The small subunit is required for functional interaction of DNA polymerase δ with the proliferating cell nuclear antigen

Jin-Qiu Zhou¹, Hua He¹, Cheng-Keat Tan², Kathleen M. Downey¹,² and Antero G. So¹,², *

Departments of ¹Biochemistry and Molecular Biology and ²Medicine, University of Miami School of Medicine, Miami, FL 33101, USA

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ABSTRACT

DNA polymerase δ is usually isolated as a heterodimer composed of a 125 kDa catalytic subunit and a 50 kDa small subunit of unknown function. The enzyme is distributive by itself and requires an accessory protein, the proliferating cell nuclear antigen (PCNA), for highly processive DNA synthesis. We have recently demonstrated that the catalytic subunit of human DNA polymerase δ(p125) expressed in baculovirus-infected insect cells, in contrast to the native heterodimeric calf thymus DNA polymerase δ, is not responsive to stimulation by PCNA. To determine whether the lack of response to PCNA of the recombinant catalytic subunit is due to the absence of the small subunit or to differences in post-translational modification in insect cells versus mammalian cells, we have co-expressed the two subunits of human DNA polymerase δ in insect cells. We have demonstrated that co-expression of the catalytic and small subunits of human DNA polymerase δ results in formation of a stable, fully functional heterodimer, that the recombinant heterodimer, similar to native heterodimer, is markedly stimulated by PCNA and that the increase in activity seen in the presence of PCNA is the result of an increase in processivity. These data establish that the 50 kDa subunit is essential for functional interaction of DNA polymerase δ with PCNA and for highly processive DNA synthesis.

INTRODUCTION

DNA polymerase δ is an essential DNA polymerase that is required to replicate chromosomal DNA in eukaryotic cells (1) and may also function in repair (2). Although studies with the reconstituted in vitro SV40 DNA replication system have suggested that DNA polymerase δ is required for leading strand synthesis at the viral replication fork as well as for completing Okazaki fragments initiated by the DNA polymerase α/primase complex (3), the role of DNA polymerase δ in cellular DNA replication is still unresolved (4).

By itself, DNA polymerase δ is a non-processive DNA polymerase and requires its accessory protein, proliferating cell nuclear antigen (PCNA), for highly processive synthesis on primed single-stranded templates such as poly(dA)·oligo(dT) (5,6). Results of kinetic and binding studies have shown that PCNA increases the activity and processivity of DNA polymerase δ by stabilizing the interaction of the enzyme with the template/primer (7,8), possibly by forming a trimERIC closed ring structure which encircles DNA and provides a sliding clamp for attachment of DNA polymerase δ (9).

When purified to homogeneity from fetal calf thymus, DNA polymerase δ was found to have a molecular weight of 173 000 and to be comprised of subunits of 125 and 48 kDa, present in equimolar amounts (7,10). Both the polymerase and 3′→5′ exonuclease activities are catalyzed by the 125 kDa subunit (11,12); the function of the small subunit, however, is unknown. Although DNA polymerase δ is usually isolated as a heterodimer from both higher and lower eukaryotes, a single subunit form of the enzyme has been purified from Drosophila melanogaster (13) and both one- and two-subunit forms of the enzyme have been isolated from mouse cells (14). Interestingly, the catalytic subunit of DNA polymerase δ isolated from either D.melanogaster or mouse cells is unresponsive to PCNA, suggesting that the small subunit is required for DNA polymerase δ–PCNA interaction.

We have recently expressed the recombinant catalytic subunit of human DNA polymerase δ in baculovirus-infected insect cells and found that it was unresponsive to stimulation by PCNA (15), similar to the catalytic subunits isolated from mouse cells and D.melanogaster embryos. Similar results were reported for the recombinant mouse catalytic subunit expressed in Escherichia coli (16) and the recombinant Schizosaccharomyces pombe catalytic subunit expressed in insect cells (17). However, our results differed from those reported for the recombinant Saccharomyces cerevisiae catalytic subunit expressed in E.coli (18) and the recombinant human protein expressed in monkey cells (19). The DNA polymerase activity and processivity of the latter polypeptides were found to be stimulated by PCNA, although the extent of...
stimulation (2- to 5-fold) was less than that seen with the native enzymes. To determine whether the lack of response to PCNA of the recombinant human catalytic subunit expressed in insect cells is due to the absence of the small subunit or to differences in post-translational modification in insect cells versus mammalian cells, we have co-expressed the two subunits of human DNA polymerase δ in baculovirus-infected insect cells and compared the properties of the recombinant heterodimer with those of the recombinant catalytic subunit and the native heterodimer isolated from calf thymus.

**MATERIALS AND METHODS**

**Materials**

Glutathione–Sepharose 4B, Source 15Q, Sephacryl S-300 and pGEX–4T-1 were obtained from Pharmacia Biotech. Protein A–Sepharose 4B Fast Flow was from Sigma. Spodoptera frugiperda cells (Sf9), wild-type baculovirus AcMNPV, recombinant β-galactosidase virus, the transfer vector pBlueBacIII, cationic liposomes and PCR primers for amplification of inserts in recombinant baculoviruses were obtained from Invitrogen Corp. Fetal bovine serum, gentamycin and pleuronic F-68 were from Gibco-BRL. Grace’s insect medium was prepared by the Cell Culture Facility, University of Miami. Calf thymus DNA polymerase δ was prepared as described by Downey and So (20). PCNA was prepared from fetal calf thymus as described in Tan et al. (5). Construction of recombinant baculovirus AcN-p125-14, which expresses the 125 kDa subunit of human DNA polymerase δ, and purification of the recombinant protein are described in Zhou et al. (15). Rabbit polyclonal antiserum to a peptide near the C-terminus of the catalytic subunit (R527) or to the recombinant small subunit (R527) of human DNA polymerase δ were prepared as described (8).

**Construction of recombinant baculovirus AcN-p50-1**

The coding sequences for the small subunit for human DNA polymerase δ were excised from an M13mp19 clone, HDSF-HDSR-4 (21), by BamHI digestion and the purified fragment was inserted into the BamHI site of the pBlueBacII transfer vector. The resulting construct, pBlueBac-p50, was co-transfected with linearized wild-type baculovirus AcMNPV DNA into Sf9 cells using cationic liposome-mediated transfection according to the supplier’s protocol and the recombinant virus was plaque purified. The presence of an insert in a putative recombinant virus was verified by PCR using primers complementary to the polymerase δ and purification of the recombinant protein are described in Zhou et al. (15). Rabbit polyclonal antiserum to a peptide near the C-terminus of the catalytic subunit (R804) or to the recombinant small subunit (R527) of human DNA polymerase δ were prepared as described (8).

**Expression of recombinant proteins**

Sf9 cells were grown to confluence in T-25 Falcon flasks at 27°C in Grace’s medium supplemented with 10% fetal bovine serum, 50 µg/ml gentamycin and 0.1% pleuronic F-68 and infected at a multiplicity of infection (MOI) of 10 with either wild-type virus AcMNPV, recombinant β-galactosidase virus, recombinant viruses AcN-p125-14 or AcN-p50-1 or co-infected at a MOI of 5 with AcN-p125-14 and AcN-p50-1. Cells were harvested at various times post-infection by centrifugation at 1200 g, washed twice with serum-free Grace’s medium and proteins analyzed by SDS–PAGE and immunoblotting.

**Purification of recombinant heterodimer**

Both AcN-p125-14 and AcN-p50-1 were used to co-infect Sf9 cells (1.5 × 10⁶ cells/ml) at a MOI of 5. After 60 h the cells were harvested, washed with serum-free Grace’s medium, resuspended in 120 ml ice-cold hypotonic buffer (20 mM HEPES, pH 7.6, 10 mM sodium bisulfite, 1 mM dithiothreitol and 1 mM EDTA) containing a mixture of protease inhibitors (10 µg/ml aprotinin, 5 µg/ml leupeptin, 10 µg/ml pepstatin A, 100 µg/ml bacitracin, 250 µg/ml soybean trypsin inhibitor, 0.4 mM phenyl-methylsulfonyl fluoride and 10 mM benzamidine hydrochloride) and subjected to Dounce homogenization. After centrifugation at 1200 g for 15 min, the supernatant was again centrifuged at 100 000 g for 75 min. The supernatant was adjusted to 100 mM NaCl, brought to 30% saturation with ammonium sulfate and left on ice for 2 h. The precipitate was collected by centrifugation at 27 000 g for 30 min, dissolved in 20 ml buffer A (20 mM HEPES, pH 7.6, 10% glycerol, 0.5 mM EDTA, 1 mM dithiothreitol and 0.4 mM phenylmethylsulfonyl fluoride) supplemented with the mixture of protease inhibitors described above, adjusted to a conductivity corresponding to 100 mM NaCl with buffer A and loaded on a Source 15Q column (1.6 x 7.5 cm) equilibrated in buffer A containing 100 mM NaCl. The column was washed with 3 bed vol of the same buffer and protein was eluted with a 130 ml linear gradient of 100–800 mM NaCl in buffer A. Recombinant heterodimer, detected by PCNA-dependent DNA polymerase activity and by immunoblot analysis, eluted between 200 and 245 mM NaCl. The peak fractions were pooled and 1.5 ml were loaded on a Sephacryl S-300 column (1.6 x 80 cm) equilibrated with buffer A containing 100 mM NaCl. The column was eluted at a flow rate of 30 ml/h and 1 ml fractions were collected. The peak fractions were pooled and concentrated on a small (1.0 x 1.3 cm) Source 15Q column.

**Immunoblot analysis**

Protein samples were separated by 10% SDS–PAGE and electroblotted onto a nitrocellulose membrane. After incubation for 30 min with blocking buffer (50 mM Tris–HCl, pH 7.4, 100 mM NaCl and 2% non-fat dry milk), the membrane was incubated overnight with rabbit polyclonal antiserum to p125 (R804) and p50 (R527) and washed three times with blocking buffer. The membrane was then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG for 1.5 h at room temperature, washed three times with blocking buffer and twice with Tris-buffered saline (50 mM Tris–HCl, pH 7.4, 100 mM NaCl). Color was developed in 3,3′-diaminobenzidine tetrahydrochloride (DAB) solution (0.4 mg/ml DAB, 0.009% H₂O₂ and 100 mM Tris–HCl, pH 7.5).

**Immunoprecipitation**

To raise antibodies against the catalytic subunit of DNA polymerase δ for immunoprecipitation studies, a 1007 bp BamHI–BglII fragment from pGEX–5X-1-p125 (15) was subcloned into the BamHI site of pGEX-4T-1 to create a fusion protein between glutathione S-transferase (GST) and the N-terminal one third (amino acids 1–335) of the catalytic subunit of human DNA polymerase δ. The fusion protein was expressed in E.coli strain DH5α, purified on glutathione–Sepharose beads and used to immunize rabbits. Antiserum was affinity purified by sequential passage through columns containing GST and GST fusion proteins.
protein respectively, cross-linked to glutathione-Sepharose beads according to Koff et al. (22). Bound antibody was eluted with 100 mM glycine, pH 2.4, and collected into a one tenth volume of 2 M Tris–HCl, pH 8.0. Affinity purified antibody to p125 (1 mg) was cross-linked to 1 ml protein A-Sepharose beads using dimethylpimelidate (23) to produce anti-p125 beads. Control beads were prepared by cross-linking pre-immune rabbit IgG to protein A-Sepharose.

Sf9 cells were grown to confluence in T-75 Falcon flasks, infected with wild-type virus, AcN-p125-14 or AcN-p50-1 or co-infected with AcN-p125-14 and AcN-p50-1, harvested 48 h post-infection and washed twice with serum-free Grace’s medium. Cells were suspended in 1 ml lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.5% NP-40, 250 µg/ml soybean trypsin inhibitor and 0.4 mM phenylmethylsulfonyl fluoride), incubated on ice for 30 min and cell extracts recovered by centrifugation at 27 000 g for 10 min. Aliquots (500 µl) of cell extracts were mixed with 10 µl anti-p125 beads or control beads and incubated at 4°C for 1 h with rocking. The beads were washed three times with lysis buffer, resuspended in 30 µl SDS–PAGE buffer and analyzed by Western blotting.

DNA polymerase assays

DNA polymerase activity was assayed with poly(dA)-oligo(dT) (10:1 nucleotide ratio) as template/primer in the presence and absence of calf thymus PCNA as described (15).

Processivity determination

Processivity was determined by measuring the sizes of products synthesized on a (dA)4000-(dT)16 (80:1 nucleotide ratio) template/primer under conditions where <1 residue of dTMP is incorporated with DNA polymerase activity was assayed with poly(dA)·oligo(dT) (10:1 nucleotide ratio) as template/primer in the presence and absence of calf thymus PCNA as described (15).

RESULTS

Expression of recombinant subunits of human DNA polymerase δ in insect cells

Recombinant baculoviruses that express the catalytic subunit (15) and the small subunit (this report) of human DNA polymerase δ were constructed and used to infect or co-infect Sf9 cells. Figure 1 shows an analysis by Western blotting (panel A) of extracts from uninfected and baculovirus-infected Sf9 cells. Cells infected with AcN-p125-14 or co-infected with AcN-p125-14 and AcN-p50-1 produced a protein of ∼125 kDa that was immunoblotted by antiserum to a peptide near the C-terminus of the catalytic subunit of mammalian DNA polymerase δ. Similarly, cells infected with AcN-p50-1 or co-infected with AcN-p50-1 and AcN-p125-14 produced a protein of ∼50 kDa that was immunoblotted by antiserum to recombinant human p50. Extracts from uninfected insect cells or cells infected with wild-type virus or recombinant β-galactosidase virus did not contain any proteins that cross-reacted with the antibodies to either the catalytic or small subunit of mammalian DNA polymerase δ.

A time course of recombinant protein expression is shown in Figure 1B. At a MOI of 10, recombinant human p125 was barely detectable in extracts of AcN-p125-14-infected cells 24 h post-infection, but was clearly visible at 36 h and the amount of recombinant protein increased until 60 h post-infection. In cells infected with AcN-p50-1 at a MOI of 10, the time course of recombinant protein expression was similar, i.e. recombinant protein accumulated up to 60 h post-infection. For both recombinant proteins, degradation products began to accumulate at 60 h post-infection. Co-infection of Sf9 cells with AcN-p125-14 and AcN-p50-1 at a MOI of 5 resulted in expression of both polypeptides with similar kinetics, although the amount of recombinant p50 exceeded that of recombinant p125.

Co-expression of recombinant p125 and p50 results in heterodimer formation

Affinity purified antibody to the catalytic subunit of human DNA polymerase δ (anti-p125) was found to immunoprecipitate both subunits of DNA polymerase δ from cell extracts. As shown in Figure 2, treatment of HeLa cell extracts with anti-p125 beads, but not with control beads, resulted in co-immunoprecipitation of the 125 and 50 kDa subunits of human DNA polymerase δ, as detected by Western blotting. Similar treatment of infected insect cell extracts showed that the anti-p125 antibody immunoprecipitated p125 from cells infected with AcN-p125-14 or co-infected with AcN-p125-14 and AcN-p50-1, but not from cells infected with...
Figure 2. Co-immunoprecipitation of p125 and p50. Immunoprecipitation of cell extracts was carried out with anti-p125 beads (lanes 3–7) or control beads (lane 2) and eluted proteins analyzed by Western blotting as described in Materials and Methods. Lane M, size markers in kDa; lane 1, 30 ng calf thymus DNA polymerase δ; lanes 2 and 3, HeLa cell extracts; lane 4, extract from wild-type virus-infected Sf9 cells; lane 5, extract from AcN-p125-14-infected Sf9 cells; lane 6, extract from AcN-p50-1-infected Sf9 cells; lane 7, extract from Sf9 cells co-infected with AcN-p125-14 and AcN-p50-1.

Figure 3. Gel filtration of recombinant human DNA polymerase δ. (A) The DNA polymerase activity of fractions from a Sephacryl S-300 column was determined with poly(dA)·oligo(dT) as template/primer in the presence (circles) or absence (triangles) of PCNA as described in Materials and Methods. Following concentration of active fractions on a Source 15Q column, the polypeptide components were analyzed by silver stained SDS–PAGE (B) and Western blotting (C). Lane M, molecular size markers; lane 1, 30 ng calf thymus DNA polymerase δ; lane 2, experimental sample.

Figure 4. Effect of PCNA on the activity and processivity of the recombinant heterodimer. (A) The effect of increasing concentrations of PCNA on the activity of the recombinant heterodimer, the recombinant catalytic subunit and native calf thymus DNA polymerase δ is shown in Figure 4A. As previously reported (15), recombinant p125 expressed in insect cells was not responsive to PCNA. However, both the recombinant heterodimer and native DNA polymerase δ were stimulated by increasing concentrations of PCNA, being maximally stimulated (45- to 50-fold) at a concentration of ∼800 ng/ml. That the stimulation of enzymatic activity is due to increased processivity is shown in Figure 4B. In the absence of PCNA all three proteins were essentially distributive, incorporating <30 nt/ enzyme binding event. In the presence of PCNA, the heterodimeric forms of DNA polymerase δ, either recombinant or native (Fig. 3, lanes 4 and 6), were highly processive, whereas the catalytic subunit (Fig. 3, lane 5) was non-processive. These reactions were carried out under conditions where the enzymes incorporated <1 residue dTMP/primer terminus and thus the sizes of the products represent the results of single binding events. The trace of short product in Figure 4B (lane 4) likely indicates the
We recently reported that the recombinant catalytic subunit of human DNA polymerase δ was found to be unresponsive to PCNA, i.e. S. cerevisiae protein expressed in E. coli (18) and human protein expressed in monkey cells (19).

In the present studies we have demonstrated that co-infection of insect cells with recombinant baculoviruses expressing the catalytic (125 kDa) and small (50 kDa) subunits of human DNA polymerase δ results in the formation of a heterodimer, as evidenced by co-elution of the 125 and 50 kDa polypeptides on gel filtration, as well as by co-immunoprecipitation of the 125 and 50 kDa polypeptides from co-infected insect cell extracts by affinity purified antibody to p125. The recombinant heterodimetric DNA polymerase δ, similar to the native heterodimer isolated from calf thymus, was found to be markedly stimulated by PCNA (40- to 50-fold) and the increase in activity seen in the presence of PCNA was found to be the result of increased processivity.

Since the recombinant catalytic subunit alone was unresponsive to PCNA, this establishes that the small subunit is essential for functional interaction of DNA polymerase δ with PCNA and for highly processive DNA synthesis. The reasons why, in some studies, recombinant catalytic subunit alone was found to be stimulated by PCNA are not obvious. In the case of human protein expressed in monkey cells (19), it is possible that the recombinant human protein formed a heterodimer with monkey cell p50, accounting for the observed stimulation by PCNA. It is more difficult to explain the effect of PCNA on the yeast catalytic subunit expressed in E. coli (18). However, this protein was denatured and renatured and it is possible that the refolded protein had undergone a conformational change which enabled it to bind to and be stimulated by PCNA. At present it is not clear whether PCNA directly interacts with p50 or whether the binding of p50 to p125 leads to an increased interaction of the catalytic subunit with PCNA, possibly as a result of a conformational change.

It has recently been reported that cdc1+ of S. pombe encodes a 51 kDa protein (27) with significant sequence similarity to the small subunit of human and bovine DNA polymerase δ (21) as well as to the Hys2 protein of S. cerevisiae (28). cdc1+ was found to interact genetically with pol3+, which encodes the catalytic subunit of S. pombe DNA polymerase δ, and physical interaction of the Cdc1 and Pol3 proteins was demonstrated in vitro, suggesting that Cdc1 is the small subunit of S. pombe DNA polymerase δ. cdc1+ was found to be essential for cell cycle progression but not for bulk DNA replication. cdc1 mutants were found to have an extended S phase and to be supersensitive to hydroxyurea and methylmethane sulfonate, consistent with a role in DNA replication and/or repair. Interestingly, cdc1+ also interacted genetically with cdc27+ and Cdc1 interacted physically with Cdc27, a protein of unknown function that is also required for cell cycle progression. Clearly, further studies on the protein–protein interactions of the small subunit of DNA polymerase δ will be necessary to clarify its role in cellular DNA replication and repair.

The subunit structure of DNA polymerase δ is very similar to that of HSV-1 DNA polymerase, i.e. both are heterodimers with subunits of similar sizes (7,10,24). The catalytic subunits, but not the small subunits, of both polymerases share significant homology (21,25). It is worth mentioning that the small subunit of HSV-1 DNA polymerase, a product of the UL42 gene, functions as an accessory factor to increase the processivity of the catalytic subunit, analogous to the effect of PCNA on DNA.

**DISCUSSION**

We recently reported that the recombinant catalytic subunit of human DNA polymerase δ expressed in insect cells is not responsive to stimulation by PCNA and suggested that the small subunit may be required for functional interaction of the enzyme with PCNA (35). These results were consistent with reports on the lack of response to PCNA of isolated native catalytic subunits from mouse cells and D. melanogaster embryos (13,14) and with reports of recombinant mouse protein expressed in E. coli (16) and recombinant S. pombe protein expressed in insect cells (17). However, they differed from two reports in which recombinant catalytic subunits were found to be responsive to PCNA, i.e. S. cerevisiae protein expressed in E. coli (18) and human protein expressed in monkey cells (19).

Figure 4. Effect of PCNA on the activity and processivity of recombinant p125, recombinant heterodimeric DNA polymerase δ and calf thymus DNA polymerase δ. (A) DNA polymerase activity was determined with poly(dA)-oligo(dT) as template/primer as described in Materials and Methods in the presence of the indicated concentration of calf thymus PCNA with either recombinant p125 (■), recombinant heterodimeric DNA polymerase (▲) or calf thymus DNA polymerase δ (●). (B) Processivity was determined as described in Materials and Methods in the absence (lanes 1, 3 and 5) or presence (lanes 2, 4 and 6) of calf thymus PCNA. Lane M, size markers; lanes 1 and 2, products of recombinant p125; lanes 3 and 4, products of recombinant heterodimeric DNA polymerase δ; lanes 5 and 6, products of calf thymus DNA polymerase δ. BPB, bromophenol blue.

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Presence of a small amount of free p125 in the recombinant heterodimer preparation. This may account for the slightly lower stimulation of the recombinant heterodimer by PCNA (45-fold) as compared with the native heterodimer (50-fold) shown in Figure 4A.
polymerase δ (26). DNA polymerase δ, on the other hand, requires PCNA for processive DNA synthesis, but its effect is dependent on association of the catalytic subunit with the small subunit of the enzyme. The finding that the processivity of DNA polymerase δ in the presence of PCNA may be modulated by the small subunit suggests the possibility that the small subunit may also modulate the interaction of DNA polymerase δ with other replication proteins.

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