Articles

Reconstitution and Characterization of the Human DNA Polymerase Delta Four-Subunit Holoenzyme†

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ABSTRACT: Mammalian DNA polymerase δ was originally characterized as a tightly associated heterodimer consisting of the catalytic subunit, p125, and the p50 subunit. Recently, two additional subunits, the third (p68) and fourth subunits (p12), have been identified. The heterotetrameric human pol δ complex was reconstituted by overexpression of the four subunits in Sf9 cells, followed by purification to near-homogeneity using FPLC chromatography. The properties of the four-subunit enzyme were shown to be functionally indistinguishable from those of pol δ isolated from calf thymus. The physicochemical properties of both the reconstituted heterotetramer and the heterodimer of the p125 and p50 subunits were examined by gel filtration and glycerol gradient ultracentrifugation. These studies show quite clearly that the heterodimer and heterotetramer complexes do not behave in solution as dimeric structures. This issue is of significance because several studies of the yeast pol δ complexes have indicated that the third subunit is able to bring about the dimerization of the pol δ complex. The heterodimer is only weakly stimulated by PCNA, whereas the heterotetramer is strongly stimulated to a level with a specific activity comparable to that of the calf thymus enzyme. These results resolve earlier, conflicting reports on the response of the heterodimer to PCNA. Nevertheless, the heterodimer does have some ability to interact functionally with PCNA, consistent with evidence that the p125 subunit itself has an ability to interact with PCNA. The functional interaction of PCNA with the pol δ complex may likely involve multiple contacts.

DNA polymerase delta (pol δ)[1] plays a central role in DNA replication and DNA repair in eukaryotic cells (*1*, *2*). Mammalian pol δ has been extensively studied as the tightly associated core heterodimer consisting of the large subunit, p125, which has both polymerase and 3′ to 5′ exonuclease activities, and a small subunit, p50 (*3*). More recently, two less tightly associated subunits have been identified in this and other laboratories. These are the third subunit, p68 (*4*−*6*), and the fourth subunit, p12 (*7*). This led to a concurrence with work in the two yeast systems, where the third subunit was described in *S. cerevisiae* (*8*, *9*) and the third and fourth subunits were found in *S. pombe* (*10*, *11*). The existence of these additional eukaroytic pol δ subunits raises important questions regarding their functions. The yeast third subunits, pol32 in *S. cerevisiae* and Cdc27 in *S. pombe*, were shown to not only increase the activity and processivity of pol δ in the presence of PCNA but also be able to dimerize pol δ complexes (*8*−*11*). The function of the fourth subunit of pol δ in *S. pombe*, Cdm1, is not fully defined, even though it was suggested to play a role in the stabilization of the DNA pol δ complex or to play a role in the processing of Okazaki fragments (*11*).

The findings that the third subunit could elicit the apparent dimerization of the pol δ heterodimer in both *S. pombe* and *S. cerevisiae* (*8*−*11*) brought attention to an important issue, as it would support a functional conservation of the mechanism of concerted DNA replication at the replication fork in eukaryotes and prokaryotes. In the case of the *S. cerevisiae* enzyme, the original claim that the pol δ heterotrimer was dimeric has been corrected (*12*). Nevertheless, a dimeric structure of pol δ, similar to T4 DNA polymerase and *E. coli* DNA polymerase III, is attractive. Using the SV40 DNA replication reconstitution system, Waga and Stillman (*13*) showed that DNA polymerase α/primase synthesizes RNA−DNA primers for the initiation of DNA replication at the origin for the priming of each Okazaki fragment. A polymerase switching mechanism was proposed, with replication factor C recruiting PCNA and pol δ, allowing two molecules of pol δ to coordinately synthesize both leading and lagging strands of the double helix. In this model, two molecules of pol δ and one molecule of pol α cooperate to synthesize DNA at the eukaryotic replication fork (*13*). Evidence that would support this model would be the ability of the pol δ enzyme to exhibit a dimeric structure, or the discovery of a protein that would lead to the formation of a dimeric pol δ

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[1] Abbreviations: pol δ, polymerase delta; PCNA, proliferating cell nuclear antigen; MOI, multiplicity of infection; FPLC, fast protein liquid chromatography.

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complex. Thus, the physicochemical behavior of the pol δ enzymes in the context of whether they are dimeric forms is of crucial interest as it relates to an important current model for the replication of eukaroytic DNA.

There also exist some issues regarding the roles of the noncatalytic subunits of pol δ, particularly in regard to their role in the PCNA response of pol δ. The catalytic subunit of DNA pol δ overexpressed in insect cells showed low activity and little response to PCNA stimulation (*14*, *15*). The role of the small subunit, p50, however is controversial. The presence of PCNA was found to only modestly increase the DNA polymerase activity compared to the native calf thymus enzyme in the case of the reconstituted p125/p50 heterodimer expressed in insect cells, or when the p50 subunit was added to the p125 subunit (*6*, *16*). In contrast, Zhou et al. (*17*) reported that p50 was able to provide for a functional interaction of pol δ with PCNA. This was based on observations that the recombinant heterodimer was markedly stimulated by PCNA and that this was accompanied by an increase in processivity (*17*). These results were contradictory to other reports, which showed that the reconstituted p125/p50 enzyme was not sensitive or poorly responsive to PCNA stimulation (*4*, *16*, *18*, *19*).

The third subunit does have one important property—the ability to bind PCNA. In fact, the initial identification of mammalian p68 arose through its binding to PCNA when pol δ complexes were overlayed with biotinylated-PCNA (*4*, *6*). Both yeast (*20*) and mammalian third subunits (*6*, *7*, *21*) contain a peptide motif that is known to mediate PCNA binding in a number of proteins (*22*, *23*). Thus, there is strong evidence that the third subunit provides a structural protein−protein interaction that mediates PCNA binding to the pol δ complex. However, recent studies of mammalian pol δ have indicated that the third subunit is insufficient to confer a full functional response to PCNA, which required the presence of the p12 subunit (*24*). This is in contrast to the *S. cerevisiae* pol δ, where a homologue of the fourth subunit does not exist (*8*, *9*).

One of the problems in the study of mammalian pol δ complexes is the difficulty in their isolation from tissues. In this study, the four-subunit human pol δ heterotetramer was reconstituted by coexpression using recombinant baculoviruses in Sf9 cells so that its properties could be examined and compared with those of the heterodimer. The purified pol δ complex was rigorously examined and shown to be functionally similar to native calf thymus pol δ, and its physicochemical properties and response to PCNA were investigated.

EXPERIMENTAL PROCEDURES

Materials. Sf9 insect cells were purchased from Invitrogen Co., and were maintained at 27 °C in SFM II medium supplemented with 2% fetal calf serum, 1 × antibiotic−antimycotic (Invitrogen Co.). BaculoGold linearized baculovirus DNA was purchased from Pharmingen. Poly(dA)$_{2000}$ was obtained from Mid-land Certificate Co. Superose 6, Superdex 200, Mono Q 5/5, and Source15 Q FPLC columns were purchased from Amersham-Pharmacia Biotech Inc. Fetal calf thymus glands were obtained from Animal Technologies Inc.

Construction of Recombinant Baculoviruses. The recombinant baculovirus for the catalytic p125 subunit was constructed as previously described (*15*). A construct encoding the p50 subunit of pol δ was generated by PCR from the p50-pET21a plasmid (*16*). The primer 5′-CAGGAGGATCCCC**ATG**TTTTCTGAGC-3′ was used for the 5′ end of the coding sequence with an engineered *Bam*HI site (underlined residues) at the initiating codon (boldface residues). The antisense primer at 5′-CCACAAGCTTGAG**TCA**GGGGCC-3′ had a *Hin*dIII site (underlined residues) after the termination codon (boldface residues). The PCR product was digested with *Bam*HI and *Hin*dIII and recloned into the p2Bac expression vector (Invitrogen). The resulting clone (p50-p2Bac) was sequenced to confirm the absence of mutations. The baculovirus was generated as previously described (*15*).

A construct encoding the p68 subunit of pol δ was generated by PCR using the cDNA clone KIAA0039 (Kazusa DNA Research Institute, Japan) as the template. The primer 5′-GAATTCTT**ATG**GCGGACCAGCTTTATC-3′ was used for the 5′ end of the coding sequence with an engineered *Eco*RI site (underlined residues) at the initiating codon (boldface residues). The antisense primer 5′-GAGATCT**TTA**TTTCCTCTGGAAGAAGCC-3′ had a *Bgl*II site (underlined residues) after the termination codon (boldface residues). The PCR product was cloned into pCR2.1 (Invitrogen Inc.) and sequenced. The coding region of p68 was excised with *Eco*RI and *Bgl*II and recloned into the pAcSG2 baculovirus transfer vector (Pharmigen). The recombinant p68 baculovirus was generated by cotransfection of pAsSG2-p68 and linearized baculovirus DNA (BaculoGold DNA, Pharmingen) into Sf9 insect cells.

A construct encoding the p12 subunit of pol δ was generated by PCR using the cDNA clone AA 402118 (ATCC) as the template. The sense primer 5′-GAATTCTT**ATG**GGCCGGAAGCGGCTC-3′ was engineered with an *Eco*RI site (underlined residues) at the initiating codon (boldface residues). The antisense primer 5′-AGATCT**TCA**TAGGGGATAGAGATGCCAGAGACTGC-3′ had a *Bgl*II site (underlined residues) after the termination codon (boldface residues). The PCR product was cloned into pCR2.1 (Invitrogen Inc.) and sequenced. The coding region of p12 was excised with *Eco*RI and *Bgl*II and recloned into the pAcSG2 vector. The recombinant p12 baculovirus was generated as described above for the p68 baculovirus. The baculovirus vector for PCNA was constructed as described previously (*4*). All of the above baculoviruses were plaque-purified.

Expression of Two-Subunit and Four-Subunit Recombinant Pol δ. For the expression of the two- or four-subunit recombinant enzyme, 200 mL of Sf9 cells (2×10^6 cells/mL) in suspension culture was co-infected either with the p125 and p50 baculoviruses for the two-subunit enzyme or with baculoviruses for all four subunits at a multiplicity of infection (MOI) of 5. The cells were collected 48 h post-infection, and the cell pellets were either treated directly with lysis buffer or stored at −80 °C.

Purification of Recombinant DNA Pol δ. All purification steps were carried out at 4 °C. The infected Sf9 cell pellet was lysed in 20 mL of lysis buffer (40 mM TrisHCl, pH 7.8, 0.25 M sucrose, 1 mM EDTA, 0.1 mM EGTA, 100 mM NaCl, 0.1% Nonidet P-40, 1 mM dithiothreitol (DTT), 2.5 μg/mL aprotinin, 5 μg/mL leupeptin, 0.4 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, 2 μM pepstatin, and

5 mM $NaHSO_3$). After passage through a French press to disrupt the cells, the lysate was centrifuged at 27000*g* for 30 min. The supernatant was filtered and adjusted to a conductivity corresponding to 100 mM NaCl with TGEED buffer (40 mM TrisHCl, pH 7.8, 10% glycerol, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 5 mM $NaHSO_3$) and loaded on a Source 15 Q column (1.6 × 7.5 cm) equilibrated with TGEED buffer. The column was washed with 3 bed volumes of TGEED buffer and eluted with a linear gradient of NaCl (140 mL, 0−1 M NaCl) in TGEED buffer. Fractions of 2 mL were collected. Recombinant enzymes were detected by PCNA-dependent DNA polymerase activity and by immunoblot analysis. The peak fractions were pooled, were adjusted to a conductivity corresponding to 100 mM NaCl with TGEED buffer, and were loaded onto a Mono Q 5/5 column equilibrated with TGEED buffer. The column was washed with 2 bed volumes of TGEED buffer and eluted with a 20 mL linear gradient (0−1 M NaCl) in TGEED buffer. Fractions of 0.5 mL were collected. Recombinant enzymes were detected by PCNA-dependent DNA polymerase activity and by immunoblot analysis. The peak fractions from the Mono Q 5/5 column were pooled and concentrated by Centricon (Millipore) centrifugation to a volume of 0.5 mL. A 250 μL sample was loaded either on a Superdex 200 or on a Superose 6 column equilibrated with 150 mM NaCl in TGEED buffer. The column was eluted at a flow rate of 0.3 mL/min, and 0.25 mL fractions were collected.

Glycerol Gradient Ultracentrifugation. Sedimentation analysis was carried out using a Beckman ultracentrifuge with a SW41 rotor. The buffer used was TGEED, pH 7.8, 150 mM NaCl. The protein standards and enzyme samples (150 μL) were layered on top of 10−45% glycerol gradients in 12 mL tubes and centrifuged at 30 000 rpm for 16 h. After centrifugation, fractions were withdrawn from the bottom of the tubes. The sedimentation velocities were calculated based on the centrifugation of standard proteins.

Coimmunoprecipitation of Individual Subunits of DNA Pol δ and PCNA. A total of 2 × 10^6 Sf9 cells were co-infected with recombinant viruses for individual subunits of DNA pol δ and PCNA at a MOI of 5. A sample (150 μL) of the crude extract (about 0.5 mg of total protein), which was precleared with mouse IgG, was incubated with 1 μg of anti-PCNA mouse monoclonal antibody (PC10, Santa Cruz) or 2 μg of control mouse IgG together with 40 μL of protein A/G beads. After rotating end-over-end overnight at 4 °C, the beads were washed 4 times with lysis buffer, and then 25 μL of 2 × SDS loading buffer was added to the beads. After boiling for 5 min at 100 °C, the proteins were subjected to SDS−PAGE (4−20% acrylamide gradients), transferred onto nitrocellulose membranes, and probed with antibodies against individual subunits.

Immunoprecipitation of p125 from HeLa Cell Extracts. HeLa cells were lysed in lysis buffer by brief sonication. The HeLa extract (2 mg of total protein precleared with normal rabbit IgG) was incubated with either 2 μg of anti-p12 rabbit polyclonal antibody or control rabbit IgG together with protein A/G beads. After rotating end-over-end overnight at 4 °C, the beads were washed 4 times with lysis buffer, and 25 μL of 2 × SDS loading buffer was added to the beads. After boiling for 5 min at 100 °C, the proteins were subjected to SDS−PAGE (10% acrylamide), transferred onto a nitrocellulose membrane, and probed with mouse monoclonal antibody against p125.

GST-p12 Fusion Proteins and GST Pull-Down Assays. pGEX-5X-3-p12 was generated by PCR using the cDNA clone AA402118 (ATCC) as template. The primer 5′-GATCGAATTCC**ATG**GGCCGG-3′ was engineered with an *Eco*RI site (underlined residues) at the initiating codon (boldface residues). The antisense primer 5′-GTTGCTCGAG**TCA**TAGGGGATAGA-3′ had a *Xho*I site (underlined residues) after the termination codon (boldface residues). The PCR product was cloned into pGEX-5X-3 (Amersham-Pharmacia Biotech) and sequenced. GST-p12 and GST were expressed in *E. coli* strain BL21 (DE3) and purified by using glutathione-Sepharose beads. For GST pull-down assays, 5 μg of GST or 5 μg of GST-p12 was incubated with 2 mg of calf thymus pol δ purified to the DE52 step (*6*), together with 50 μL of glutathione-Sepharose beads in GST-binding buffer (50 mM TrisHCl, pH 7.8, 1 mM EDTA, 150 mM NaCl, 1% NP-40, 0.2 mM PMSF, 10 μg/mL aprotinin) for 6 h at 4 °C. Beads were washed 5 times with 1 × PBS (10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4, 130 mM NaCl, 260 mM KCl), and bound proteins were subjected to SDS−PAGE (10% acrylamide), transferred to nitrocellulose membrane, and detected by the use of 78F5 anti-p125 mouse monoclonal antibody.

Western Blot Analysis. Western blotting was performed using a pol δ monoclonal antibody, 78F5 (*25*), and a p50 subunit monoclonal antibody, 13D5 (*16*). Rabbit polyclonal antibodies against p12 and p68 (*6*, *7*) were also used. Nitrocellulose blots were blocked with 5% w/v nonfat dry milk in TBST (20 mM TrisHCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20), for 1 h at 25 °C. The blot was then incubated with the primary antibody for 1 h at 25 °C or overnight at 4 °C. After three 10 min washes in TBST, the blot was incubated with HRP-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (Pierce, Rockford, IL) for 1 h at 25 °C and washed with TBST 4 times for 10 min. SuperSignal West Pico chemiluminescence substrate (Pierce) was used for signal production.

Protein Determination. Protein was determined by the Bradford method with bovine serum albumin as a standard (*26*).

DNA Polymerase Assays. Sparsely primed poly(dA)/oligo(dT) was used as the template. Standard reactions for the poly(dA)/oligo(dT) assay contained 0.25 OD unit/mL poly(dA)/oligo(dT) (20:1), 200 μg/mL BSA, 5% glycerol, 10 mM $MgCl_2$, 25 mM HEPES, pH 6.0, 100 cpm/pmol of [^{3}H]TTP, and 0.2 unit of pol δ in the presence of 0.1 μg of PCNA in a total volume of 50 μL. Reaction mixtures were incubated for 30 min at 37 °C and were terminated by spotting onto DE 81 papers which were washed 4 times with 0.3 M ammonium formate, pH 7.8, and once with 95% ethanol and counted as previously described (*3*, *6*). Processivity assays and pol δ holoenzyme assays on M13mp18 were performed as previously described (*27*).

RESULTS

Expression and Purification of the Recombinant Human Pol δ Four-Subunit Enzyme. To examine the properties of the human pol δ four-subunit enzyme in well-defined preparations, we used recombinant baculoviruses for all four

subunits (p125/p50/p68/p12) and to reconstitute the heterotetrameric form of pol δ by their coexpression in Sf9 insect cells. In previous studies, we had developed methods for the expression of the p125 subunit as well as the recombinant p125/p50 heterodimer in insect cells, and had shown that this expression system allows for expression of functional enzymes (*4*, *6*, *15*). For these studies, we chose to use the unmodified subunits, rather than his-tagged proteins that could result in the assembly of complexes which might have modified or compromised properties. To facilitate the purification of the intact complexes, we developed a procedure which uses FPLC chromatography on Source15Q, MonoQ, and Superose 6 or Superdex 200 gel filtration columns (Experimental Procedures). This procedure allowed the purification of the four-subunit (p125/p50/p68/p12) complex in an intact form to near-homogeneity. The purification was monitored by both assays of pol δ using poly(dA)/oligo(dT) as the template and by Western blot analysis.

When pol δ activity was purified from lysates of Sf9 cells co-infected with recombinant baculoviruses for all four subunits, the peak of pol δ activity was eluted between 300 and 350 mM NaCl (estimated from the elution position) from the Source 15Q column. The peak contained all four subunits as judged by Western blot analysis, and the activity was stimulated by PCNA about 10-fold (not shown). The peak fractions were combined and loaded onto a MonoQ 5/5 column. The peak activity eluted between 350 and 450 mM NaCl (not shown). The behavior of the four-subunit enzyme was examined on a Superose 6 gel filtration column. The activity for the four-subunit recombinant enzyme was eluted between fractions 46 and 48 (Figure 1A), between ferritin (445 kDa) and catalase (232 kDa) with an M_r of ca. 280 000, based on a semilog plot of M_r vs elution volume (not shown). The activity of the four-subunit enzyme at this stage of purification was ca. 21 000 units/mg and was stimulated over 50-fold by PCNA. Western blot analysis of the fractions across the peak on the Superose 6 column (Figure 1B) shows that all four subunits coeluted. Protein staining of the peak fractions showed that all four subunits were present as the major protein bands (Figure 1A, inset). However, there remained major contaminant bands of ca. 90 kDa (Figure 1A, inset). These were contaminants as they could be removed by immunoaffinity purification using anti-pol δ antibodies coupled to agarose beads (Figure 1C). The specific activity of the enzyme after this step was ca. 25 000 units/mg of protein. These results show that the use of recombinant baculoviruses for the coexpression of the four subunits allows for the reconstitution and subsequent isolation of an intact four-subunit pol δ enzyme. Thus, the reconstitution was successful, and the preparation behaved as a heterotetrameric complex of all four subunits.

The pol δ heterodimer was expressed and purified by the same procedure to provide for a direct comparison of the properties of the two complexes. The purification protocol was the same, but it was noted that the heterodimer eluted at slightly lower salt concentrations than the heterotetramer on Source15Q (between 200 and 280 mM NaCl) and on Mono Q 5/5 (between 300 and 350 mM NaCl) columns (data not shown). The concentrated peak fractions from the Mono Q 5/5 column were loaded onto a Superose 6 column. The peak activity eluted at fraction 55, at a position that indicated that it is larger than aldolase (Figure 2). The pol δ activity

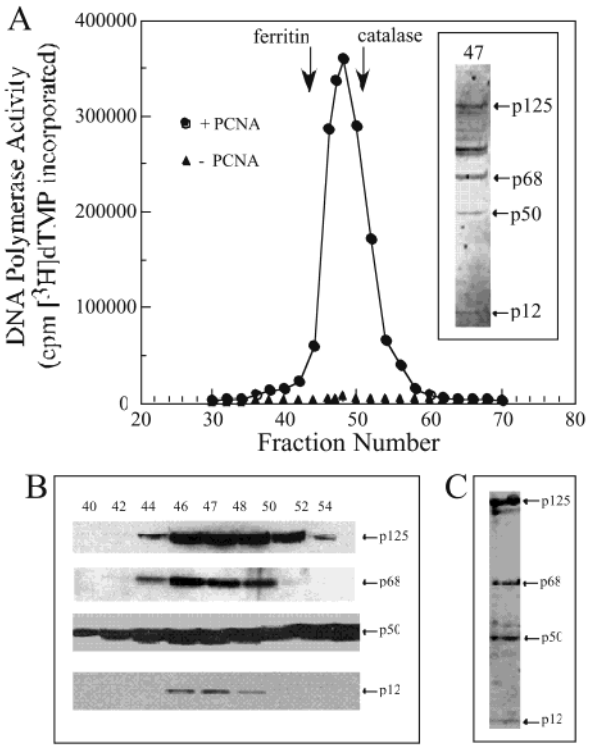

FIGURE 1: Purification of the recombinant human pol δ heterotetramer (p125/p50/p68/p12). Panel A: The peak fractions (fractions 14−18) from the Mono Q chromatography step were combined and concentrated to a volume of 0.5 mL (Experimental Procedures); 250 μL was passed through a Superose 6 column precalibrated with molecular weight standards (arrows). The fractions were assayed for DNA polymerase activity using poly(dA)/oligo(dT) as a template in the presence (circles) and absence (triangles) of PCNA. The inset shows the Coomassie Blue stain of the peak fraction (fraction 47) after SDS−PAGE. Panel B: Western blot analysis of the four-subunit enzyme after Superose 6 gel filtration. The fractions across the peak of polymerase activity were run on 4−20% SDS−PAGE and Western-blotted with the indicated antibodies. Panel C: Aliquots of 100 μL each from fractions 46−48 were combined and further purified by anti-p125 antibody coupled beads (Experimental Procedures), and stained with Coomassie Blue for protein. The specific activity of the enzyme was about 25 000 units/mg of protein after this step. Densitometric analysis of the gel image (Alpha Innotech Imager) indicated an approximate stoichiometry of the p125, p50, p68, and p12 subunits of 1:1:1.2:1.15.

was stimulated at most 2−3-fold by PCNA using poly(dA)/oligo(dT) as template under optimal assay conditions (Figure 2). The preparation was near-homogeneous as the major protein bands were 125 and 50 kDa as shown by Coomassie blue staining of the peak fraction on SDS−PAGE (Figure 2, inset).

The overall purification schemes for the pol δ heterotetramer and heterodimer enzymes are summarized in Tables 1 and 2, respectively. The specific activity of the pol δ heterotetramer (20 830 units/mg) was about 8 times higher than that of the heterodimer (2600 units/mg), when assayed on poly(dA)/oligo(dT) in the presence of PCNA. (Assay conditions were optimized by variation of the salt concentration, enzyme concentration, PCNA amounts, pH, incubation time, and template concentration.)

Physicochemical Analysis of Recombinant Human DNA Pol δ Complexes. The molecular weights of the purified pol δ four-subunit and two-subunit enzymes were determined from their Stokes radii on gel filtration and their sedimenta-

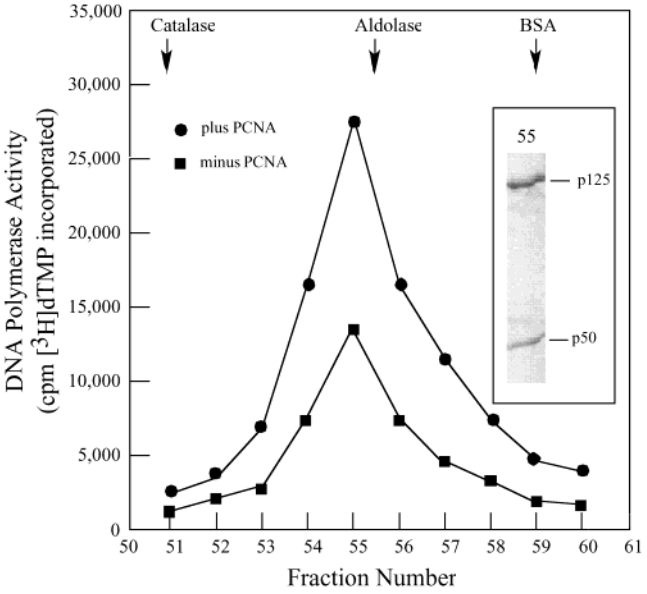

FIGURE 2: Purification of the recombinant human pol δ heterodimer (p125/p50). The peak fractions (fractions 12−14) from the Mono Q chromatography step were combined, and 250 μL was passed through a Superose 6 column precalibrated with molecular weight standards (arrows). The fractions were assayed for DNA polymerase activity using poly(dA)/oligo(dT) as a template in the presence (solid circles) and absence (solid squares) of PCNA. The inset shows the Coomassie stain of the peak fraction (fraction 55) after SDS−PAGE.

Table 1: Purification of the Recombinant Human Pol δ Heterotetramer (p125/p50/p68/p12)[a]

purification step	protein (mg)	activity (units)	specific activity (units/mg)	recovery (%)
cell extract	280	8400	30	100
Source15Q	16.9	4225	250	50
Mono Q HR 5/5	1.5	1176	784	14
Superose 6 (peak fraction)	0.0094	196	20830	2.4

[a] Assays were performed using poly(dA)/oligo(dT) template.

Table 2: Purification of the Recombinant Human Pol δ Heterodimer (p125/p50)[a]

purification step	protein (mg)	activity (units)	specific activity (units/mg)	recovery (%)
cell extract	226	542	2.4	100
Source15Q	6.8	261	38.4	48
Mono Q HR 5/5	0.73	110	151	20
Superose 6 (peak fraction)	0.01	26.4	2639	5

[a] Assays were performed using poly(dA)/oligo(dT) template.

tion coefficients by glycerol gradient centrifugation. For the determination of the Stokes radii, both enzymes were also analyzed on Superdex 200 in addition to the analyses performed on Superose 6 (see above). The calibration curves are shown in Figure 3. The value for the Stokes radius for the recombinant pol δ heterotetramer was 61 Å on Superose 6 and 57 Å on Superdex 200. For the two-subunit enzyme, a Stokes radius of 48 Å was obtained on Superose 6 and 47 Å on Superdex 200.

The purified pol δ heterotetramer was also analyzed by glycerol gradient ultracentrifugation (Figure 4). Western blot analysis of the collected fractions showed that all four

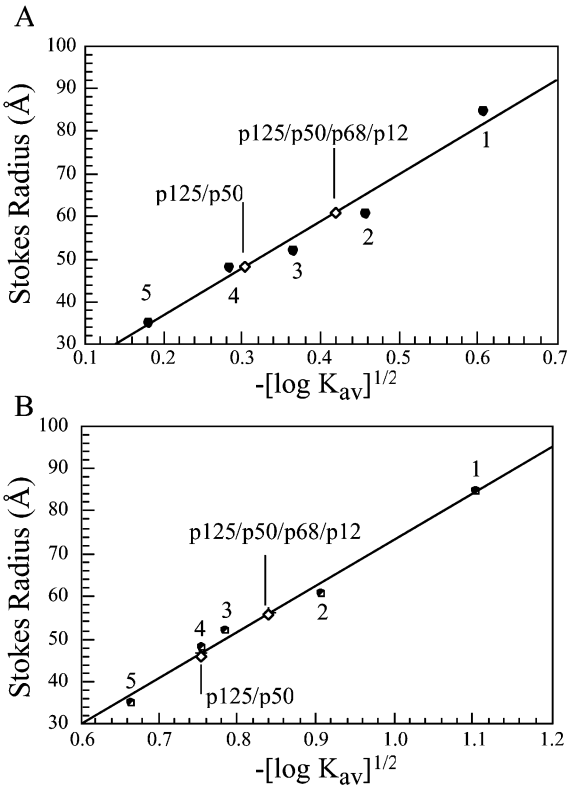

FIGURE 3: Determination of the Stokes radii for the heterodimer and heterotetramer forms of pol δ. Panel A: The purified two-subunit and four-subunit forms of recombinant pol δ were chromatographed on Superose 6 (Experimental Procedures). The diagram shows the calibration of the column that was used to estimate the Stokes radii. The standards used to calibrate both size exclusion columns were: 1, thyroglobulin (667 000, 85 Å); 2, ferritin (445 000, 61 Å); 3, catalase (232 000, 52.2 Å); 4, aldolase (158 000, 48.1 Å); and 5, bovine serum albumin (67 000, 35.5 Å). The elution positions of the recombinant enzyme complexes are indicated by the vertical lines and open diamonds. The estimated Stokes radii are given in Table 3. Panel B: The purified two-subunit and four-subunit forms of recombinant pol δ were chromatographed on Superdex 200 (Experimental Procedures). The diagram shows the calibration of the column that was used to estimate the Stokes radii. The standards used to calibrate the column were the same as for panel A. The elution positions of the recombinant enzyme complexes are indicated by the vertical lines and open diamonds.

subunits co-sedimented (not shown); i.e., there was no apparent loss of subunits during this procedure. The sedimentation coefficients for the two-subunit enzyme and four-subunit enzymes were determined, and found to be 7.8 and 9.2 S, respectively, by calibration with protein standards (Figure 4, inset). The molecular weights calculated from the Stokes radii and the sedimentation coefficients were 155 000 and 230 000 for the two- and four-subunit enzymes, respectively (Table 3). These values for the recombinant proteins are similar to those previously reported (Table 3) for the native calf thymus two-subunit core (*3*) and the calf thymus DNA pol δ complex containing all four subunits (*6*). The molecular masses of the pol δ heterotetramer and heterodimer estimated from their amino acid sequences are 239 and 175 kDa, respectively. Our results showed that both the purified two-subunit recombinant enzyme and the four-subunit recombinant enzyme behaved in a manner consistent with their being monomeric; i.e., there was no evidence for dimerization of either the heterodimer or the heterotetramer.

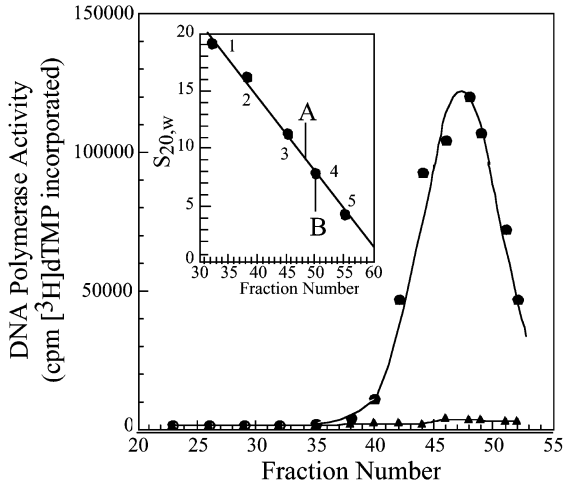

FIGURE 4: Glycerol gradient ultracentrifugation of the recombinant human pol δ complexes. The peak fractions from the Mono Q column were concentrated to 500 μL on a Centricon-30 filter, and a sample (150 μL) was subjected to glycerol gradient ultracentrifugation (Experimental Procedures). After centrifugation, fractions of 0.2 mL each were withdrawn from the bottom of tubes and assayed for polymerase activity in the presence (solid circles) and absence (solid triangles) of PCNA using poly(dA)/oligo(dT) as the template. The inset shows the calibration curve using the following protein standards: 1, thyroglobulin (19 S); 2, ferritin (16.2 S); 3, catalase (11.2 S); 4, aldolase (7.8 S); and 5, bovine serum albumin (4.3 S). The migration position of the four-subunit pol δ is shown by the vertical line marked "A". In the same experiment, the purified recombinant two-subunit form was also subjected to glycerol gradient ultracentrifugation (data not shown), and its migration is shown by the vertical line marked "B". The estimated *s* values are given in Table 3.

Table 3: Molecular Weights of Mammalian DNA Polymerase δ Enzymes

source	Stokes radius (Å)	sedimentation coefficient (S)	calcd mol wt	ref
two-subunit pol δ				
calf thymus	53	7.9	173000[a]	Lee et al. (*3*)
recombinant human	48	7.8	155000[a]	this work
			160000[b]	this work
four-subunit pol δ				
calf thymus	57	9.2	215000[a]	Mo et al. (*6*)
recombinant human	61	9.2	230000[a]	this work
			280000[b]	this work

[a] Calculated from the Stokes radii and sedimentation coefficient (*3*). [b] M_r determined by comparison to protein standards on Superose 6 gel filtration using a semilog plot of M_r vs elution volume.

Characterization of the Recombinant Four-Subunit Human DNA Pol δ. The specific activity of the reconstituted human pol δ heterotetramer (Table 1) was comparable to that of native calf thymus enzyme we obtained previously (*6*). The behavior of the purified reconstituted pol δ heterotetramer was compared to that of the native calf thymus pol δ isolated by immunoaffinity chromatography (*6*, *25*). The activities of both enzymes were inhibited by KCl, *N*-ethylmaleimide, and aphidicolin (Figure 5). These properties are similar to those reported for the native enzyme purified from calf thymus (*3*, *6*). Thus, in our hands, the reconstituted pol δ heterotetramer preparation appeared to be indistinguishable from the native enzyme, providing an assurance that the recombinant subunits had been properly folded and assembled in a native state.

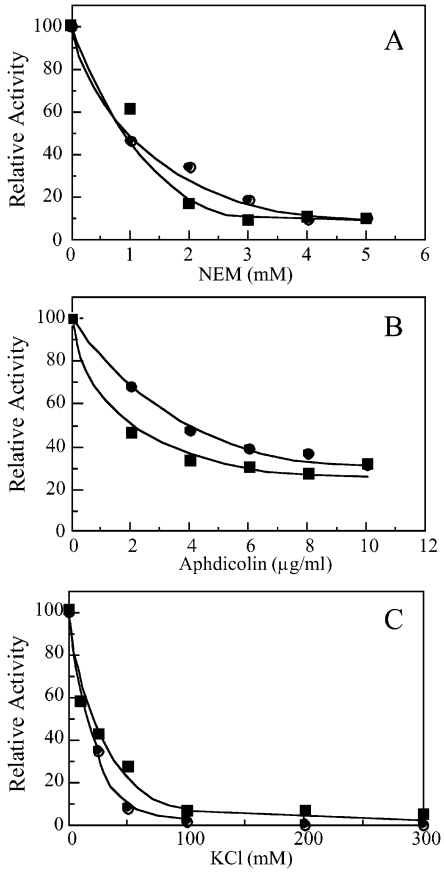

FIGURE 5: Characterization of recombinant four-subunit DNA pol δ: comparison with native calf thymus DNA pol δ. Effects of different compounds and conditions were assayed using poly(dA)/oligo(dT) as a template. Assay conditions were as described under Experimental Procedures. The activities of purified recombinant four-subunit DNA pol δ (circles) and purified native calf thymus pol δ (squares) were compared at the indicated concentrations of *N*-ethylmaleimide (panel A), aphidicolin (panel B), and KCl (panel C).

The Responses of the Two- and Four-Subunit Recombinant Enzymes to PCNA. The activity and processivity of the two- and four-subunit enzymes were compared in the presence and absence of various amounts of PCNA on sparsely primed poly(dA)/oligo(dT) template/primer. The two-subunit enzyme was relatively insensitive to the PCNA stimulation; the maximal stimulation was about 2−5-fold. Under the same assay conditions, the pol δ heterotetramer was far more sensitive to PCNA, and its activity was stimulated by over 50-fold (Figure 6A). The processivity of both forms of recombinant enzyme was also examined. The two-subunit enzyme was not processive even when excess PCNA was provided, while the heterotetramer was processive under the same conditions (Figure 6B). The heterotetramer was also assayed using a singly primed M13 template, an assay in which pol δ activity is dependent on the presence of RFC for the loading of PCNA. The activity of the pol δ heterotetramer was dependent on PCNA and RFC in the M13 holoenzyme assay, while two-subunit recombinant enzyme was not active under the same assay conditions (Figure 7).

It should be noted that despite a poorer stimulation by PCNA, there was nevertheless a consistent and measurable increase in activity of the pol δ heterodimer when assayed on sparsely primed poly(dA)/oligo(dT) template/primer. This did not, however, result in a shift to a highly processive

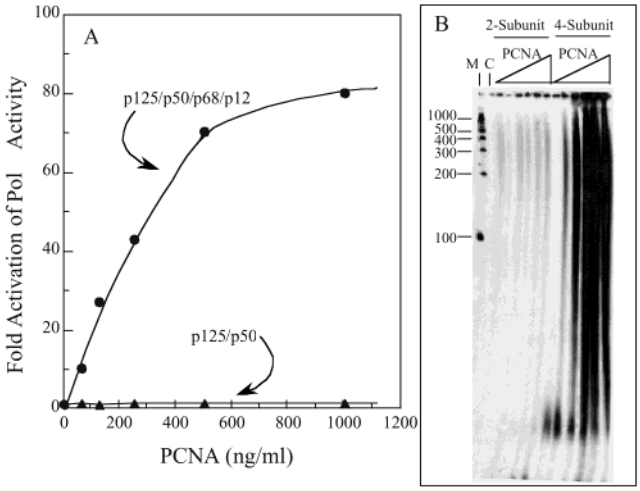

FIGURE 6: Effects of PCNA on the activity and processivity of two-subunit and four-subunit forms of purified recombinant pol δ. Panel A: DNA polymerase activity was determined in the presence of the indicated amounts of recombinant PCNA with 0.2 unit of two-subunit enzyme (triangles) or four-subunit enzyme (circles). Enzymes used were the peak fractions after Superose 6 chromatography. Panel B: Processivity was determined by electrophoretic examination of the reaction products (Experimental Procedures). The assay contained 0.2 unit of either the two-subunit or the four-subunit recombinant enzyme, plus the following amounts of purified recombinant human PCNA (from left to right): 0, 1, 10, 100, 250, and 500 ng. The lane marked "M" shows the DNA standards, and the lane marked "C" shows a reaction mixture containing 500 ng of PCNA but no added enzyme.

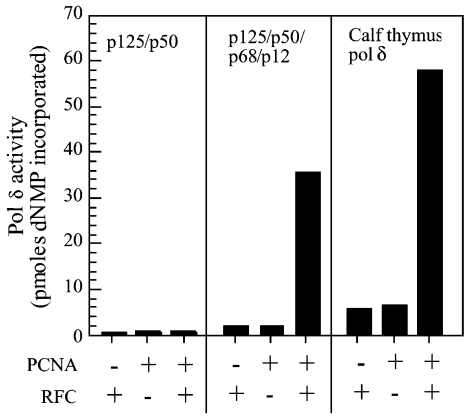

FIGURE 7: Assay of two-subunit, four-subunit recombinant, and native calf thymus pol δ enzymes using M13 DNA as the template. The M13 assay was performed as described under Experimental Procedures. The standard 30 μL reaction mixtures contained equimolar amounts (40 fmol) of the purified recombinant pol δ enzymes after FPLC gel filtration chromatography or the immunoaffinity-purified native calf thymus pol δ enzyme. The reaction mixtures also contained RFC (25 ng), PCNA (250 ng), and RPA (850 ng), 40 mM TrisHCl (pH 7.8), 8 mM magnesium acetate, 0.2 mg/mL bovine serum albumin, 1 mM dithiothreitol, 100 μM each of dATP, dCTP, and dGTP, 25 μM [^{3}H]dTTP (100 cpm/pmol), 0.5 mM ATP, and 100 ng of singly primed M13mp18 DNA. The complete reaction mixtures were incubated at 37 °C for 30 min.

synthesis. This would argue for some ability of the p125/p50 complex to interact with PCNA, although it is clear that in the absence of the p68 and p12 subunits a functional response to PCNA is lost; this is consistent with the known ability of the p68 subunit to bind to PCNA, but argues that additional interactions between p125 and p50 subunits with PCNA may be involved.

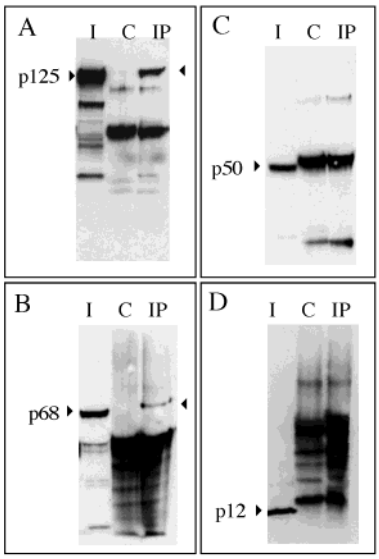

FIGURE 8: Interactions between PCNA and the individual subunits of human DNA pol δ. Cell lysates of Sf9 cells coinfected with recombinant baculoviruses for PCNA and individual subunit were immunoprecipitated with anti-PCNA mouse monoclonal antibody (PC10, Santa Cruz). The input lysates are indicated as "I"; mouse IgG was used as a control antibody for mock immunoprecipitation and is indicated as "C". The immunoprecipitates with PCNA are indicated as "IP". The presence or absence of pol δ subunits in the immunoprecipitates was analyzed by Western blotting with antibodies against p125 (panel A), p68 (panel B), p50 (panel C), and p12 (panel D). The presence of the subunits is indicated by the arrowheads.

Interaction of PCNA with Individual Subunits of DNA Pol δ. The interaction of PCNA with the individual recombinant subunits of human DNA pol δ was investigated by co-immunoprecipitation studies. Previous reports have given conflicting results as to whether there is an interaction between PCNA and the p125 subunit (*4*, *19*, *21*, *28*, *29*). One reason for this could be a variability in the expression of the recombinant p125 subunit in a native state. The interaction of PCNA with individual pol δ subunits was examined by their coexpression in insect cells, followed by immunoprecipitation with a monoclonal antibody against PCNA. PCNA antibody could co-immunoprecipitate p125 (Figure 8A) as well as p68 (Figure 8B), which is consistent with previous observations of interactions between PCNA with both p125 (*4*, *6*, *27*) and p68 (*4*, *6*, *21*). Under the same conditions, an interaction between PCNA and p50 (Figure 8C) or p12 (Figure 8D) could not be demonstrated.

p12 Is Part of the Pol δ *Complex.* The fourth subunit of mammalian DNA pol δ was originally identified by sequencing a 12 kDa polypeptide associated with highly purified calf thymus DNA pol δ (*7*). However, there have been difficulties showing that p12 is consistently associated with pol δ in a stoichiometric manner. Anti-p12 antibody was used to co-immunoprecipitate the other subunits of DNA pol δ from human HeLa cell lysates. The anti-p12 antibody efficiently coprecipitated the p125 catalytic subunit, while control rabbit IgG could not (Figure 9A). The anti-p12 antibody could also coprecipitate p50 and PCNA from HeLa cell extracts (data not shown). Furthermore, GST-p12 could pull-down p125 from a partially purified pol δ complex, while GST alone could not (Figure 9B). These results clearly indicated that p12 is a part of the mammalian DNA pol δ holoenzyme in vivo.

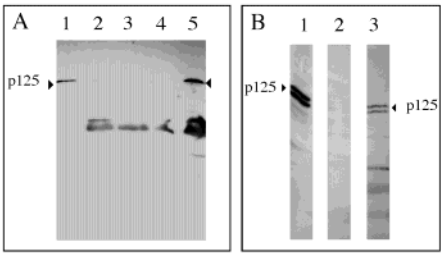

FIGURE 9: Interaction of the p12 subunit with the pol δ complex in vivo. Panel A: HeLa cells were lysed by sonication and immunoprecipitated with anti-p12 polyclonal antibody (Experimental Procedures). About 2 mg of total protein was immunoprecipitated with 2 μg of anti-p12 rabbit polyclonal antibody together with protein A/G beads. Normal rabbit IgG was used as control. The bound proteins were analyzed by Western blot using anti-p125 mouse monoclonal antibody. Lane 1, input cell lysate; lanes 2−4, 2, 1.5, and 1 μg of control rabbit IgG; lane 5, immunoprecipitate with anti-p12 antibody. Arrowheads show the position of p125. Panel B: Pull-down assays using a GST-p12 fusion protein. GST pull-down assays were performed as described under Experimental Procedures. Lane 1, input calf thymus pol δ purified to the DE 52 step (*6*); lane 2, control pull-down assay using 5 μg of GST; lane 3, pull-down assay using 5 μg of GST-p12 fusion protein. The pull-down assays were analyzed by Western blotting with an antibody against the p125 subunit of pol δ. Arrowheads show the position of p125.

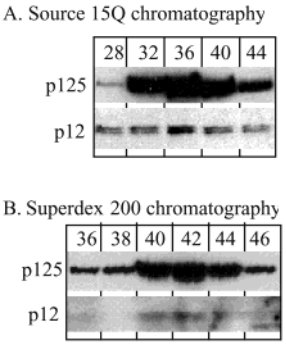

FIGURE 10: Interaction of the p12 subunit with the p125 subunit. Panel A: Sf9 cells (200 mL, 2×10^6 cells/mL) were infected with p125 and p12 baculoviruses, and the lysates were chromatographed on a Source 15Q column (Experimental Procedures). Eluted fractions (1 mL each) were analyzed on 4−20% SDS−PAGE and Western-blotted using anti-p125 monoclonal antibody and anti-p12 polyclonal antibody. Panel B: The peak fraction (number 36) from the Source 15Q column was chromatographed on a Superdex 200 gel filtration column. Fractions of 250 μL were collected. The fractions were run on 4−20% SDS−PAGE and Western-blotted using anti-p125 monoclonal antibody and anti-p12 polyclonal antibody.

Sf9 cells were co-infected with baculoviruses for p12 and p125 in order to determine if these two subunits interacted. The lysate was chromatographed on a Source 15Q column (Experimental Procedures), and the elution of p125 and p12 was monitored by Western blotting. The results (Figure 10A) showed that these two subunits coeluted. The peak fraction from the Source 15Q column was then subjected to gel filtration, and it was found that p12 and p125 coeluted (Figure 10B) at a position between aldolase (M_r 158 000) and bovine serum albumin (M_r 67 000). These experiments indicate that p12 interacts directly with the p125 subunit.

DISCUSSION

While it has been possible to isolate well-defined multisubunit pol δ complexes from yeast, this has been extraordinarily difficult in mammalian tissues (*3*, *5*, *25*), particularly in the case of the p68 subunit, which is prone to proteolytic nicking (*6*, *7*). In this study, the four-subunit human pol δ enzyme was reconstituted by overexpression in the insect cells. Rigorous isolation of the pol δ activity from insect cell lysates that had been co-infected with recombinant baculoviruses for all four human pol δ subunits led to the isolation of a near-homogeneous pol δ heterotetramer. No degradation (proteolytic nicking) of the p68 subunit was noticed. In these studies, we used the unmodified pol δ subunits, and avoided the use of histidine tags. While these tags facilitate isolation, they can potentially alter both functions and properties of the tagged proteins (*30*, *31*), and were of particular concern for their potential to alter functions that involve complex protein−protein interactions. This would have left some room for doubt in regard to the issues that were being examined in this work, viz., whether the pol δ complex is able to dimerize, and its interaction with PCNA. The studies reported here provide the first report of the expression of the pol δ heterotetramer in a native, unmodified (i.e., untagged) state, and the isolation of the intact complex to near-homogeneity. The properties of the pol δ heterotetramer were rigorously examined, and our results show that the reconstituted enzyme is functionally indistinguishable from the native calf thymus enzyme (*3*, *6*, *25*), by a direct side-by-side comparison with the most highly purified calf thymus pol δ preparations available. This includes comparable specific activities and response to PCNA, and similar responses of activity to KCl, aphidicolin, and the alkylating agent *N*-ethylmaleimide. The response of pol δ activity to these reagents is a classical set of properties that have been well established for pol δ (*32*).

The molecular weights of the purified recombinant two- and four-subunit forms of pol δ were determined by gel filtration and glycerol gradient ultracentrifugation. As noted in the introduction, this issue is of significance because the ability of pol δ to form dimeric complexes would provide supporting evidence for a mechanism involving pol δ dimers that could function in coordinated leading and lagging strand synthesis. However, previous evidence for a dimeric structure has been contradictory in yeast and inconsistent in mammalian systems. Previous studies based on gel filtration experiments provided evidence that the third subunit led to the dimerization of the yeast pol δ complexes in both *S. cerevisiae* (*8*, *9*) and *S. pombe* (*11*). In the case of the *S. cerevisiae* enzyme, a more careful reinvestigation has shown that the pol δ complex is not dimeric (*12*), and the initial findings (*8*, *9*) were attributed to the result of an anomalous migration of the pol δ complex on gel filtration due to an asymmetric structure. In our laboratory, the isolated p125/p50 heterodimer from calf thymus was shown to be a monomer, as was a highly purified complex of the heterotetramer (Table 3; *3*, *6*). However, it was also found that highly purified pol δ enzymes isolated by immunoaffinity chromatography are polydisperse on gel filtration, with apparent molecular weights in the range of 250 000 to >500 000 (*6*). Examination of the size of pol δ, by native gel electrophoresis, gave results which indicated the existence of discrete complexes of >500 000 (*6*), consistent with the existence of a dimeric form, either through an intrinsic property of the heterotetramer or through the agency of another protein.

In this study we have carefully examined the molecular size of the pol δ heterotetramer. The calculated molecular weight of the purified reconstituted human pol δ heterotetramer, based on our gel filtration and glycerol gradient results, is 230 000 (Table 3). The elution position on gel filtration alone is consistent with this molecular weight when estimated simply using size standards, indicating also that the pol δ heterotetramer did not behave anomalously. Thus, our results are unequivocal and show that the pol δ heterotetramer does not dimerize in solution. This is consistent with values we have previously obtained by determination of the Stokes radii and sedimentation coefficients for the rigorously purified native enzyme isolated from calf thymus either by conventional methods or by immunoaffinity chromatography (*3*, *6*). Our present studies of the reconstituted pol δ heterotetramer, as well as those of the calf thymus pol δ, indicate that both enzymes behaved normally on gel filtration. This is in contrast to the *S. cerevisiae* pol δ, which behaved as an asymmetric protein complex on gel filtration (*8*, *9*). A heterotetrameric (*24*) pol δ complex reconstituted using his-tagged subunits has been reported, but its physical properties were not examined.

The responses of the recombinant pol δ heterodimer and heterotetramer forms to PCNA were compared. The recombinant pol δ heterotetramer was found to be much more active and processive in the presence of PCNA than the two-subunit (p125/p50) enzyme. The maximal stimulation of the pol δ heterodimer by PCNA was found to be about 5-fold. The specific activities of the heterodimer never exceeded 3000 units/mg, which is much lower than that of four-subunit recombinant enzyme (ca. 25 000 units/mg). In addition, it may be noted that the specific activities of the heterodimer and heterotetramer in the absence of PCNA were similar, so that the behavior of the heterodimer cannot be explained on the basis of improper folding of the subunits. These findings resolve an issue that resulted from a previous report, which suggested that the mammalian pol δ heterodimer was fully active and processive when assayed in the presence of PCNA (*17*). Our results are consistent with our previous work on the immunoaffinity-purified calf thymus native enzyme in that the activities of these preparations were always much higher than that of the near-homogeneous calf thymus heterodimer (p125/p50) purified through single-stranded DNA cellulose columns (*6*). Podust et al. (*24*) have reconstituted a recombinant pol δ heterotetramer containing a his-tagged p68 subunit. This preparation was reported to have specific activities in the presence of PCNA (15 000 units/mg), comparable to those that we observed for the unmodified pol δ heterotetramer, but the level of stimulation by PCNA was only 5−7-fold, suggesting a much higher basal activity. This could be due to the use of the his-tagged p68, or to some difference in the assay conditions.

The requirement for p68 for a PCNA response by pol δ is consistent with the fact that p68 is a PCNA binding protein and possesses a well-defined PCNA binding motif at its C-terminus as has been demonstrated in a number of studies (*6*, *7*, *21*). While PCNA did not induce any detectable processivity when added to the reconstituted pol δ heterodimer, we have nevertheless found a consistent and reproducible stimulation of activity. These findings on the response of the pol δ heterodimer were representative of more than 10 preparations that were examined. Furthermore, care was taken to ensure that endogenous insect polymerases or PCNA were not present in the preparations as described previously (*15*). The *S. cerevisiae* pol δ heterodimer consisting of the p125 and p50 subunits without the third subunit was reported to be stimulated and to carry out a processive reaction when PCNA was present in high concentrations (*9*). In summary, we have shown that while the pol δ heterodimer does not carry out processive synthesis in the presence of PCNA, it is nevertheless stimulated by factors of 2−5-fold when assayed on sparsely primed poly(dA)/oligo(dT) template. When M13 templates are used, no response to PCNA was observed. The results clearly indicate that the pol δ heterodimer does have an intrinsic ability to interact with PCNA, but that this interaction is much weaker than that of the heterotetramer and does not lead to a highly processive synthesis although it may increase the level of DNA synthesis by promoting a greater interaction of pol δ with the template/primer.

The issue of whether the p125 subunit interacts with PCNA has been controversial. The evidence that the pol δ p125 subunit could interact with PCNA came from studies which showed that the two proteins could be co-immunoprecipitated when they were coexpressed in Sf9 cells (*4*). This interaction could be abrogated by deletion of the N-terminus of pol δ, and a synthetic peptide from residues 129 to 149 could inhibit the PCNA stimulation of pol δ (*4*, *28*). This was followed by the demonstration of a physical complex between p125 and PCNA by chemical cross-linking and gel filtration studies (*4*). Our findings that recombinant human p125 and PCNA can be co-immunoprecipitated have been confirmed by an independent study (*19*). In yeast, no evidence for an interaction between PCNA and the pol δ catalytic subunit was found by co-immunoprecipitation or yeast two-hybrid assays (*29*), and in a recent study of human p125 expressed in Sf9 cells, no interaction between PCNA and p125 was observed by far Western analysis, or by PCNA pull-down assays of the reconstituted heterodimer containing p125 and a his-tagged p50 subunit (*21*). A potential source of the differences could lie in variations in the folding of the p125 subunits under the specific conditions that were used for its expression. We have reexamined this issue, since our present expression system clearly allows for the reconstitution of a pol δ complex which is functionally indistinguishable from the native calf thymus enzyme. The interaction of PCNA with all four of the individual subunits of pol δ expressed in insect cells was examined. Our co-immunoprecipitation experiments showed that both p125 and p68 subunits interacted with PCNA, while p50 and p12 did not, which agrees with our previous PCNA overlay studies showing that both p125 and p68 could interact directly with PCNA (*4*, *6*). Thus, there seems little argument that there is an interaction of PCNA with p125.

Taking these facts into consideration, a model for the interaction of PCNA with the pol δ complex emerges which involves at least two sites of protein−protein interaction, i.e., between p68 and PCNA, and between p125 and PCNA. The latter interaction could explain the ability of PCNA to stimulate the activity of the pol δ heterodimer, while having no detectable effect on processivity. In addition, as we have previously noted (*27*), the fact that PCNA is a trimeric structure provides for a model in which multisite interactions between a single molecule of pol δ may involve more than

one of the monomers of PCNA. This would suggest that p125 and p68 could interact with different PCNA monomers within the PCNA homotrimer.

The homologue of p12 in *S. pombe* is Cdm1, which is nonessential to cell viability (*10*). No functional role for this subunit has been clearly defined in either the yeast or the mammalian system. The ability of anti-p12 antibody to precipitate other subunits of DNA pol δ from HeLa cell extracts and the fact that GST-p12 could pull-down calf thymus p125 shown here indicate that p12 is indeed part of mammalian DNA pol δ holoenzyme. The subunit with which p12 interacts has not been previously determined, and the ability of p12 and p125 to coelute on gel filtration provides the first evidence for a direct interaction between these two subunits. However, the specific role(s) of p12 is (are) at present uncertain. Rough comparisons of recombinant human pol δ complexes containing his-tagged subunits indicated that a complex which contained only the p125, p50, and p12 subunits was fully active in a PCNA-dependent assay, while the heterotrimer (p125/p50/p68) was much less active than the heterotetramer (*24*). The activity of the latter heterotrimer could be stimulated by addition of the p12 subunit (*24*). It was suggested that p12 may play a structural or stabilizing role in the formation of the pol δ complex. The activity of the heterotrimer containing p12 is paradoxical given the fact that the PCNA binding subunit, p68, was absent, but might be explained if p12 enhances the interaction between p125 and PCNA, as an interaction between PCNA and p12 seems unlikely based on the studies reported here.

In summary, our findings show the pol δ heterotetramer does not behave as a dimeric complex, and leave open the issue of whether a dimeric pol δ complex is involved in mammalian DNA replication. The possibilities remain that another protein factor is required for the dimerization of pol δ, or it could suggest that the mechanism of replication in the eukaryotes does not involve a dimeric form of pol δ. In this regard, there has been evidence that the DNA polymerase ϵ is involved in chromosomal DNA replication and is localized at the replication fork, as it could be cross-linked to nascent DNA in replicating mammalian chromosomes (*33*). There is evidence that yeast pol δ and pol ϵ replicate different DNA strands in yeast (*34*). In *Xenopus* egg extracts, polymerase ϵ is required for coordinated and efficient chromosomal DNA replication, and the elongation step of DNA replication is impaired in this system when the egg extracts were immunodepleted of pol ϵ (*35*).

REFERENCES

1. Zeng, X. R., Jiang, Y., Zhang, S. J., Hao, H., and Lee, M. Y. W. T. (1994) *J. Biol. Chem. 269*, 13748−13751.
2. Burgers, P. M. J. (1998) *Chromosoma 107*, 218−227.
3. Lee, M. Y. W. T., Tan, C. K., Downey, K. M., and So, A. G. (1984) *Biochemistry 23*, 1906−1913.
4. Zhang, P., Mo, J., Perez, A., Leon, A., Liu, L., Mazloum, N., Xu, H., and Lee, M. Y. W. T. (1999) *J. Biol. Chem. 274*, 28847−26653.
5. Hughes, P., Tratner, I., Ducoux, M., Piard, K., and Baldacci, G. (1999) *Nucleic Acids Res. 27*, 2108−2114.
6. Mo, J., Liu L., Leon, A., Mazloum, N., and Lee, M. Y. W. T. (2000) *Biochemistry 39*, 7245−7254.
7. Liu, L., Mo, J., Rodriguez-Belmonte, E., and Lee, M. Y. W. T. (2000) *J. Biol. Chem. 275*, 18739−18744.
8. Gerik, J., Li, X., Pautz, A., and Burgers, P. M. J. (1998) *J. Biol. Chem. 273*, 19747−19755.
9. Burgers, P. M. J., and Gerik, K. J. (1998) *J. Biol. Chem. 273*, 19756−19762.
10. Zuo, S., Gibbs, E., Kelman, Z., Wang, T. S. F., O'Donnell, M., MacNeill, S. A., and Hurwitz, J. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 11244−11249.
11. Zuo, S., Bermudez, V., Zhang G., Kelman Z., and Hurwitz, J. (2000) *J. Biol. Chem. 275*, 5153−5162.
12. Johansson, E., Majka, J., and Burgers, P. (2001) *J. Biol. Chem. 276*, 43824−43828.
13. Waga, S., and Stillman, B. (1994) *Nature 3699*, 207−212.
14. Zhou, J., Tan, C., So, A., and Downey K. (1996) *J. Biol. Chem. 271*, 29740−29745.
15. Wu, S., Zhang, P., Zeng, X., Zhang, S., Mo, J., Li, B., and Lee, M. Y. W. T. (1998) *J. Biol. Chem. 273*, 9561−9569.
16. Sun, Y., Jiang, Y., Zhang, P., Zhang, S., Zhou, Y., Li, B., Toomey, N., and Lee, M. Y. W. T. (1997) *J. Biol. Chem. 272*, 13013−13018.
17. Zhou, J. Q., He, H., Tan, C., Downey, K. M., and So, A. G. (1997) *Nucleic Acids Res. 25*, 1094−1099.
18. Suzuki, S., Suzuki, M., and Yoshida, S. (2000) *Nagoya J. Med. Sci. 63*, 99−113.
19. Shikata, K., Ohta, S., Yamada, K., Obuse, C., Yoshikawa, H., and Tsurimoto, T. (2001) *J. Biochem. 129*, 699−708.
20. Reynolds, N., Warbrick, E., Fantes, P., and MacNeill, S. (2000) *EMBO J. 19*, 1108−1118.
21. Ducoux, M., Urbach, S., Baldacci, G., Hubscher, U., Koundrioukoff, S., Christensen, J., and Hughes, P. (2001) *J. Biol. Chem. 276*, 49258−49266.
22. Warbrick, E. (1998) *Bioessays 20*, 195−199.
23. Xu, H., Zhang, P., Liu, L., and Lee, M. Y. W. T. (2001) *Biochemistry 40*, 4512−4520.
24. Podust, V., Chang, L., Ott, R., Dianov, G., and Fanning, E. (2001) *J. Biol. Chem. 277*, 3894−3901.
25. Jiang, Y., Zhang, S., Wu, S., and Lee, M. Y. W. T. (1995) *Arch. Biochem. Biophys. 320*, 297−304.
26. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
27. Zhang, P., Sun, Y., Hsu, H., Zhang, L., Zhang, Y., and Lee, M. Y. W. T. (1998) *J. Biol. Chem. 273*, 713−719.
28. Zhang, S. J., Zeng, X. R., Zhang, P., Toomey, N. L., Chuang, R.-Y., Chang, L. S., and Lee, M. Y. W. T. (1995) *J. Biol. Chem. 270*, 7988−7992.
29. Tratner, I., Piard, K., Grenon, M., Pederiset, M., and Baldacci, G. (1997) *Biochem. Biophys. Res. Commun. 231*, 321−328.
30. Wu, J., and Filutowicz, M. (1999) *Acta Biochim. Pol. 46*, 591−599.
31. Huang, Q., Woods, L., Feiss, M., and Catalano, E. E. (1999) *J. Biol. Chem. 274*, 15305−15314.
32. Lee, M. Y. W. T., Tan, C. K., Downey, K. M., and So, A. G. (1981) *Prog. Nucleic Acid Res. Mol. Biol. 26*, 83−96.
33. Zlotkin, T., Kaufmann, G., Jiang, Y., Lee, M. Y. W. T., Uitto, L., Syvaoja, J., Dornreiter, I., Fanning, E., and Nethanel, T. (1996) *EMBO J. 15*, 2298−2305.
34. Karthikeyan, R., Vonarx, E. J., Straffon, A. F., Simon, M., Faye, G., and Kunz, B. A. (2000) *J. Mol. Biol. 299*, 405−409.
35. Waga, S., Masuda, T., Takisawa, H., and Sugino A. (2001) *Proc. Natl. Acad. Sci. U.S.A. 98*, 4978−4983.

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