis is only observed with the three-subunit DNA polymerase δ complex containing p66. The presence of p66 stimulated DNA synthesis by about 3–4-fold in the presence but not in the absence of PCNA. This synthesis was completely abolished by the p21^{Cip1} peptide but not by the mutated p21^{Cip1} peptide. This indicates that PCNA-dependent DNA synthesis by DNA polymerase δ relies on the PCNA-binding domain of p66. Also, these results further stress the importance of p66 for observing the inhibitory action of p21^{Cip1}. On the other hand, basal DNA synthesis, which required the two-subunit complex (data not shown), was neither stimulated by PCNA nor inhibited by the p21^{Cip1} peptide, a result that argues against the presence of a functional PCNA-binding domain in the large subunit. The result agrees with data showing PCNA-independent DNA synthesis by the recombinant large subunit alone (35, 41) but is at variance with results showing that the recombinant two-subunit complex depends on PCNA for activity (29).

Phosphorylation of p66—During the purification of DNA polymerase δ complexes from mouse cells we noticed that the p66 band on Western blots interacted with antibodies directed against phosphorylated threonine residues but not with antibodies directed against phosphorylated serine (Fig. 6A, compare lane 2 in middle and bottom panels). As expected the recombinant p66 protein expressed in *E. coli* failed to interact with either antibody (Fig. 6A, compare lane 1 in middle and bottom panels). To test whether p66 is a substrate for Cdk phosphorylation, purified p66 expressed in *E. coli* or histone H1 was incubated with purified complexes of CycE-Cdk2, CycA-

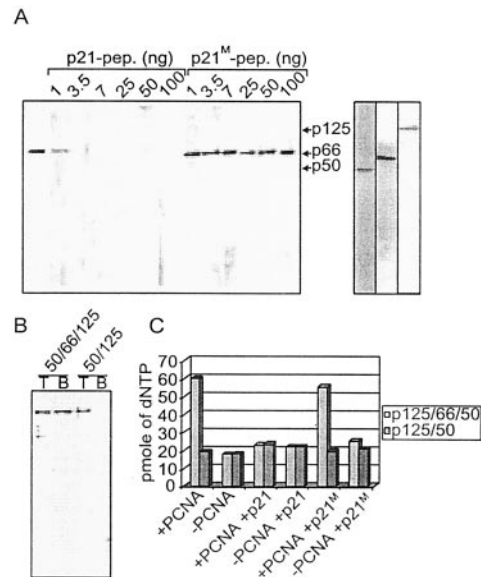


FIG. 5. p66 protein is essential for the PCNA-binding and PCNA-dependent DNA synthesis activities of human DNA polymerase δ . Recombinant three-subunit DNA polymerase δ was partially purified as described under "Materials and Methods." *A*, partially purified DNA polymerase δ , obtained from four 175-cm² flasks of insect cells infected with baculoviruses expressing the three subunits of DNA polymerase δ , was loaded across the entire width of a 12.5% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was cut into 13 equal strips. Each strip was incubated with 1 μ g/ml of PCNA and different amounts of p21 (p21-pep.) or mutant p21 (p21^M-pep.) peptides. PCNA binding to the blot was revealed using PCNA monoclonal antibody PC10 antibody. *Inset*, Western blotting analysis of the Ni-NTA column fraction used in the far Western blots. The presence of the three subunits of DNA polymerase δ was revealed by incubating the blot successively with monoclonal antibody against p66 and polyclonal antibodies against p50 and p125 as indicated. *B*, pull-down assays of the binding of the large subunit of DNA polymerase δ to PCNA in the presence and absence of p66. Partially purified two-subunit or three-subunit DNA polymerase δ was incubated with biotinylated PCNA (200 ng), and bound materials were pulled down with streptavidin-coated beads as described under "Materials and Methods." *Lanes T*, one-tenth of the total amount of purified fraction added to the assays; *lanes B*, all of the eluted material from the streptavidin beads. The immunoblot was probed with polyclonal p125 antibody (see "Materials and Methods"). *C*, *in vitro* DNA replication assays of partially purified two-subunit and three-subunit DNA polymerase δ . Ni-NTA column fractions were dialyzed against DNA polymerase assay buffer, and 2 μ l of either the two-subunit or three-subunit DNA polymerase δ fraction was added to each assay along with 0.1 μ g of PCNA where indicated (see "Materials and Methods"). When present, p21 or mutant p21 (p21^M) peptide was added at 3.5 ng/assay.

Cdk1, or CycA-Cdk2 in the presence of [γ -³²P]ATP, and the reaction products were run on a 12% SDS-polyacrylamide gel. Autoradiography of the dried gel showed that p66 protein was phosphorylated under these conditions irrespective of the nature of the Cdk-cyclin complex employed. These results indicate that p66 is a substrate for Cdk phosphorylation, at least *in vitro*. To determine whether PCNA binding had any effect on p66 phosphorylation by CycE-Cdk2, increasing concentrations of PCNA were added to the p66 or histone phosphorylation reactions. Fig. 6C shows that PCNA inhibited the phosphorylation of p66 at lower concentrations than those that inhibited histone H1 phosphorylation, demonstrating that PCNA interferes with the CycE-Cdk2 phosphorylation of p66. Similar results were obtained with the other cyclin-dependent Cdk complexes (data not shown). Conversely, however, phosphorylation did not block p66 binding to PCNA (data not shown). In accordance with previous results, PCNA was not phosphorylated under these conditions (Fig. 6C) (42).

p66 Monoclonal Antibodies Co-immunoprecipitate p66 and

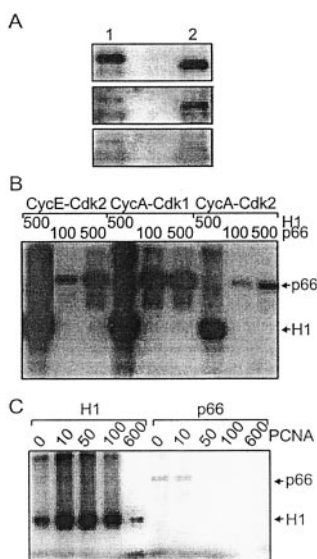


FIG. 6. Phosphorylation of p66. *A*, immunoblots of recombinant and native p66 protein probed with monoclonal p66 antibody and antibodies against various phosphorylated amino acids. *Top panel*, recombinant p66 (75 ng) from *E. coli* (lane 1) and native p66 (lane 2) purified as part of the mouse DNA polymerase δ complex (see *inset* in Fig. 4*B*) were probed with p66 monoclonal antibody. *Middle panel*, the same blot probed with anti-phosphothreonine antibody. *Bottom panel*, the same blot probed with anti-phosphoserine antibody. *B*, phosphorylation of recombinant p66 protein (*E. coli*) and histone H1 by cyclin-Cdk complexes (CycA-Cdk1, CycA-Cdk2, and CycE-Cdk2). The numbers above the lanes indicate the amount of p66 protein (in ng) or histone H1 added to the assays. See "Materials and Methods" for details. *C*, PCNA inhibits p66 phosphorylation by CycE-Cdk2. The indicated amounts (in ng) of PCNA were added to the standard phosphorylation assay.

the p125 Subunit of DNA Polymerase δ —To demonstrate unequivocally that p66 is part of native DNA polymerase δ , we attempted to co-immunoprecipitate the p125 subunit of DNA polymerase δ by incubating a 0.4 M phosphocellulose fraction from mouse cells, which was shown previously to be enriched in DNA polymerase δ (30), with monoclonal antibodies against p66. Fig. 7 shows that the p125 was effectively co-immunoprecipitated with p66 under these conditions, demonstrating that both proteins form a complex *in vivo*. However, it should be noted that only about 10% of the amount of p125 in the extract was immunoprecipitated, whereas over 50% of p66 was precipitated under the same conditions (data not shown; compare the intensity of the p125 band in the supernatant (one-tenth of input) and precipitated fractions in Fig. 7), perhaps indicating that p125 and p66 exist as free entities or form complexes with other proteins *in vivo*.

Co-localization of p66 with PCNA and p125 within Nuclear Structures Resembling S Phase DNA Replication Foci—PCNA has been shown to form discrete foci within the nucleus during the S phase of the cell cycle. These foci have been shown to correspond to active centers of DNA replication (43, 44). Other proteins directly involved in DNA replication or post-replicative events, including the single-stranded DNA-binding protein RP-A, DNA ligase 1, deoxycytosine methyltransferase, and chromatin assembly factor 1 also co-localize with PCNA to these replication foci, also referred to as "replication factories" (44). In a growing number of instances, co-localization of certain of these proteins has been shown to depend on the presence of a consensus PCNA-binding motif in their extreme C-terminal or N-terminal regions, leading to the interpretation that PCNA, besides its direct role in DNA synthesis, also functions to localize proteins to the replication factories where they are required either during or after the replication process (45). We were thus interested in seeing whether p66 would also be

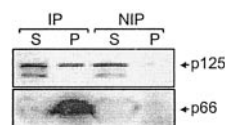


FIG. 7. The large subunit of native three-subunit DNA polymerase δ co-immunoprecipitates with p66. Co-immunoprecipitation assays were performed as described under "Materials and Methods." *IP*, extracts were incubated with protein G-Sepharose beads and anti-p66 monoclonal antibody. *NIP*, extracts were incubated with protein G-Sepharose beads alone. *Lanes S*, one-tenth of the extract added to the assays; *lanes P*, all of the protein G-Sepharose-bound material. Failure to observe p66 in the *NIP* supernatant(s) was due to occlusion of the p66 band by the large amount of bovine serum albumin in the p66 hybridoma supernatants, which migrates to nearly the same position as p66 during SDS-PAGE electrophoresis.

co-localized to these replication factories. The histochemical immunolocalization studies shown in Fig. 8 show this to be effectively the case. In the *two upper rows of panels*, fluorescein isothiocyanate-conjugated monoclonal antibodies against PCNA reveal the typical pattern of PCNA localization within nuclei of HeLa cells. As expected, most, but not all, of these foci co-localized with the large subunit of DNA polymerase δ (Fig. 8, *top panels*), confirming that PCNA and polymerase δ act together to accomplish DNA replication in these cells. Monoclonal antibodies against p66 monoclonal also gave a punctuate pattern within nuclei (Fig. 8, *middle and bottom panels*); however, only a limited number of these foci co-localized with PCNA and p125 foci (Fig. 8, *middle and bottom panels*). Although these results demonstrate that p66 is part of a limited number of replication foci, the results also suggest that p66 may be engaged in other activities occurring in the absence of PCNA or p125. This will be discussed in more detail below.

DISCUSSION

Taken together, the findings presented here strongly suggest that p66 is the subunit that bridges the catalytic activity of DNA polymerase δ to PCNA for processive DNA synthesis at the replication fork in human cells. As expected from the presence of a canonical PCNA-binding domain present in the C terminus of p66, the subunit interacted with PCNA, and the interaction was almost entirely dependent on the PCNA-binding domain (Figs. 1 and 2). From this point of view, p66 strongly resembles the third subunit of *S. pombe* DNA polymerase δ , Cdc27, whose binding to PCNA relies on a PCNA-binding domain located in its C terminus, a region that shows significant homology to the PCNA-binding region of p66 (23). The interaction between the C-terminal region of p66 and PCNA was compromised by the same mutations within the domain-interconnecting loop region that compromised DNA synthesis by three-subunit DNA polymerase δ *in vitro* (Fig. 3) (18). So far, only proteins containing the canonical PCNA-binding domain, such as Fen1 and p21^{Cip1}, have been shown to depend on this region for their interaction with PCNA (46). *In vitro* results obtained with two *S. pombe* thermosensitive p125 mutants showed that only a limited decrease in PCNA stimulation at the restrictive temperature, probably because of overall modification of the DNA polymerase δ complex (47). Because neither p125 nor p50 contain a canonical PCNA-binding domain, these results suggest that p66 is the main mediator of PCNA-dependent synthesis by DNA polymerase δ . These conclusions were further substantiated and confirmed by experiments employing the C-terminal domain of p21^{Cip1} and reconstituted DNA polymerase δ . As expected the PCNA-binding domain of p21^{Cip1} competed with p66 binding to PCNA and inhibited DNA replication by the native three-subunit DNA polymerase δ *in vitro* (Fig. 4). Thus, a major target for the inhibitory action of p21^{Cip1} on DNA synthesis would be the

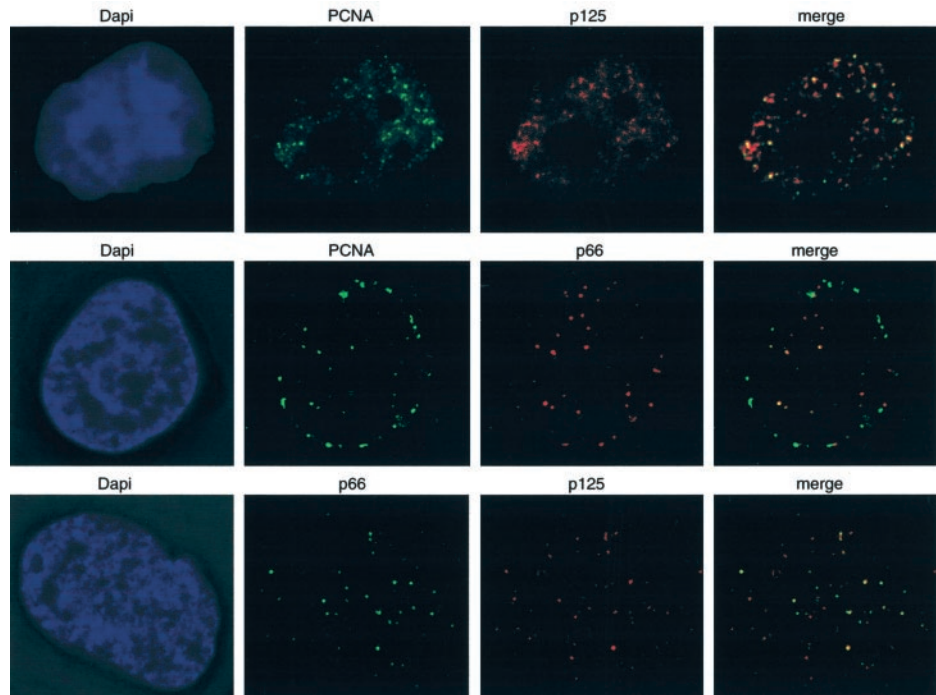


FIG. 8. Immunolocalization of p66 with PCNA and p125 within the replication foci of HeLa cells. *Top row*, nuclear immunolocalization of PCNA and p125. *Middle row*, nuclear immunolocalization of PCNA and p66. *Bottom row*, nuclear immunolocalization of p66 and p125. *Left-hand side frames*, 4,6-diamidino-2-phenylindole staining. For details about the experimental procedures and the identity of the various antibodies employed, see "Materials and Methods."

interaction between p66 and PCNA. The reconstitution experiments confirm this conjecture. PCNA-dependent synthesis was only observed with the three-subunit complex, and only the PCNA-dependent replication of the three-subunit complex could be inhibited by the p21^{Cip1} peptide. We could neither stimulate the two-subunit complex with PCNA nor inhibit its activity with the p21^{Cip1} peptide (Fig. 5C). Furthermore, only recombinant p66 on far Western blots interacted with PCNA (Fig. 5A) and p66 was absolutely required to obtain pull-down of the catalytic subunit (p125) using biotinylated PCNA-coated streptavidin-agarose beads (Fig. 5B). Although these results further stress the fundamental importance of p66 for observing PCNA-dependent DNA synthesis by DNA polymerase δ , they contradict previous results obtained by other investigators using native and reconstituted DNA polymerase δ . Prominent among these is the observation that native DNA polymerase δ has been consistently published as a two-subunit complex whose activity depends on PCNA. However, as recently shown for purified calf thymus DNA polymerase δ (31), these complexes may have contained p66 or small proteolytic fragments of p66 that may not necessarily have been visible or recognizable even on silver-stained SDS-polyacrylamide gels. Our failure to observe PCNA stimulation of the two subunit complex also seems to be in conflict with previous results showing that PCNA stimulates the activity of a two-subunit complex reconstituted from recombinant baculovirus infected insect cells but agrees with data showing that PCNA is not required to activate the large subunit alone (35, 41). Our results are also in accord with those of Shikata *et al.* (32), who recently reported the successful reconstitution and purification of an active three-subunit but not an active two-subunit DNA polymerase δ . These discrepancies may reflect differences under "Experimental Procedures" or contamination of recombinant enzymes with insect proteins that functionally complement one or more of the subunits of human DNA polymerase δ .

Arguing for the existence of at least one more PCNA-binding site in human DNA polymerase δ , DNA polymerase δ from *S. cerevisiae* and *S. pombe* can be reconstituted as active two- or three-subunit complexes (21, 24). The two-subunit complexes lacking Pol32 or Cdc27 depend on PCNA for their activity,

although much higher amounts of PCNA are required compared with three-subunit complexes (21, 24). Thus, yeast DNA polymerases must possess alternative PCNA-binding domain(s) distinct from those found in the extreme C termini of Pol32 and Cdc27. Indeed, the *pol32* gene of *S. cerevisiae* is not essential (25). An answer as to whether human DNA polymerase δ relies solely on p66 for interaction with PCNA might be obtained by studying the activity of reconstituted DNA polymerase δ complexes containing p66 derivatives mutated for the PCNA-binding domain.

These considerations have important implications for nucleotide excision repair carried out by DNA polymerase δ . Although this DNA repair is widely accepted to be dependent on PCNA, the effect of p21^{Cip1} on the resynthesis step has been controversial. Some laboratories have reported almost no effect (15), whereas others have equal or nearly equal (48, 49) inhibition of replicative DNA synthesis and repair synthesis by p21^{Cip1}. Our results showing p21^{Cip1} inhibition of p66 binding to PCNA and specific inhibition of PCNA-dependent DNA synthesis dependent on p66 (Fig. 5C) would seem to argue that p21^{Cip1} inhibits both repair and replicative DNA synthesis equally well by abrogating p66 binding to PCNA. However, the situation may be more complicated. Only very few assays of p21^{Cip1} inhibitory activity have employed purified DNA polymerase δ and PCNA preparations. Most assays have been done in crude cell extracts, *in vivo*, or in reconstituted systems containing many other factors that have been shown to interact with PCNA and whose activities could be potentially inhibited by p21^{Cip1}. Thus, the differential effects of p21^{Cip1} on excision repair synthesis may reflect the varying amounts of these factors in the assays and not the presence of multiple sites with different affinities for PCNA on DNA polymerase δ .

Figs. 7 and 8 show that p66 is an integral part of DNA polymerase δ and co-localizes with PCNA and the catalytic subunit of DNA polymerase δ within replication foci. Surprisingly, however, most p66 foci did not co-localize with PCNA or DNA polymerase δ . Also, not all PCNA or p125 foci co-localized with p66. Although the latter may have been due to the stringent conditions used to fix the cells, which might have resulted in the loss of loosely associated p66 from the replication foci,

the former might suggest that p66 plays other roles in S phase nuclei. In agreement with this interpretation, there exists mounting evidence to suggest that the functional homologue of p66, Pol32, plays other important roles in DNA repair and perhaps in other events during S phase. In *S. cerevisiae*, Pol32 has been shown to be involved in the RAD6/REV3 pathway for UV radiation-induced mutagenesis where it may facilitate coupling of SRS2 helicase activity to DNA synthesis by DNA polymerase δ (50). Also, Pol32 is required for coupling DNA synthesis by DNA polymerase δ to WRN helicase unwinding of tetraduplex and hairpin structures (51). Indeed, WRN has been shown to interact directly with DNA polymerase δ (52). Implication of *pol32* in DNA helicase activity is reminiscent of the τ subunit of DNA polymerase III of *E. coli*, which plays the dual role of bridging the catalytic core of the functional equivalent of PCNA, subunit β , to DnaB, the replicative DNA helicase (53). The p66 protein may play a similar dual role in mammalian cells.

p66 protein isolated from mouse cells interacted specifically with anti-phosphothreonine antibodies, suggesting that it is phosphorylated *in vivo* (Fig. 6A). *In vitro* phosphorylation assays also showed that it was a substrate for phosphorylation by Cdk1 and Cdk2 kinases (Fig. 6B). However, there exists only one possible Cdk phosphorylation site in p66 located within the sequence ²⁷³KLATPAGL²⁸¹, although the sequence is not conserved in other putative p66 homologues from other species (data not shown). The *in vitro* phosphorylation of p66 was inhibited by PCNA (Fig. 6C), suggesting that p66 may be maintained in an unphosphorylated state when complexed to PCNA. The unphosphorylated state of DNA ligase I was also shown to depend on the PCNA-binding site located within the sequence that targets DNA ligase I to replication foci during S phase. Dephosphorylation was suggested to be due to PCNA occlusion of the phosphorylated serine residue, which lies close to the PCNA-binding domain, and to be important for the establishment of the prereplicative form of DNA ligase I (54). A similar mechanism might control p66 activity and/or modulate p66 targeting to replication complexes. The nature of the eventual role of p66 phosphorylation might be forthcoming from experiments designed to identify the residue(s) involved, the phosphorylated state of p66 during the cell cycle, and the relative activities of reconstituted DNA polymerase δ complexes containing phosphorylated or unphosphorylated p66.

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