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The Human Homologue of Fission Yeast cdc27, p66, Is a Component of Active Human DNA Polymerase δ[1]

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An essential eukaryotic DNA polymerase, DNA polymerase δ (pol δ), synthesizes DNA processively in the presence of proliferating cell nuclear antigen (PCNA). Recently, a 66 kDa polypeptide (p66) that displays significant homology within its PCNA binding domain to that of fission yeast cdc27 was identified as a component of mouse and calf thymus pol δ. Our studies show that p66 interacts tightly with other subunits of pol δ during size fractionation of human cell extracts, and co-immunoprecipitates with these subunits along with PCNA-dependent polymerase activity. Active human pol δ could be reconstituted by co-expressing p125, p50, and p66 recombinant baculoviruses, but not by co-expressing p125 and p50 alone. Interaction studies demonstrated that p66 stabilizes the association between p125 and p50. Pull-down assays with PCNA-linked beads demonstrated that p66 increases the overall affinity of pol δ for PCNA. These results indicate that p66 is a functionally important subunit of human pol δ that stabilizes the pol δ complex and increases the affinity of pol δ for PCNA.

Key words: cdc27, DNA polymerase δ, monoclonal antibody, PCNA, reconstituted enzyme.

DNA polymerase δ (pol δ) plays pivotal roles in chromosomal replication (*1*) and DNA repair synthesis (*2–4*) in eukaryotic cells. *In vitro* DNA replication studies with SV40 DNA have revealed that mammalian pol δ, which synthesizes DNA strands processively in the presence of proliferating cell nuclear antigen (PCNA) (*5–7*) and replication factor C (RFC) (*8–10*), is involved in both leading- and lagging-DNA strand syntheses (*11*, *12*). Mammalian pol δ was originally purified as a two subunit-complex comprising a catalytic 125 kDa polypeptide (p125) and a 50 kDa polypeptide of unknown function (p50) (*13*, *14*). Zhou *et al.* (*15*) reported that these two subunits, expressed using the baculovirus system, are sufficient to reconstitute active pol δ. However, recent studies have indicated the existence of additional subunits for pol δ. For example, pol δ in *Saccharomyces cerevisiae* is composed of three subunits, Pol3p, Pol31p, and Pol32p, and that of *Schizosaccharomyces pombe* consists of at least four subunits, Pol3, Cdc1, Cdc27, and Cdm1. The two large subunits, Pol3p and Pol3, contain the catalytic activity and are homologous to mammalian p125. Pol31p and Cdc1 are yeast homologues of mammalian p50, both of which are known to be essential for cell growth. Pol32p and Cdc27 are the third, and Cdm1 is the fourth subunit. Interestingly, the third subunit in budding yeast is not essential for growth, unlike that in fission yeast (*16–20*).

Recently, a 66 kDa polypeptide (p66) and two polypeptides of 12 and 68 kDa (p12 and p68) were identified as novel components of mouse and calf thymus pol δ, respectively (*21–23*). Peptide sequencing revealed that both p66 and p68 are coded by the genes similar to the human cDNA, KIAA0039, and that p12 is the product of the gene homologous to the human cDNA clone AA402118. Since p66/p68 and p12 display similarities to Pol32p/Cdc27 and fission yeast Cdm1, respectively, they were suggested to be the third and fourth subunits of mammalian pol δ (*21*, *22*). The similarity between the third subunits of yeast and human pol δ is especially high in the C-terminal regions where the putative PCNA binding motif is located (*18*, *21–24*).

With respect to the function of the third subunit of yeast pol δ, several groups have demonstrated that reconstituted DNA polymerase complexes containing the third subunit exist as dimers of a heterotrimeric complex of Pol3p/Pol31p/Pol32p in the case of budding yeast, or as a dimer of a heterotetrameric complex of Pol3/Cdc1/Cdc27/Cdm1 in the case of fission yeast (*18*, *20*). This is in contrast to complexes lacking this subunit, which exist as monomers of these subunits. Furthermore, the presence of the third subunit increases the processivity and efficiency of DNA synthesis by these pol δs (*20*, *25*). Thus, the third subunit of pol δ from yeast may connect two pol δ complexes, allowing coupled DNA synthesis on the lagging and leading strands during DNA replication *in vivo*. However, neither the subunit configuration of functional human pol δ nor the role of

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the putative third subunit has yet to be established in mammalian cells.

In this work, we show that p66 is a functionally important component of human pol δ, although the resulting trimeric complex appears to be monomeric rather than dimeric as in yeast. Studies of the interactions between the different subunits and their interactions with PCNA revealed that p66 is crucial for the formation of a stable pol δ complex and for the interaction between pol δ and PCNA.

MATERIALS AND METHODS

Construction of Recombinant Baculoviruses to Express pol δ Subunits—The cDNAs coding for the p125 and p50 subunits of human DNA polymerase δ were amplified with mRNA prepared from human 293 cells by RT-PCR using a First-Strand cDNA Synthesis Kit (Amarsham Pharmacia Biotech) and specific primers [5′-GGCTGTGGCTCGAGACGCTGTTTGAAGCGGG-3′ (p125 forward), 5′-AGAACTTTATGAATTCTCCCTGGTCC-3′ (p125 reverse), 5′-GTGGACCAAGGAATTCAGGAGTGTGG-3′ (p50 forward), and 5′-AATGAACAGCCTCGAGCTGGGCCTCT-3′ (p50 reverse)]. *Xho*I and *Eco*RI sites were generated at the 5′ and 3′ ends of the p125 cDNA, respectively, and *Eco*RI and *Xho*I sites at the 5′ and 3′ ends of p50 cDNA, respectively. The amplified p125 and p50 cDNA fragments were inserted into *Eco*RI and *Xho*I digested pBacPAK8 and pBacPAK9 (Clontech), respectively. To generate the histidine tagged p50 (His-p50) sequence, the *Eco*RI–*Xho*I fragment of the p50 cDNA was inserted into *Eco*RI and *Xho*I digested pET28a(+) plasmid (Novagen), and the *Nco*I–*Xho*I fragment of the resulting plasmid harboring His-p50 was inserted into *Nco*I and *Xho*I digested pBacPAK-His1 (Clontech). A cDNA, KIAA0039, coding for the putative p66 subunit was obtained from Kazusa DNA Research Institute, Japan, and the *Nco*I–*Bgl*II fragment carrying the entire coding region from the first codon ATG to the 3′ untranslated region was inserted into *Nco*I–*Bgl*II digested pBacPAK-His1 to obtain untagged p66. The histidine tagged p66 (His-p66) was obtained by inserting the *Nco*I–*Bgl*II fragment of p66 cDNA into the *Hin*dIII–*Bam*HI site of pBacPAK-His1. In this case, the *Nco*I and *Hin*dIII sites were joined through end filling. The generated transfer vectors were co-transfected with *Bsu*36I-digested BacPAK6 DNA (Clontech) into sf9 insect cells, and the recombinant baculoviruses were isolated.

Preparation of sf9 Cell Lysates Expressing pol δ Subunits—Sf9 insect cells grown at 27°C to 1.5 × 10^6 cells/ml in Grace's medium supplemented with 10% fetal bovine serum in a spinner flask were infected with various combinations of recombinant baculoviruses at a multiplicity of infection (MOI) of 5 for each baculovirus. After a 60 h incubation at 27°C, the infected cells were harvested and washed three times with ice-cold phosphate-buffered saline (PBS). The washed cells (from a 100 ml culture) were resuspended in 4 ml ice-cold hypotonic buffer (20 mM HEPES, pH 7.6, 10 mM sodium bisulfite, 0.05 mM EDTA) containing 2 μg/ml leupeptin, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and subjected to Dounce homogenization. After centrifugation at 1,200 ×*g* for 15 min, the cell lysates were obtained by centrifuging the supernatant at 100,000 ×*g* for 75 min.

Protein Preparations—Human RPA, pol δ from calf thymus and bacterially expressed recombinant human PCNA were prepared according to previously published methods with slight modifications (*26*). Human RFC was obtained from sf9 cells infected with baculoviruses carrying human RFC cDNAs as described by Shiomi *et al.* (*27*).

Antibodies Specific for Human pol δ Subunits—A polyclonal antibody directed against the human DNA pol δ p125 subunit, #43303, was obtained by immunizing rabbits with a synthetic peptide containing the last 20 amino acid residues from the C-terminal region of the p125 subunit. A monoclonal antibody, 8A5-E3, directed against the p125 subunit, was prepared from BALB/c mice immunized with a bacterially expressed peptide containing the region from Val_{248} to the C-terminus of p125. A monoclonal antibody, 2A1-C11, directed against the p66 subunit, was prepared from BALB/c mice immunized with a bacterially expressed full-length p66 polypeptide. A polyclonal antibody, δss, directed against the p50 subunit was purchased from Santa Cruz Biotechnology.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)—Protein samples were mixed with equal volumes of 1 × Laemmli SDS sample buffer, and loaded onto 12.5% or 4–20% SDS-polyacrylamide gels (TEFCO, Tokyo).

Immunoblot Analysis—Samples were separated by 12.5% SDS-PAGE and transferred to PVDF transfer membranes (Amersham Pharmacia Biotech) using a semidry transfer apparatus in transfer solution (100 mM Tris-HCl, 192 mM glycine, pH 8.3) at 3 mA/cm^2 for 45 min. The membranes were blocked with Tris-buffered saline (TBS) containing 1% dry milk (Snow Brand, Sapporo) for 30 min, and then incubated with first antibodies in TBS containing 1% dry milk for 1 h, followed by peroxidase-labeled second antibodies in the same solution for 1 h. Antibody reactive protein bands were visualized using the ECL system (Amersham Pharmacia Biotech).

Protein Quantification—Proteins were quantified by the Bradford method using a protein assay kit (Bio-Rad Laboratories). Stained or immunoblotted proteins were quantified with a KODAK 1D image analysis system Ver. 3.5 (Eastman Kodak).

Preparation of Native Polymerase δ from Human Cytoplasmic S100 Extracts—An S100 extract (930 mg), prepared as described in Refs. *28* and *29*, was adjusted to 0.1 M NaCl and loaded onto a 1 ml BSA (8 mg of BSA) column joined in tandem to a 2 ml PCNA (8 mg of PCNA) column, both of which were pre-equilibrated with buffer $A_{7.5}$ [25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.01% Nonidet P40 (NP40), 10% glycerol, 1 mM DTT, 2 μg/ml leupeptin, and 0.1 mM PMSF] containing 0.1 M NaCl (*23*). The columns were washed with 40 ml of the same buffer, and the PCNA-bound proteins were eluted with 20 ml of the same buffer containing 0.3 M NaCl. The eluted fraction (6 ml) was mixed with 14 ml of buffer $A_{8.0}$ (the same as buffer $A_{7.5}$ except pH 8.0) without NaCl, and charged onto a monoQ column (PC 1.6/5, Amersham Pharmacia Biotech) equilibrated with buffer $A_{8.0}$ containing 0.1 M NaCl with the SMART system (Amersham Pharmacia Biotech). The column was washed with 1 ml of buffer $A_{8.0}$ containing 0.1 M NaCl, and then 75 μl fractions were eluted with a 2 ml linear NaCl gradient from 50 to 500 mM in buffer $A_{8.0}$. Fractions displaying DNA synthesis activity in the presence of PCNA (fraction 14) were pooled.

Immunoprecipitation of pol δ from S100 Extracts—Pro-

tein A Sepharose beads (Amersham Pharmacia Biotech) crosslinked to p66 monoclonal antibody 2A1-C11 were obtained by mixing the beads with ascitic fluid containing the anti-p66 monoclonal antibody, and then incubating with dimethylpimelimidate as described in Ref. *30*. Then 2.5 or 5 μl of the 2A1-C11–bound protein A Sepharose beads were incubated with 0.1 ml of S100 extracts (7.5 mg/ml) in 0.4 ml of PBS for 3 h on ice with occasional mixing. After incubation, the beads were washed three times with PBS containing 0.1% NP40 and bound proteins were eluted with 30 μl of 1× Laemmli sample buffer. The extract and the eluted fractions, obtained from 6.7 times more extract than added to the gel, were immunoblotted after electrophoresis on a 12.5% SDS polyacrylamide gel. For the DNA polymerase assay, one-tenth of the washed beads were mixed with 5 μl of polymerase assay mixture, and DNA synthesis was measured as described below.

Size Fractionation of Human Cell Extracts—A human 293 cell S100 extract with a protein concentration of 9 mg/ml was concentrated to 15 mg/ml by successive dialyses against polyethylene glycol (PEG) #20000 and buffer H (25 mM HEPES, pH 7.5, 1 mM EDTA, 100 mM NaCl, 0.01% NP40, 10% glycerol, 1 mM DTT, 2 μg/ml leupeptin, and 0.1 mM PMSF). For gel filtration, 1 ml of the sample was mixed with 1 ml of buffer H and loaded onto a Sephacryl S-300 HR column (1.5 × 42 cm, Amersham Pharmacia Biotech), equilibrated in buffer H, and 1 ml fractions were collected. An aliquot (0.1 ml) of the same sample was loaded onto a 15–35% glycerol gradient (4.2 ml) in buffer H to study the sedimentation profile. The tubes were centrifuged in a Beckman SW 55 Ti rotor at 49,000 rpm for 18 h at 4°C, and 0.2 ml fractions were collected from the bottom of the tubes. Fractions from these two sizing chromatographies were subjected to 12.5% SDS-PAGE followed by immunoblotting, or to DNA polymerase assay with or without 10 μg/ml of human PCNA.

Purifications of Recombinant pol δ-Isolation of the 3-Subunit Complex—An sf9 cell lysate from 1 liter of cell cultures expressing p125, p66, and His-p50 subunits was prepared as described above. The lysate (40 ml, 175 mg) was mixed with 30 ml of His-Bind buffer (20 mM Tris-HCl, pH 7.9, 250 mM NaCl, and 0.01% NP40) containing 5 mM imidazole, and loaded onto a 5 ml column of Ni^{2+} resin (Novagen) equilibrated with the same buffer. The column was washed with 50 ml of the same buffer, and bound proteins were eluted with His-Bind buffer containing 60 mM imidazole. The eluate was dialyzed against buffer $A_{8.0}$ containing 50 mM NaCl (5 ml, 2.5 mg of proteins), and loaded onto a monoQ column (HR 5/5, Amersham Pharmacia Biotech) in buffer $A_{8.0}$ containing 50 mM NaCl. Proteins were eluted by a 5 ml linear gradient of 50 to 600 mM NaCl in buffer $A_{8.0}$ and 0.1 ml fractions were collected. PCNA-dependent DNA synthesis was eluted at 270 mM NaCl and the active fractions were pooled (0.3 ml; 0.034 mg protein). A 50 μl portion of the pooled fractions was further fractionated on a 2.4 ml 15–35% glycerol gradient in buffer H in a Beckman TLS 55 rotor at 50,000 rpm for 12 h at 4°C. Fractions (0.1 ml) were collected from the bottom of the tube after centrifugation.

Isolation of the 2-Subunit Complex—A 40 ml lysate containing 174 mg protein was prepared as for the 3-subunit complex, except that the cells were infected with viruses expressing only p125 and His-p50 subunits. After Ni^{2+} resin column chromatography, fractions (5 ml; 3.3 mg protein) containing PCNA-dependent polymerase activity were pooled and dialyzed, and charged onto a monoQ column. A portion (50 μl) of the eluted fractions from the column (0.3 ml, 0.05 mg) was subjected to glycerol gradient sedimentation as above.

DNA Synthesis Assay for pol δ—Assays of pol δ DNA synthetic activity using poly(dA)/oligo(dT) as a template/primer were performed according to the previously published method (*31*) with or without 100 ng PCNA and the indicated amounts of pol δ. One unit (U) of pol δ activity supported the incorporation of 1 pmol of dTMP under the reaction conditions used in the assay with poly(dA)/oligo(dT) (30 min at 37°C). For assays with singly-primed M13 DNA as template, reaction mixtures (5 μl) containing 30 mM HEPES, pH 7.6, 7 mM $MgCl_2$, 0.5 mM DTT, 0.1 mg/ml BSA, 25 mM each of dATP, dCTP, and dGTP, 25 μM [α-^{32}P]dTTP (3,000 cpm/pmol), 2 mM ATP, 10 fmol of singly-primed M13 DNA, 150 ng of human RPA, 25 ng of human RFC, 12 ng of human PCNA and the indicated amounts of pol δs were incubated at 37°C for 30 min. The incorporated dTMP was measured as the DE81 paper-absorbed count after washing with 0.5 M Na_2HPO_4. The synthesized DNA product was analyzed by alkaline-agarose gel electrophoresis followed by autoradiography as described in Ref. *10*.

Pull-Down Assays—To study the interactions between the different pol δ subunits, 50 μl of sf9 cell lysates infected with various combinations of pol δ subunit, including His-p50 or His-p66, were mixed with either 5 μl of Ni^{2+}-charged resin (experimental) or uncharged (negative control) beads (Novagen) and incubated for 3 h on ice with occasional mixing. The beads were washed three times with 100 μl of modified His-Bind buffer (20 mM Tris-HCl, pH 7.9, 500 mM NaCl, 5 mM imidazole, and 0.1% NP40), and the bound proteins were eluted with 30 μl of elution buffer (20 mM Tris-HCl, pH 7.9, 500 mM NaCl, and 1 M imidazole). An aliquot (3 μl) of the cell lysate and 5 μl of the eluted fraction, obtained from 2.8 times more cell lysate than added to the gel, were subjected to immunoblotting after electrophoresis through a 12.5% SDS polyacrylamide gel. The interactions between PCNA and pol δ subunits were studied with the same sf9 lysates using either 5 μl PCNA beads or BSA beads (*27*) instead of Ni^{2+} beads. The bead-lysate mixtures were incubated for 3 h on ice with occasional mixing, and the bound proteins were eluted with 40 μl of 1× Laemmli SDS sample buffer after three washes with 100 μl of buffer $A_{7.5}$ at 0.1 M NaCl without DTT. An aliquot of the cell lysate (3 μl) and 5 μl of the eluted fractions, obtained from 2 times more cell lysate than added to the gel, were subjected to immunoblotting as described above.

RESULTS AND DISCUSSION

Detection of the Human Homologue of Fission Yeast cdc27, p66, in Active Human pol δ Fractions—Previous reports have demonstrated that pol δ from mouse cells and calf thymus contain a third subunit with apparent molecular masses of 66 and 68 kDa, respectively (*21*, *22*). We have found independently that a 66 kDa polypeptide co-elutes with the p125 and p50 subunits of pol δ from a PCNA-affinity column charged with a human S100 cytoplasmic extract (Fig. 1A, lane 1).

The PCNA-bound fraction was subjected to monoQ column chromatography (Fig. 1, A and B), and a peak of PCNA-dependent DNA synthesis activity was detected in fraction 14 (lane 4). The staining pattern of the fraction again indicated the presence of a 66 kDa polypeptide that co-eluted with two other proteins of p125 and p50, whose intensities also peaked in this active fraction. The three subunits were the major components and exhibited almost equal stoichiometry in this fraction (Fig. 1A). To confirm that the eluted p66 protein was the product of the KIAA0039 cDNA clone previously identified as a homologue of p66 or p68 by other groups, we prepared a monoclonal antibody, 2A1-C11, raised against the bacterially expressed polypeptide encoded by the KIAA0039 DNA, and found that the antibody interacted specifically with the p66 protein in the eluted fractions from the PCNA-affinity column (Fig. 1A, lane 7).

We also obtained polyclonal antibodies, #43303 and δss (Santa Cruz Biotechnology), raised against the C-terminal 20 amino acid residues of p125 and the C-terminal amino acid residues of p50, respectively. Immunoblotting analysis with these antibodies revealed that this fraction also contained the p125 and p50 subunits of pol δ (Fig. 1A, lane 7). Therefore, the highly purified pol δ fraction contained the polypeptide encoded by the KIAA0039 gene and will be referred to hereafter as human p66.

We further tested for co-immunoprecipitation of p125 and p50 with the 2A1-C11 antibody, since this antibody could precipitate p66 efficiently from human cell extracts (Fig. 1C). We detected p125 and p50 bands in the precipitates (Fig. 1C, lanes 2 and 3), as well as PCNA-dependent polymerase activity (Fig. 1D). Although the recovered polymerase activity was lower than that of purified pol δ from human cells (Fig. 1, A and B), the activity roughly paralleled the band intensities of the pol δ subunits recovered in these experiments (Fig. 1, C and D). This result indicates that p66 is part of an active pol δ complex containing p125 and p50 in human cells.

Active pol δ Complexes Are Composed of Sub-Populations of the Three Subunits—As shown in Fig. 1C, p66 in the immunoprecipitate was concentrated efficiently (lanes 2 and 3), although it could hardly be detected by immunoblot analysis in the human cell extract (lane 1). In contrast, only a limited amount of p125 could be detected in the p66 immunoprecipitate compared to its efficient detection in the cell extract. These results suggest that active pol δ complexes contain only sub-populations of p125 in human cells. To elucidate the status of the three pol δ subunits in human cells, an S-100 extract was fractionated by Sephacryl S-300 column chromatography, and the elution profiles of p125, p66, and p50 were examined by immunoblotting using specific antibodies (Fig. 2A). Except for the broader elution profile of the p125 signal, all three subunits peaked around fraction 42, which corresponds to a native molecular mass of approximately 430 kDa. When we measured DNA synthesis using poly(dA)/oligo(dT) as a template in the presence and absence of PCNA, both activities were detected around fraction 42 and their difference peaked at fractions 40–42. Since we could detect other DNA polymerases, for example, pol α and ε, in these fractions by immunoblotting (data not shown), the significant DNA-synthesis activity without PCNA seems to be due to the pres-

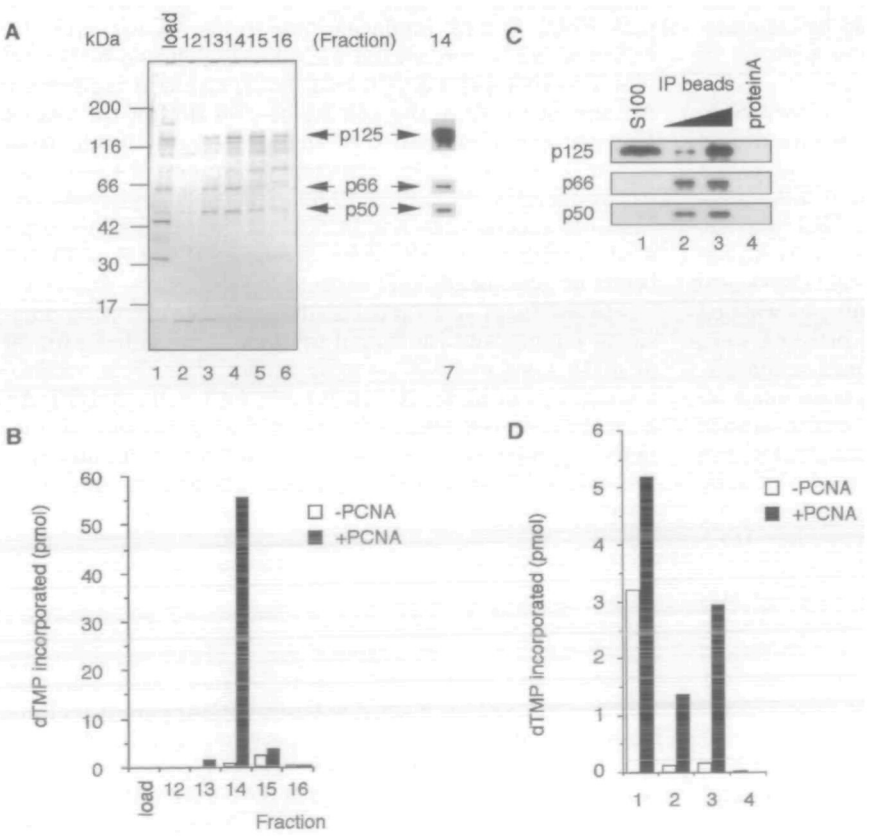

Fig. 1. **Subunit configuration of a highly purified pol δ fraction.** (A and B) Fractions from a monoQ column charged with a highly purified pol δ fraction obtained by PCNA-affinity chromatography of a human cytoplasmic extract (S100). (A) 1 μl aliquots of the monoQ fractions (fractions 12–16) were subjected to electrophoresis in a 4–20% SDS polyacrylamide gradient gel and stained with silver (lanes 1–6) or immunoblotted with anti-p125 polyclonal antibody #43303, anti-KIAA0039 gene product (p66) monoclonal antibody 2A1-C11 or anti-p50 polyclonal antibody δss (lane 7). (B) 0.3 μl aliquots of the fractions were tested for DNA polymerase activity using poly(dA)/oligo(dT) as a template in the presence (black bars) or absence (white bars) of PCNA. (C and D) Co-immunoprecipitation of pol δ subunits from an S100 extract using antibody 2A1-C11–bound protein A Sepharose beads (2A1-C11 beads). (C) The antibody-bound proteins from an S100 extract were detected by immunoblotting as in (A). Lane 1, 5 μl of extract; lanes 2 and 3, eluted fractions (10 μl) from 2.5 or 5 μl of 2A1-C11 beads; lane 4, eluted fractions (10 μl) from 5 μl of antibody-free protein A beads. (D) The samples in (C) were tested for DNA synthesis on poly(dA)/oligo(dT) in the presence (black bars) or absence (white bars) of PCNA. Column 1, activities with 0.1 μl of extract; columns 2 and 3, activities of the precipitates with 0.5 and 1 μl of 2A1-C11 beads; and column 4, activity of the precipitates with 1 μl of protein A beads.

ence of these other polymerases. Thus, both PCNA-dependent DNA synthesis activities, represented by the difference, and the three pol δ subunits were recovered in the same fraction. The broader elution profile of p125 indicates that p125 may also exist in a free form, dissociated from p66 and p50.

Next, we subjected the same extract to glycerol gradient centrifugation (Fig. 2B). The three polypeptides peaked in fraction 9, corresponding to a molecular mass of 210 kDa. PCNA-dependent polymerase activity, as the difference in the two measurements, peaked in the same fraction. This result again indicates that p66 sediments with the active pol δ complex containing the two other subunits. During centrifugation, some p125 molecular populations sedimented faster and some p50 molecular populations more slowly than the peak fraction containing the three subunits, confirming the existence of different forms of pol δ in these extracts. Apparently the elution profile of p125 is affected by the method used to separate the extract as the p125 population free from p66 and p50 gave a different molecular mass by each method. One possibility is that this is due to the unusual shape of p125, which might show different migration profiles depending on the method of separation. The intensity of the p125 band in the fractions around the peak was relatively weak compared to the intensity of the p125 subunit in the peak fractions, although the immunoprecipitation experiments in Fig. 1C show that more than half of the p125 population exists separately from p66. This discrepancy may be caused by the aggregation of p125 during the fractionation steps.

The apparent molecular masses of native pol δ as determined by gel filtration and glycerol gradient centrifugation differ significantly. The native molecular mass of 430 kDa determined by S-300 size fractionation is almost twice that of the 241 kDa mass calculated from the sum of the molecular weights of the three subunits. This might indicate that pol δ exists as a dimeric complex in these cell extracts. Indeed, the presence of dimeric pol δ in budding yeast (*25*) and fission yeast (*20*) has been reported. However, since we could not detect any pol δ complex or PCNA-dependent DNA synthesis activity in the glycerol gradient fractions above 400 kDa, this interpretation seems unlikely. Another possibility might be irregularity in the shape of the pol δ complex. Previous studies have reported hydrodynamic irregularities for proteins during gel filtration and sedimentation experiments (*32*). If we take these irregularities into account, the native molecular mass would be 230 kDa, which is almost equal to the sum of the masses of the three subunits. Therefore, pol δ in human cell extracts is a monomer of a heterotrimeric complex composed of p125, p66, and p50. Recent work has also shown that the calculated molecular mass of pol δ from calf thymus is about 215 kDa (*23*).

Recent studies have demonstrated that pol δ has a fourth subunit in fission yeast and bovine tissue. Although the presence of the fourth subunit in these fractions is an important question, we were unable to test for its presence because no antibodies specific for the AA402118 peptide were available.

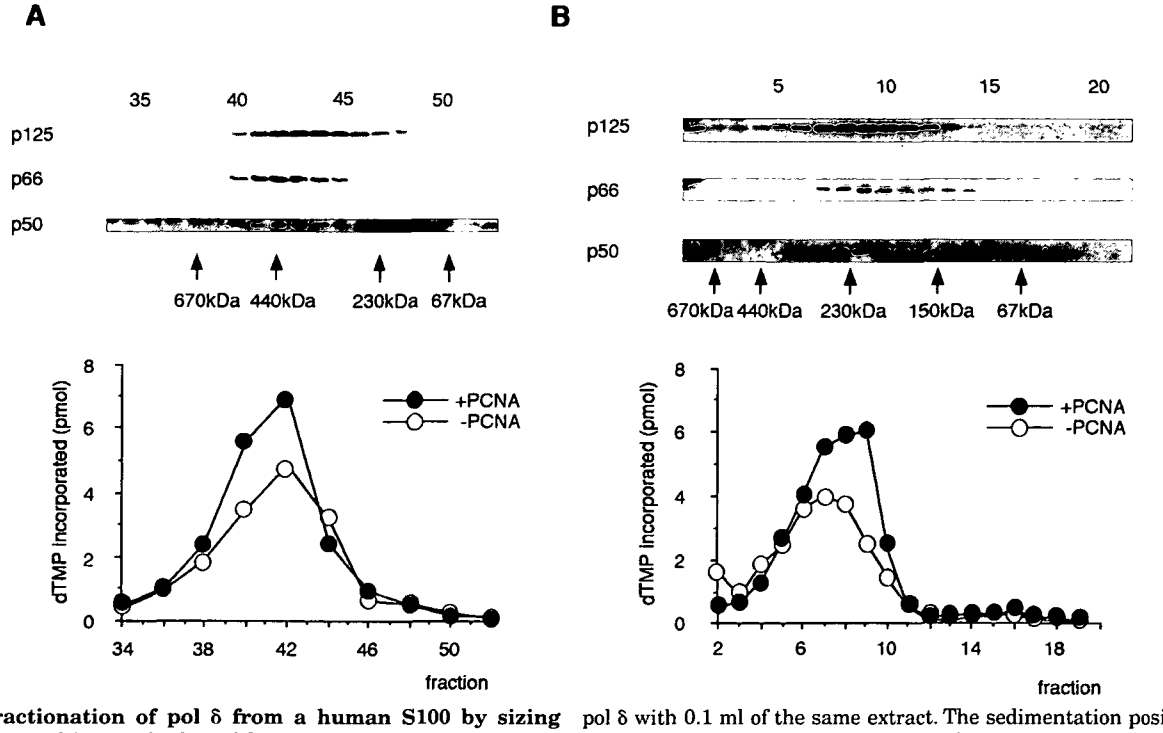

Fig. 2. **Fractionation of pol δ from a human S100 by sizing chromatographies.** (A) Sephacryl S-300 gel filtration analysis of pol δ with a human S100 extract (15 mg proteins). P125, p66, and p50 polypeptides eluted from the column were detected by immunoblot analyses as in Fig. 1 (upper panel). DNA synthesis on poly(dA)/oligo(dT) was assayed with 0.5 μl aliquots of the fractions in the presence or absence of 50 ng PCNA (closed and open circles, respectively, in the lower graph). (B) Glycerol gradient sedimentation analysis of pol δ with 0.1 ml of the same extract. The sedimentation positions of the pol δ subunits and their corresponding DNA synthesis activities are indicated as in (A). 0.25 μl aliquots of the fractions were used to measure DNA polymerase activity. The positions of size marker proteins [thyroglobulin (670 kDa), ferritin (440 kDa), catalase (230 kDa), alcohol dehydrogenase (150 kDa), and BSA (67 kDa)] used for calibration are indicated in both panels.

Reconstitution of Human pol δ with 2- or 3-Subunit Components—Previous reports have demonstrated that p125 and p50, expressed by the baculovirus expression system, are sufficient to reconstitute active human pol δ (*15*). To explore the role of p66 in pol δ DNA synthesis, we constructed baculoviruses carrying intact human p125, p50, or p66 coding sequences, one of which, p50, was tagged with 6 × (His) at the amino terminus (His-p50). The tagged p50 seemed to be functional, since we were able to obtain the same level of PCNA-dependent DNA synthesis in insect cell lysates using both tagged and untagged p50 in combination with p125 (data not shown). We prepared cell lysates co-infected with viruses carrying only p125 and His-p50 or all three components. The 3- or 2-subunit pol δ complexes were obtained by sequential chromatography of the cell extract on Ni^{2+} resin and mono Q columns, followed by glycerol gradient centrifugation.

PCNA-dependent polymerase activity was eluted at 270 mM NaCl (fraction 39) from the monoQ column if the 3-subunit pol δ fraction from the Ni^{2+} resin column was employed (Fig. 3, A and B), although the band intensities of the three subunits did not peak in this fraction. This indicates that the reconstitution of pol δ by co-infection with multiple baculoviruses is not efficient, and that heterogeneous complexes such as p125-p50 and p66-p50 predominate.

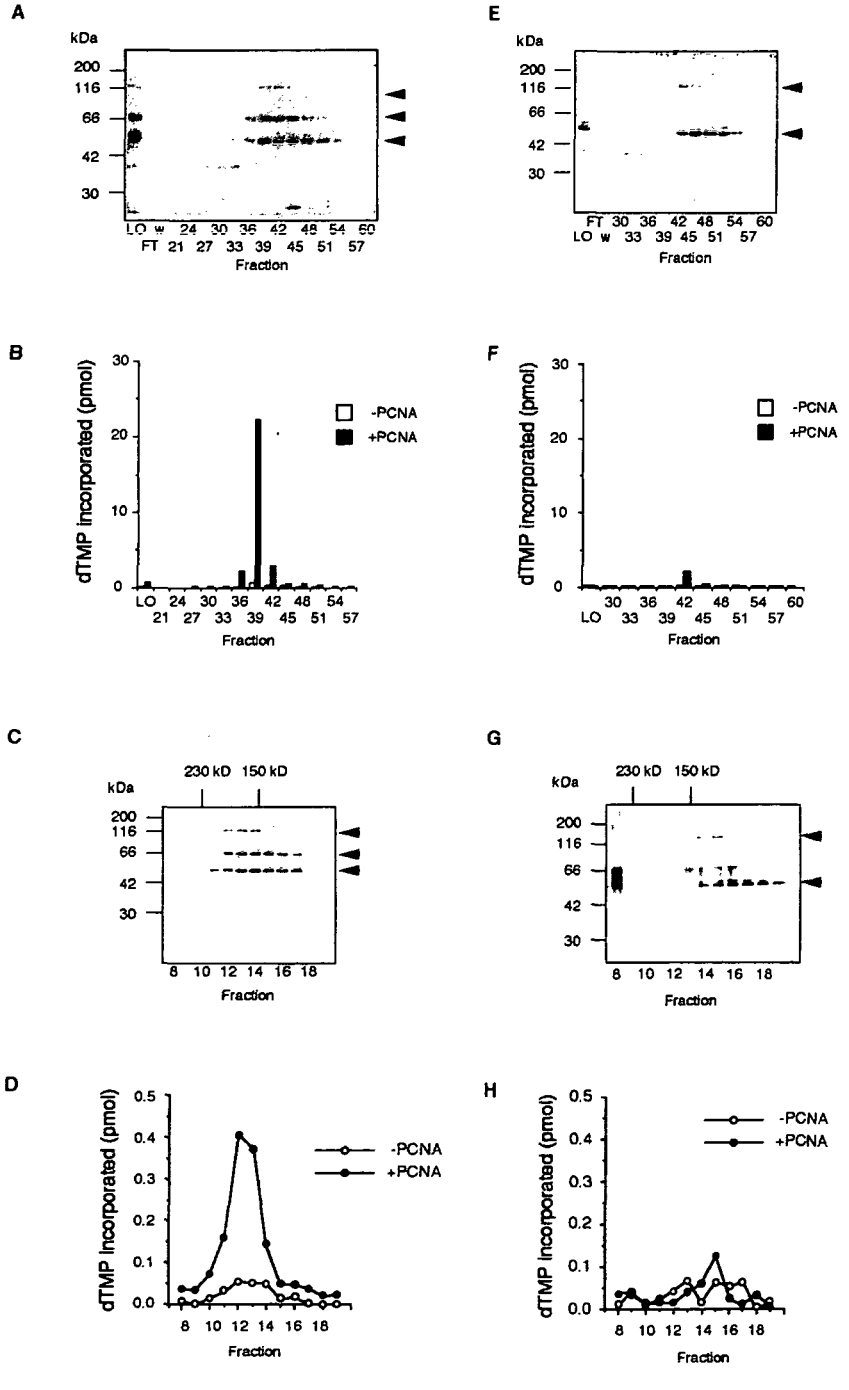

Fig. 3. **Fractionation of the recombinant 3- and 2-subunit human Pol δ.** Fractionation of the Ni^{2+} resin bound fractions obtained from cells co-expressing p125, p66, and histidine-tagged p50 (His-p50) (A–D) or from cells co-expressing p125 and His-p50 (E–H). Proteins in the Ni^{2+} resin bound fractions were separated by applying them to a mono Q column and eluting the column with a linear NaCl gradient (A, B, E, and F). Aliquots of the eluted fraction (5 μl) were subjected to 12.5% SDS-PAGE and the proteins were stained with Coomasie Blue (A, E). The DNA polymerase activities in the fractions (0.25 μl) (B, F) were assayed using poly(dA)/oligo(dT) in the presence (black bars) or absence of PCNA (white bars). The active fractions were pooled and subjected to glycerol gradient centrifugation (C, D, G, and H). Silver-staining patterns with 10 μl aliquots (C, G), and corresponding DNA polymerase activities with 0.3 μl aliquots (D, H) in the presence (filled circles) and absence (open circles) of PCNA are indicated. P125, p66, and His-p50 bands are indicated by arrowheads in (A, C, E, and G). LO; loading fraction, FT; flow through fraction and w; wash fraction. The standard proteins used to estimate the molecular mass of pol δ on the glycerol gradient were catalase (230 kDa) and alcohol dehydrogenase (150 kDa).

PCNA-dependent polymerase activity was recovered in fractions 12 and 13 after glycerol gradient centrifugation. This position on the gradient corresponds to a molecular mass of 180 kDa (Fig. 3, C and D), which is slightly slower than that of native pol δ from human cells (210 kDa, Fig. 2B). This difference might be due to differences in the subunit compositions of recombinant and native pol δ. For example, recombinant pol δ might lack the fourth subunit p12 (*22*). PCNA-dependent polymerase activity decreased significantly after glycerol gradient centrifugation (Fig. 3D).

When extracts expressing only p125 and p50 were subjected to the same purification scheme, only a limited amount of PCNA-dependent polymerase activity was recovered after Ni^{2+} chromatography, although larger amounts of p125 and p50 polypeptides were present than in the case of the 3-subunit pol δ (Fig. 3, E and F). PCNA-dependent polymerase activity eluted in fraction 42 at 310 mM NaCl, which apparently differs from the position of the 3-subunit pol δ. This result indicates that the 2-subunit and 3-subunit DNA polymerase fractions are physically different, which is probably a reflection of their different subunit configurations. After glycerol gradient centrifugation, PCNA-dependent polymerase activity of the 2-subunit pol δ could hardly be detected, and most of the 125 kDa and 50 kDa subunits seemed to sediment separately at positions corresponding to their individual molecular masses (Fig. 3, G and H). Thus, it seems to be difficult to obtain active pol δ in a highly purified form from the 2-subunit expression system. This result seems to be in conflict with a previous report (*15*), which indicated that only the p125 and p50 subunits are necessary to reconstitute active pol δ from baculovirus-infected insect cells. In fact, we could detect PCNA-dependent activity in lysate and cruder fractions containing 2-subunit pol δ (data not shown and Fig. 3F). Thus, p125 and p50 alone may be able to form an active pol δ complex, but this interaction may be unstable without additional factor(s), such as p66. Indeed, the third subunit of *S. cerevisiae* is dispensable for cell viability and seems to have only an accessory function in this organism (*18*). If this were the case, factor(s) supplied by the insect cells may have partially substituted for p66, and may have been present in the pol δ preparation described in Ref. *15*. To obtain active pol δ in a highly purified form, our data clearly show that p66 is necessary. As shown by glycerol gradient sedimentation analyses of the two pol δ forms, p66 appears to be necessary for maintaining the stability of the p125–p50 interaction. This point is further supported by the subunit-interactions described below.

Our data also indicate that active pol δ could be reconstituted without the fourth subunit. Indeed, *S. pombe* cdm1 is not an essential gene (*19*) and its function may not always be necessary for pol δ activity. However, we also observed a significant loss of pol δ activity between the Ni^{2+} resin column and glycerol gradient chromatographic steps without the fourth subunit (data not shown and Fig. 3, B and D). Thus, this subunit may stabilize pol δ in the active configuration. However, full elucidation of the role played by the fourth subunit must await future study.

Characterization of 3-Subunit pol δ—As described above, the recombinant 3-subunit pol δ has DNA polymerase activity but behaves slightly differently than native human pol δ during glycerol gradient centrifugation. We asked whether this 3-subunit recombinant human pol δ has polymerase activity comparable to that of native pol δ from human cells or calf thymus. As a source of purified recombinant pol δ, we used the monoQ fraction pool, which contained 15 μg/ml pol δ as estimated from the band intensity of the p125 band. As a source of native pol δ, we prepared pol δ from a human cell lysate by PCNA-affinity chromatography (76 μg/ml: Fig. 1) and a calf thymus extract by the conventional method (11 μg/ml). It should be noted that these pol δ preparations still contained impurities and free forms of p66 and p50. Therefore, we estimated the concentrations of pol δ in these fractions by comparing the intensities of their p125 bands with those of a series of BSA bands of known concentration. Taking these concentrations into account, the specific activities of recombinant 3-subunit pol δ, native human pol δ, and calf thymus pol δ were estimated to be 2×10^5, 2×10^6, and 6×10^7 U/mg, respectively (Fig. 4B).

The recombinant pol δ had only one-tenth and one three-hundredth of the specific activities of human native pol δ and calf thymus pol δ, respectively. The results also show that native human pol δ has one-thirtieth the activity of calf thymus pol δ. There are several possible explanations for these different specific activities. First, recombinant pol δ may still lack certain subunits, such as the fourth subunit p12, that are important for maximum activity. Second, extracts from insect cells may contain inhibitors or immature complex(es) of pol δ that interfere with DNA synthesis. Third, cell type-specific modifications of certain subunits of recombinant pol δ may be necessary to obtain the high activities of pol δ purified from human cells or calf thymus. Although we do not have any evidence at the moment in favor of either of these hypothesis, the disparities in the activities of pol δ isolated from different sources raises the interesting question of how pol δ activity is regulated. There may be some mechanism that regulates pol δ activity depending on the cell type or cell growth conditions. In addition, several sub-complexes of pol δ with distinct properties may exist. From this point of view, pol δs from human cells and calf thymus display clear differences in their processivity and p66 content as described below.

Next, we analyzed the DNA products synthesized by these polymerases (Fig. 4, C and D). Processivity was measured using poly(dA)/oligo(dT) (average length is 400 nucleotides) as a template/primer under conditions in which fewer than one nucleotide was synthesized per input primer end on average. It should be noted that we were unable to measure processivity when the size of the synthesized strand exceeded 400 nucleotides. In the presence of PCNA, recombinant human pol δ and native human pol δ synthesized DNA of up to 1,000 nucleotides with an average size of 300 nucleotides (Fig. 4C, lanes 1 and 3). On the other hand, pol δ from calf thymus synthesized slightly shorter products (up to 500 nucleotides with an average size of 200 nucleotides) in the presence of PCNA (lane 5). Since we analyzed uniformly labeled products in these experiments, the actual average sizes must be shorter than these values. Even without accurate processivity, it is apparent that the two human pol δs were judged to be about 100 nucleotides more processive than calf thymus pol δ. The shorter length of the products synthesized by calf thymus pol δ was not due to the heterogeneous combination of PCNA (human) and pol δ (bovine), because the addition of calf thymus instead of human PCNA did not change the

size of the products (data not shown). Thus, these differences in processivity may be an intrinsic characteristic of their polymerization activities.

We propose that differences in the subunit compositions of calf thymus pol δ and the human enzyme (both recombinant and native) may be why these polymerases display different processivities. For example, full-length p66 was clearly present in purified recombinant and native human pol δ fractions (Figs. 1A and 4A, 1 and 2), but not in the calf thymus pol δ fraction (Fig. 4A, 1 and 2). Pol δ from calf thymus has also been reported to lack the full-length p66 subunit (*22*). Instead, some degraded p66 forms, which may not have the PCNA binding motif, were detected in the calf thymus fractions. Actually, we could detect smaller polypeptide bands at 34 and 25 kDa (*22*, *23*) in the purified calf thymus fraction by silver staining, although these bands did not react with the 2A1-C11 antibody, probably because they had lost the epitope recognized by this antibody. If p66 contributes to the polymerization activity of pol δ, we suggest that an active p66 processing mechanism might regulate pol δ function *in vivo*. Through such processing, pol δ activity could be modified to preferentially synthesize, for example, DNA on the leading or lagging strand, or to carry out short patch synthesis in DNA repair. This point could be verified by studying the activities of recombinant pol δ containing variously truncated forms of p66.

Next, we studied the abilities of these polymerases to synthesize long stretches of DNA on a singly-primed M13 DNA in the presence of RPA and RFC, conditions that mimic the replication elongation reaction *in vivo* (*10*). As shown in Fig. 4D, recombinant pol δ could synthesis DNA longer than 1 kb, as is the case for native human and calf thymus pol δs. These results indicate that recombinant pol δ is as functional as native pol δs for DNA synthesis in the presence of other replication factors.

Interactions between the Three pol δ Subunits—To study the functions of the p66 subunit in the pol δ complex, its interactions with other pol δ subunits were analyzed by pull down assays employing His-tagged polypeptides. For this purpose, we prepared histidine-tagged p66 (His-p66) in addition to His-p50. His-p66 was also active, since we could obtain the same activity with this subunit as with native p66 (data not shown). Insect cells infected with various combinations of baculoviruses expressing each of the three subunits were prepared and their lysates were incubated with Ni^{2+} resin beads. The bead-bound fractions were recovered and analyzed by immunoblotting with antibodies

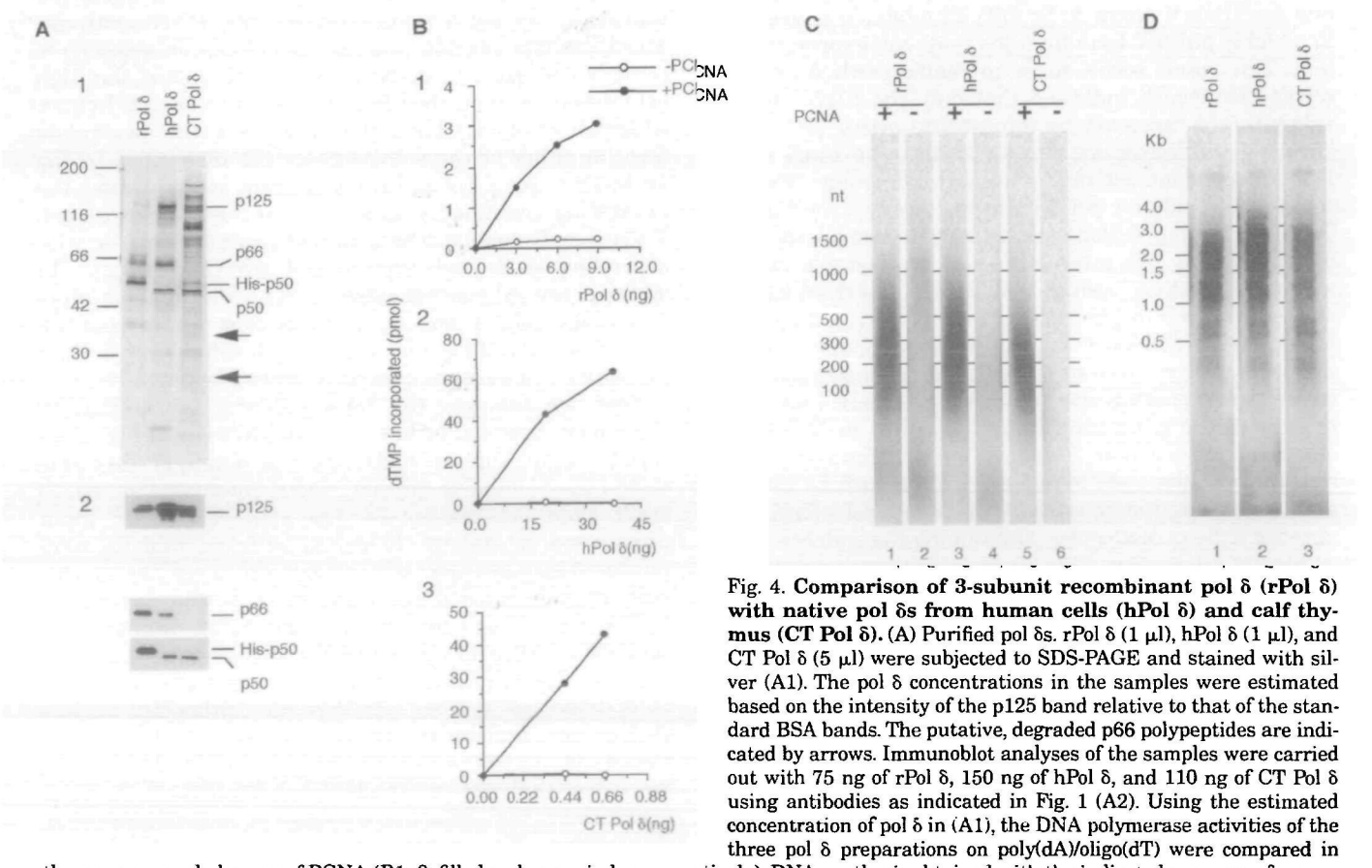

Fig. 4. **Comparison of 3-subunit recombinant pol δ (rPol δ) with native pol δs from human cells (hPol δ) and calf thymus (CT Pol δ).** (A) Purified pol δs. rPol δ (1 μl), hPol δ (1 μl), and CT Pol δ (5 μl) were subjected to SDS-PAGE and stained with silver (A1). The pol δ concentrations in the samples were estimated based on the intensity of the p125 band relative to that of the standard BSA bands. The putative, degraded p66 polypeptides are indicated by arrows. Immunoblot analyses of the samples were carried out with 75 ng of rPol δ, 150 ng of hPol δ, and 110 ng of CT Pol δ using antibodies as indicated in Fig. 1 (A2). Using the estimated concentration of pol δ in (A1), the DNA polymerase activities of the three pol δ preparations on poly(dA)/oligo(dT) were compared in the presence and absence of PCNA (B1–3, filled and open circles, respectively). DNA synthesis obtained with the indicated amounts of recombinant pol δ (B1), human pol δ (B2), and calf thymus pol δ (B3) are shown. In (C), the products of DNA synthesis on poly(dA)/oligo(dT) with the different pol δs are shown. The products were separated by 1.5% alkaline agarose gel electrophoresis and visualized by autoradiography. Products obtained with 0.4 U of rPol δ (lanes 1 and 2), 0.4 U of hPol δ (lanes 3 and 4), or 0.5 U of CT Pol δ (lanes 5 and 6) in the absence (lanes 2, 4, and 6) and presence (lanes 1, 3, and 5) of PCNA are shown in (C). In (D), the DNA synthesis products obtained with 0.1 U of rPol δ (lane 1), 0.1 U of hPol δ (lane 2), or 0.1 U of CT Pol δ (lane 3) on singly-primed M13 DNA were separated by 1.2% alkaline agarose gel electrophoresis and autoradiographed as indicated in (C).

against the individual subunits (Fig. 5). p125 was recovered with His-p50, but not with His-p66 (lanes 2 and 5). p50 was precipitated efficiently with His-p66 (lane 8), as seen in yeasts (*18*, *20*, *24*). Both p125 and p66 were recovered with His-p50 upon co-expression (lane 11). Judging from the band intensities of the subunits on the immunoblots after one exposure, the ratios of the recovered amounts of p125 to p50 varied between 3.3 and 1.0 depending on whether or not p66 was present in the extracts (lanes 11 and 2). These results indicate that p125 has significant binding affinity for p50, and that p66 further increases the stability of the interaction. We have obtained consistent results with respect to the reconstitution of pol δ with or without p66 (Fig. 3). Therefore, one of the roles of p66 may be to stabilize the interaction between p125 and p50. Since p66 has apparent affinity only for p50, it may induce some conformational change in p50 that favors its interaction with p125.

Pol δ Interacts with PCNA through p66 and p125—The interaction of pol δ with PCNA is crucial for its DNA synthesis activity. It has been reported that PCNA binds specifically to p125 (*23*, *33*, *34*). Interestingly, mammalian p66 and its homologues, cdc27 in *S. pombe* and Pol 32p in *S. cerevisiae*, all possess the highly conserved PCNA binding motif, and the yeast homologues interact with PCNA (*18*, *24*). To understand the importance of the two potential PCNA interactions in pol δ for its activity, we studied the association between PCNA and individual human pol δ subunits.

Insect lysates expressing human pol δ subunit(s) in various combinations were incubated with PCNA-linked beads, and the PCNA-bound fractions were studied by immunoblot analyses (Fig. 6). p50 did not interact with PCNA at all (lane 2), but p66 (lane 5) and p125 (lane 8) were observed to bind to PCNA independently. The same result could be seen with the lysate co-expressing p125 and p66 (lane 14), in which the two subunits exist in their free forms as shown in Fig. 5, lane 5. With the lysate co-expressing p125 and p50, only the p125 subunit was precipitated with PCNA beads, although p125 and p50 formed a complex (lane 11, Fig. 5, lane 2). This suggests that the binding of p125 to PCNA destabilizes its interaction with p50 or that the interaction between p125 and p50 is not strong enough to survive the pull-down procedure. When p50 and p66 were co-expressed, they formed a complex as shown above (Fig. 5, lane 8), which resulted in their co-precipitation by the PCNA beads (lane 17). When the three subunits were co-expressed together, they were all precipitated more efficiently by the PCNA beads (lane 20). For example, p66 in the three-subunit complex was recovered more efficiently (lane 20; 37% of the input shown in lane 19) than in other subunit combinations, such as p66 alone, p66 plus p125, or p66 plus p50, whose recoveries were 12, 11, and 16%, respectively (lanes 5, 14, and 17). Similarly, the recovery of the p125 subunit in the 3-subunit complex was higher at 22% (lane 20) than at other subunit combinations, which yielded only 5, 15, and 2% of the input p125 in lanes 8, 11, and 14, respectively. p50 was also precipitated more efficiently (lane 20, 32%) as part of the three subunit complex than as part of the p66-p50 complex (lane 17, 6%). All these data indicate that the three subunit pol δ complex interacts more efficiently with PCNA than other subunit combinations.

Since p125 and p66 interact with PCNA independently, they must have their own PCNA binding motifs as reported for other PCNA-binding proteins. Indeed, the third subunits of yeast, cdc27 and Pol32p, interact with PCNA specifically (*18*, *24*). However, the yeast p125 homologues have not been shown to interact with PCNA (*18*, *35*, *36*), although mammalian p125 can bind to PCNA as shown by others (*23*, *33*, *34*) and by this work. The significance of the differences in PCNA binding modes of mammalian and yeast pol δs is unclear at this stage, but may suggest multiple roles for pol δ in higher eukaryotes, possibly mediated by p66 processing as mentioned above.

In summary, we have shown that p66, the human counterpart of yeast cdc27 and Pol32p, is a functional component of purified native DNA polymerase δ. Indeed, the p66 subunit was found to be required for the reconstitution of

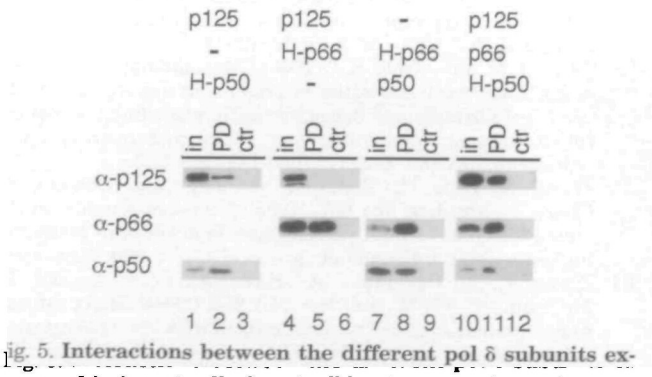

Fig. 5. **Interactions between the different pol δ subunits expressed in insect cells.** Insect cell lysates expressing various combinations of the pol δ subunits as indicated at the top were incubated with Ni^{2+} resin beads, and the bound fractions were analyzed by immunoblotting using antibodies. Bands detected by Western blot analysis with specific antibodies, performed as described in Fig. 1, except for 8A5-E3 for anti-p125 antibody, are indicated with the label "α-p125," "α-p66," or "α-p50." "in," "PD," and "ctr" represent the cell lysate, the fraction that bound to the Ni^{2+}-charged beads and the fraction that bound to the Ni^{2+}-uncharged beads, respectively. The histidine tagged peptide is indicated as H-p50 or H-p66 at the top.

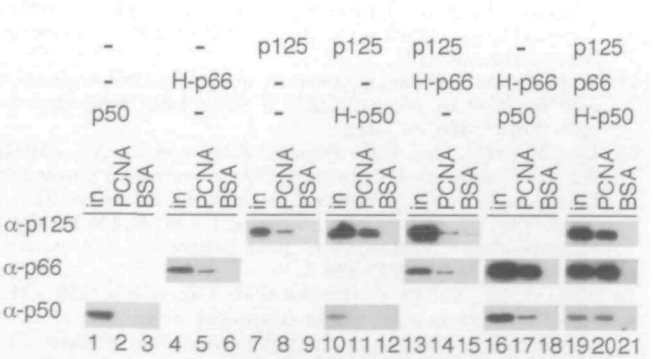

Fig. 6. **Interactions between PCNA and recombinant pol δ subunits.** PCNA-bound proteins were studied by a pull-down assay using PCNA-bound beads. Insect cells expressing various combinations of recombinant pol δ subunit(s) as indicated at the top were mixed with PCNA-bound beads or BSA-bound beads. The fractions that bound to the beads were subjected to immunoblot analysis using specific antibodies against α-p125, α-p66, and α-p50. Samples; cell lysate, the fraction that bound to the PCNA-beads, and the fraction that bound to the BSA-beads are indicated by the labels "in," "PCNA," and "BSA," respectively. The histidine tagged peptide is indicated at the top as H-p50 or H-p66.

active pol δ from recombinant pol δ subunits expressed in a baculovirus expression system. Although the recombinant 3-subunit pol δ had a lower specific activity compared to that of native human pol δ, it was able to synthesize DNA as well as native pol δs in the presence of RPA and RFC. Pull-down assays showed that p66 interacts with p50, and stabilizes the weak interaction between p125 and p50. Both p125 and p66 interacted with PCNA independently, and the presence of p66 increases the affinity of pol δ for PCNA.

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