Reconstitution of Human DNA Polymerase δ Using Recombinant Baculoviruses

THE p12 SUBUNIT POTENTIATES DNA POLYMERIZING ACTIVITY OF THE FOUR-SUBUNIT ENZYME*

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Eukaryotic DNA polymerase δ is thought to consist of three (budding yeast) or four subunits (fission yeast, mammals). Four human genes encoding polypeptides p125, p50, p66, and p12 have been assigned as subunits of DNA polymerase δ. However, rigorous purification of human or bovine DNA polymerase δ from natural sources has usually yielded two-subunit preparations containing only p125 and p50 polypeptides. To reconstitute an intact DNA polymerase δ, we have constructed recombinant baculoviruses encoding the p125, p50, p66, and p12 subunits. From insect cells infected with four baculoviruses, protein preparations containing the four polypeptides of expected sizes were isolated. The four-subunit DNA polymerase δ displayed a specific activity comparable with that of the human, bovine, and fission yeast proteins isolated from natural sources. Recombinant DNA polymerase δ efficiently replicated singly primed M13 DNA in the presence of replication protein A, proliferating cell nuclear antigen, and replication factor C and was active in the SV40 DNA replication system. A three-subunit subcomplex consisting of the p125, p50, and p66 subunits, but lacking the p12 subunit, was also isolated. The p125, p50, and p66 polypeptides formed a stable complex that displayed DNA polymerizing activity 15-fold lower than that of the four-subunit polymerase. p12, expressed and purified individually, stimulated the activity of the three-subunit complex 4-fold on poly(dA)-oligo(dT) template-primer but had no effect on the activity of the four-subunit enzyme. Therefore, the p12 subunit is required to reconstitute fully active recombinant human DNA polymerase δ.

A family of eukaryotic DNA-dependent DNA polymerases (pol)¹ has greatly expanded in the last few years (1–3). Among polymerases known today, polα, polδ, and polε are viewed as major replicative polymerases in chromosomal DNA synthesis (4, 5). The replicative functions of polα and polδ have been defined primarily from studies of the SV40 DNA replication system (6). polα and its associated DNA primase synthesize RNA-DNA primers that initiate DNA synthesis on the leading strand and synthesis of each Okazaki fragment on the lagging strand (7, 8). The subsequent processive elongation of the leading strand and completion of Okazaki fragments are catalyzed by polδ (9–11). It has recently been suggested that polδ not only completes the gaps between pre-Okazaki fragments synthesized by pol α-primase but may also displace downstream RNA-DNA primers and refill the gaps so that no DNA products polymerized by polα are left on the newly replicated strand (12). The role of polε in eukaryotic DNA replication is more obscure. It appears to play no role in SV40 DNA replication (13, 14). However, polε, like polα and polδ, can be cross-linked to nascent cellular DNA (14), and neutralizing antibodies against polε inhibited replicative DNA synthesis (15). On the other hand, although the budding yeast pol catalytic subunit Pol2 is encoded by an essential gene (16), the catalytic domain within this polypeptide is dispensable for cell viability (17, 18). Point mutations in the catalytic domain of Pol2 are dominant-negative, suggesting that polε normally participates in DNA replication but another polymerase can substitute in its absence (18).

Consistent with the role of polδ in lagging strand DNA synthesis, polδ, but not polε, is required for telomerase-mediated telomere addition in vivo (19). Three temperature-sensitive mutants within the catalytic subunit of Schizosaccharomyces pombe polδ have been identified that exhibited a typical cell division cycle terminal phenotype. These data suggested that polδ is also involved in cell cycle control (20). polδ appears to be not only the major replicative enzyme but also the primary polymerase for most DNA repair pathways. Biochemical and genetic studies implicate polδ in mismatch repair (21), nucleotide excision repair (22), base excision repair (23, 24), double-strand break repair (25, 26), and trans-lesion DNA synthesis (26–28).

The subunit composition of polδ has been controversial and may vary among different eukaryotes. Saccharomyces cerevisiae polδ consists of three subunits 125, 58, and 55 kDa. These subunits are encoded by two essential genes, POL3 and POL31, and a non-essential gene POL32 (29, 30). S. pombe polδ was isolated as a complex of four subunits (31, 32). The fission yeast subunits Pol3, Cdc1, and Cdc27 are encoded by essential genes homologous to S. cerevisiae POL3, POL31, and POL32, respectively. The smallest non-essential subunit Cdm1 has no apparent homologue in budding yeast (33). Mammalian polδ was...
originally purified as a two-subunit complex of 125 and 50 kDa (34–36). cDNAs encoding both subunits have been cloned (37–40). The p125 subunit is homologous to budding yeast POL3 and fission yeast Pol3, whereas the p50 subunit is a homologue of POL31 and Cdc1. However, several recent studies suggest that mammalian polδ may consist of four subunits similar to the S. pombe enzyme. Analysis of PCNA-interacting proteins in murine cell extracts identified a novel protein p66, whose human homologue, a hypothetical protein KIAA0039, displayed some similarity to POL32 and Cdc27 (41). More directly, an association of KIAA0039 protein, also called p68, with highly purified polδ was demonstrated by Lee and co-workers (42, 43). Finally, an additional small subunit p12 encoded by the human EST clone AA402118 was proposed to be the fourth subunit of mammalian polδ (43). This conclusion was based on co-purification of this polypeptide with earlier characterized p125, p50, and p68 subunits of polδ and significant homology of p12/AA402118 to S. pombe Cdm1 (43).

Attempts to produce recombinant polδ have met with variable success. The human catalytic subunit p125 was expressed in insect cells using a vaccinia virus vector system. The recombinant protein was active on poly(dA)-oligo(dT)模板, and its activity could be stimulated 4.5-fold by PCNA, whereas native polδ was stimulated 10-fold by the same amount of PCNA (44). Recombinant murine p125 produced in bacteria as a glutathione S-transferase fusion displayed some DNA polymerizing activity, but no stimulatory effect of PCNA could be detected (45). Human p125 expressed in insect cells using a recombinant baculovirus displayed DNA polymerase activity, which was also not stimulated by PCNA (46–48). Co-expression of p125 and p50 subunit in insect cells resulted in formation of functional heterodimer. Its activity was stimulated 40–50-fold by PCNA, and the processivity of the heterodimer on poly(dA)-oligo(dT) was increased in the presence of PCNA like that of native calf thymus polδ (49). However, these data were not confirmed in another study of recombinant co-expressed with p50 in insect cells (48). Recent results suggest a possible explanation for the discrepancy. Co-expression of p125 and p50 in insect cells yielded a labile complex that possessed very low DNA polymerase activity and dissociated upon glycerol gradient centrifugation (50). Co-expression of three subunits p125, p50, and p66 resulted in a more stable complex. This complex displayed PCNA-stimulated activity on poly(dA)-oligo(dT) template and was active on singly primed complex. This complex displayed PCNA-stimulated activity on three subunits p125, p50, and p66 resulting in a more stable complex that possessed very low DNA polymerase activity and dissociated of p125 and p50 in insect cells yielded a labile complex that possessed very low DNA polymerase activity and dissociated upon glycerol gradient centrifugation (50). Co-expression of three subunits p125, p50, and p66 resulted in a more stable complex. This complex displayed PCNA-stimulated activity on poly(dA)-oligo(dT) template and was active on singly primed M13 DNA in the presence of RP-A, RF-C, and PCNA. Nevertheless, the specific activity of the three-subunit polδ was 10-fold lower than that of polδ isolated from human 293 cells by PCNA-affinity chromatography and 300-fold lower than that of enzyme purified from calf thymus (50).

Here we present reconstitution of human polδ in insect cells infected with four recombinant baculoviruses encoding subunits p125, p50, p66, and p12. Protein preparations purified to near-homogeneity by two different protocols contained each of the expected polypeptides. Characterization of the recombinant polδ demonstrated that it possesses specific activity comparable with the native human, bovine, and S. pombe polδ and is active on singly primed M13 DNA in the presence of RP-A, RF-C, PCNA, as well as in the SV40 DNA replication system.

**MATERIALS AND METHODS**

*Proteins and Nucleic Acids—*Bovine topoisomerase I was purified from calf thymus as described previously (51). Purification procedures for recombinant proteins, human PCNA (52), human RFC-3 (53), human pol α-primase (54), human RP-A (55), and SV40 T antigen (56), have been described. *Poo DNA polymerase was purchased from Roche Molecular Biochemicals; other enzymes were from Promega. A polyclonal antibody against human polδ catalytic subunit was obtained by immuno- 

**Recruitment of Recombinant polδ—**Four-subunit polδ was expressed by insect cells with four recombinant baculoviruses, each encoding a subunit of human polδ. 6 × 10^6 High Five cells were infected for 48 h with corresponding viruses, then harvested, and lysed in 20 ml of buffer A (20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.2% (v/v) Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml each of aprotinin and leupeptin). Cell debris was removed by centrifugation, and the supernatant fraction was bound to 1 ml of Ni-NTA resin. The suspension was mixed for 1.5 h at 4 °C, and the resin was pelleted by centrifugation, packed into a column, and washed with 10 ml of buffer A. Proteins were eluted with buffer B (20 mM Tris-HCl (pH 7.5), 200 mM imidazole HCl (pH 7.5), 100 mM NaCl, 0.02% (v/v) Nonidet P-40, 10% (v/v) glycerol), and 0.5-mM fractions were collected. The eluted fractions were analyzed by SDS-PAGE and Coomassie Blue staining. Most of the protein eluted in fractions 2–5. Pooled fractions were dialyzed 5-fold with buffer Q (20 mM Tris-HCl (pH 8.0), 10% glycerol, 1 mM DTT, 0.02% Nonidet P-40, 1 μg/ml each of aprotinin and leupeptin), and loaded onto a 1-mL Mono Q column (Amersham Biosciences). Proteins were eluted with a 20–400-mM gradient of NaCl in buffer Q, and fractions of 0.5 ml were collected. Eluted fractions were analyzed by SDS-PAGE and Coomassie Blue staining. pOδ eluted from the Mono Q column at 300 mM NaCl. In another protocol, fractions eluted from Ni-NTA resin were pooled, dialyzed 5-fold with buffer Q, and loaded onto a 1-mL Mono S column (Amersham Biosciences). Proteins were eluted with a 20–500-mM gradient of NaCl from 20 to 500 mM in buffer Q. pOδ eluted from the Mono S column at 400 mM NaCl.
Purification of Recombinant polδ—To produce a four-subunit human polδ, we constructed recombinant baculoviruses encoding four polypeptides (p125, p50, p66, and p12) proposed to be subunits of the human enzyme. Recombinant viruses encoding the three small subunits as N-terminal His tag fusion proteins were also constructed. Several combinations of untagged and His-tagged subunits were then tested to determine the optimal combination for isolation of the reconstituted enzyme. Insect cells were infected with the untagged p125 baculovirus, together with baculoviruses encoding each of the small subunits, of which one expressed the His-tagged form (either p50, p66, or p12). The co-expressed proteins were purified by chromatography on Ni-NTA resin. As a control, we infected cells with a baculovirus constructed from unmodified vector pBacHisC (59) and purified proteins from cells by the same protocol. The resulting preparations were analyzed by Western blotting using an antibody against the p66 catalytic subunit (Fig. 1A) and in a replication assay using poly(dA)-oligo(dT) as template-primer (Fig. 1B). Preparations isolated by Ni-NTA chromatography from cells infected with the control virus did not react with anti-p125 antibody (Fig. 1A, lane C) and showed no DNA polymerizing activity (Fig. 1B, column C). Infections with all three combinations of four baculoviruses with one His-tagged subunit yielded protein complexes that contained the p125 subunit (Fig. 1A, lanes 1–3) and were about equally active in the poly(dA)-oligo(dT) assay (Fig. 1B, columns 1–3).

To explore the roles of the recently described p66 and p12 subunits, we also produced three-subunit complexes lacking one of these subunits. When either p50 or p12 was His-tagged, the virus encoding the p66 subunit could be omitted during cell infection without any apparent loss of the catalytic subunit (Fig. 1A, lanes 5 and 6) or poly(dA)-oligo(dT) polymerizing activity (Fig. 1B, columns 5 and 6). Surprisingly, a combination of viruses encoding p125, p50, and p66-his, but lacking the p12-encoding virus, yielded a protein preparation that contained the p125 subunit (Fig. 1A, lane 4) but displayed sharply reduced DNA polymerizing activity (Fig. 1B, column 4).

To test the feasibility of a large scale preparation of the four-subunit polδ and to make an initial characterization of the recombinant protein, we chose a virus combination with the p66 subunit His-tagged (Fig. 1B, column 2). Rigorous isolation of natural mammalian polδ resulted in protein preparations with no detectable p66 (34–36). Even in the protocol which demonstrated co-purification of p66 with p125 and p50, the p66 subunit was subject to proteolytic degradation and stoichiometrically under-represented in the purified preparation (43). By placing a His tag on the p66 subunit most susceptible to proteolysis and/or denaturation, we hoped to optimize the yield of four-subunit complex.

The four-subunit polδ complex was isolated on a preparative scale using Ni-NTA chromatography as a rapid first step and further purified using either Mono Q (Fig. 2) or Mono S (Fig. 3) column chromatography. In both cases, a protein complex containing four subunits of the expected molecular masses was...
obtained. polδ complex purified by Mono Q chromatography was slightly contaminated with polypeptides of 100 and 30 kDa in fractions 27–29 (Fig. 2). These polypeptides resemble those that co-purified with RF-C four-subunit subcomplex p40-his/p38/p37/p36 expressed in insect cells (60). However, the 100- and 30-kDa proteins did not co-purify with the five-subunit RF-C complex purified by Mono Q chromatography. Four-subunit polδ was expressed in insect cells infected with baculoviruses encoding p125, p50, p66-his, and p12. Proteins were purified on Ni-NTA resin and Mono Q as described under “Materials and Methods.” Eluted fractions were analyzed by 10% SDS-PAGE (A) or 17% SDS-PAGE (B), and stained with Coomassie Blue.

FIG. 2. Purification of the four-subunit polδ by Mono Q chromatography. Four-subunit polδ was expressed in insect cells infected with baculoviruses encoding p125, p50, p66-his, and p12. Proteins were purified on Ni-NTA resin and Mono Q as described under “Materials and Methods.” Eluted fractions were analyzed by 10% SDS-PAGE (A) or 17% SDS-PAGE (B), and stained with Coomassie Blue.

The small size of the p12 subunit of human polδ (about 2-fold smaller than Cdm1) made detection of this subunit difficult. Because p12 migrated with the dye front in 10% SDS-PAGE (not shown), we used 17–20% SDS-PAGE to detect the polypeptide. This subunit was very weakly stained by Coomassie Blue in preparations of recombinant polδ (Figs. 2 and 3) and in polδ from calf thymus (43). Also, p12 was very poorly stained by silver (data not shown). The difficulties in detecting the fourth mammalian polδ subunit suggest that this polypeptide might have been overlooked in the so-called “two-subunit” preparations. We reanalyzed highly purified calf thymus polδ used for previous studies (53, 61) using 20% SDS-PAGE and indeed detected, in addition to p125 and p50 subunits, a polypeptide migrating in the gel exactly as the p12 from the recombinant polδ preparations (data not shown). However, a more careful analysis using p12-specific antibodies will be required to document the presence of the p12 subunit in the highly purified bovine polδ.

We also overexpressed and purified a two-subunit subcomplex containing the p50 and p66-his subunits. The p50/p66-his complex was purified by Ni-NTA chromatography followed by either Mono Q (not shown) or Mono S chromatography (Fig. 4A). The existence of this subcomplex is consistent with earlier demonstrations of homologous subcomplexes POL31-POL32 from budding yeast and Cdc1-Cdc27 from fission yeast (29, 32). Purification of the p50/p66-his subcomplex was also used to rule out a possible contamination of the four-subunit polδ preparations with other polymerases, either from insect cells or encoded by the baculovirus vector. DNA polymerizing activity was monitored during isolation of the p50/p66 complex, and only traces of polymerase activity were found in fractions eluted from Ni-NTA resin. When the Ni-NTA-purified p50/p66 complex was further purified by Mono Q chromatography, no detectable activity was observed in the fractions corresponding to the elution positions for the p50/p66-his subcomplex and the four-subunit polδ (data not shown).

Finally, a three-subunit complex containing p125, p50, and p66-his was overexpressed and isolated. The p125, p50, and p66-his subunits co-expressed in insect cells formed a stable soluble complex, which was purified by Ni-NTA and Mono Q chromatography (Fig. 4B). Two minor bands of 100 and 30 kDa similar to those seen in the four-subunit preparations were also detectable (Fig. 2). The isolation of the three-subunit complex...
clearly indicates that p12 is dispensable for assembly of the p125-p50-p66 complex in the baculovirus expression system. In contrast to our results with recombinant human polδ, Pol3-Cdc1, and Pol3-Cdc1-Cdc27 subcomplexes of fission yeast polδ expressed in insect cells could not be isolated in the absence of Cdm1 (32). Moreover, fission yeast polδ could not be purified from a fully viable strain in which the non-essential cdm1 gene was deleted, suggesting that fission yeast polδ may be unstable in the absence of Cdm1, both in the baculovirus expression system and in yeast cells (32).

Characterization of DNA Polymerizing Activity of Recombinant polδ—The four-subunit polδ complexes isolated by either Mono Q or by Mono S chromatography were active in the poly(dA)-oligo(dT) assay and showed identical specific activities (Fig. 5A). Under the conditions described under “Materials and Methods,” one molecule of the four-subunit polδ incorporated 60 molecules of dTMP in 1 min. By using the commonly accepted definition of 1 unit as an incorporation of 1 nmol of dTMP/h (62), the specific activity of our recombinant polδ purified by Mono S, open circles, four-subunit polδ purified by Mono Q, filled circles, three-subunit polδ purified by Mono Q, open circles, four-subunit complex indicates that the smallest subunit p12 greatly potentiates the polymerizing activity of the enzyme.

We also tested the activity of the three-subunit polδ (p125/p50/p66) on poly(dA)-oligo(dT) template-primer (Fig. 5A). Assayed under exactly the same conditions as the four-subunit complex, one molecule of three-subunit polδ incorporated only 4.1 molecules of dTMP in 1 min. The 15-fold lower specific activity of the three-subunit complex in comparison with the four-subunit complex indicates that the smallest subunit p12 greatly potentiates the polymerizing activity of the enzyme.

The hallmark of polδ is the dependence of its polymerizing activity on the auxiliary factor PCNA (62). Recombinant four-subunit polδ was stimulated by PCNA 5–7-fold on a poly(dA)-oligo(dT) template-primer (Fig. 5B). This level of stimulation was somewhat lower than the values reported for native two-subunit polymerases: 16- (36), 34- (35), and 50-fold (49). It is possible that the 5–7-fold stimulation indicates an impaired interaction between PCNA and recombinant polδ. On the other hand, it is also possible that the residual PCNA-independent activity of the four-subunit recombinant polδ is higher than that of native two-subunit polδ, thus causing an apparent reduction of PCNA stimulation.

To examine more directly the interaction of the recombinant polδ with PCNA, we analyzed its activity in the holoenzyme assay, which included singly primed M13 DNA, RP-A, RF-C, and PCNA (57, 63, 64). Recombinant four-subunit polδ efficiently replicated primed M13 DNA in the presence of the auxiliary proteins (Fig. 6). As expected, activity of polδ was completely dependent on simultaneous addition of RP-A, RF-C,
and PCNA (Fig. 6A). DNA synthesis by increasing amounts of recombinant polδ was tested at saturating amounts of the auxiliary proteins (Fig. 6B). 0.15 units (42 fmol) of polδ, equimolar to the amounts of 3'-OH ends of the primer in the reaction mixture, catalyzed incorporation of 75 pmol of dNMP into DNA, about 60% of the maximal DNA synthesis. At a 5-fold molar excess of enzyme over 3'-OH ends, polδ was no longer a limiting factor for DNA synthesis (Fig. 6B). Under the experimental conditions used, efficient dNMP incorporation was observed for 15 min and reached a maximum at about 1 h (Fig. 6C). Taken together, these results indicate that the recombinant polδ efficiently interacts with PCNA in the holoenzyme assay.

The products generated in the holoenzyme assay were analyzed by denaturing gel electrophoresis and autoradiography (Fig. 7). In a 30-min incubation in the presence of auxiliary factors, 50 fmol of the four-subunit polδ replicated singly primed M13 DNA, mostly to completion (Fig. 7, lane 3). However, 5 fmol of polδ synthesized notably shorter products (Fig. 7, lane 1), indicating that the four-subunit polδ was not fully processive, but replicated M13 DNA through a number of dissociating and reloading steps as proposed for mammalian polδ isolated from natural sources (61, 65). We also analyzed DNA products synthesized by three-subunit polδ (Fig. 7, lanes 4–6). Amounts of this enzyme equimolar to the amounts of the four-subunit polδ synthesized much less product (Fig. 7, compare lanes 4-6 to lanes 1–3). These results demonstrate a potentiating role of the smallest polδ subunit in the holoenzyme assay.

The four-subunit and three-subunit polδ complexes were then tested in an in vitro SV40 DNA replication system, which included SV40 origin-containing DNA and purified proteins as follows: T antigen, pol α-primase, topoisomerase I, RP-A, PCNA, and RF-C. 20 and 50 fmol of the four-subunit polδ (0.072 and 0.18 units, respectively) efficiently extended short DNA products synthesized by pol α-primase (Fig. 8, compare lane 1 and lanes 3 and 4) to the full size of the pUC-HS plasmid (2886 base pairs) and even greater, due to the strand displacement DNA synthesis. As was observed in the poly(dA)-oligo(dT) and holoenzyme assays, the three-subunit polδ was not efficient in SV40 DNA replication (Fig. 8, lanes 5–7). 50 fmol of the three-subunit enzyme synthesized about the same amount of product as 5 fmol of the four-subunit enzyme (Fig. 8, compare lanes 2 and 7).

In three different assays, a recombinant polδ that lacks the p12 subunit was at least an order of magnitude less efficient in DNA synthesis than the four-subunit enzyme (Figs. 5, 7, and 8). These results strongly suggest a potentiating role for p12 subunit in polδ activity. However, it is also possible that the three-subunit complex generated in the absence of p12 differs in some more subtle way from the four-subunit enzyme, for example by post-translational modifications. If the presence of p12 subunit is the sole difference between three- and four-subunit complexes, it might be possible to restore full activity of the purified three-subunit complex by adding back the purified p12 subunit. To test this idea, p12-his was expressed in insect cells and purified using Ni-NTA resin (Fig. 9A). Addition of purified p12-his to the reactions containing 50 fmol of the three-subunit polδ stimulated DNA synthesis on poly(dA)-oligo(dT) template-primer from 240 to 890 fmol dTMP/min (Fig. 9B). Under the same conditions, DNA synthesis by 50 fmol of the four-subunit polδ (3.4 pmol dTMP/min) was not affected by addition of p12-his (Fig. 9B). These data clearly demonstrate that the low activity of the three-subunit complex in DNA synthesis could be stimulated by p12 subunit. Still, at saturating amounts of p12-his, the pol(dA)-oligo(dT) polymerizing activity of the three-subunit polδ was not fully restored to the level observed for the four-subunit enzyme. Furthermore, the p12-his subunit failed to stimulate the three-subunit polδ activity in the holoenzyme assay (data not shown). The features of the holoenzyme assay that distinguish it from the poly(dA)-oligo(dT) assay are that polδ must elongate a relatively low number of 3'-OH primer ends using the enzymatically loaded PCNA clamp, and it must replicate a template covered with...
RP-A. These more stringent replication conditions may require more accurate assembly of the four-subunit pol, which apparently was not achieved in vitro by adding p12 protein to the p125-p50-p66 complex. For optimal activity of the recombinant pol, all four subunits must be co-expressed in insect cells.

Pol α is a key enzyme in DNA replication and repair, and defects in pol α have been linked to genomic instability. Mutations that affect the 3′-5′-exonuclease activity of pol α increased genomic instability in S. cerevisiae (66) and resulted in cancer susceptibility in mice (67). pol α, p125-p50-p66 complex. For optimal activity of the recombinant DNA polymerase, all four subunits must be co-expressed in insect cells.

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