Identification of a Fourth Subunit of Mammalian DNA Polymerase δ*

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A 12-kDa and two 25-kDa polypeptides were isolated with highly purified calf thymus DNA polymerase δ by conventional chromatography. A 16-mer peptide sequence was obtained from the 12-kDa polypeptide which matched a new open reading frame from a human EST (AA402118) encoding a hypothetical protein of unknown function. The protein was designated as p12. Human EST AA402118 was identified as the putative human homologue of Schizosaccharomyces pombe Cdm1 by a tBlastn search of the EST data base using S. pombe Cdm1. The open reading frame of human EST AA402118 encoded a polypeptide of 107 amino acids with a predicted molecular mass of 12.4 kDa, consistent with the experimental findings. p12 is 25% identical to S. pombe Cdm1. Both of the 25-kDa polypeptide sequences matched the hypothetical KIAA0039 protein sequence, recently identified as the third subunit of pol δ. Western blotting of immunoaffinity purified calf thymus pol δ revealed the presence of p125, p50, p68 (the KIAA0039 product), and p12. With the identification of p12 mammalian pol δ can now be shown to consist of four subunits. These studies pave the way for more detailed analysis of the possible functions of the mammalian subunits of pol δ.

DNA polymerase δ (pol δ) is the key polymerase that is involved in the replication of chromosomal DNA in eukaryotic cells. Studies of the in vitro replication of SV40 DNA have established that pol δ plays a central role in mammalian DNA replication (1). Proliferating cell nuclear antigen (PCNA), which is involved in both initiation and elongation of DNA synthesis, is present (7, 8). The current view of DNA replication at the replication fork is that the pol δ complex is responsible for synthesis of the leading strand and that pol δ also participates in synthesis of the lagging strand (1). DNA polymerase α/primase is primarily involved in the synthesis of RNA primers plus short stretches of DNA primers on the lagging strand, and the elongation of the primers is performed by DNA polymerase δ in a process that requires “polymerase switching” (9). Additional proteins, including topoisomerase and helicase activities, are also involved in the movement of the replication fork (1).

Mammalian pol δ has been rigorously isolated by conventional methods as a heterodimer consisting of two subunits, p125 and p50 (3). The subunit structure of pol δ has been the focus of recent investigations in yeast, and these have led to the identification of additional subunits. In Schizosaccharomyces pombe, pol δ is believed to consist of at least four subunits: a large catalytic subunit (Pol3) and three smaller subunits (Cdc1, Cdc27, and Cdm1) (10, 11). Pol δ purified from Saccharomyces cerevisiae is composed of three subunits: Pol3p, Pol31p/Hys2p, and Pol32p (12–14). The pol δ core purified from calf thymus consists of two subunits: p125 and p50 (3). However, we have found that recombinant p125 catalytic subunit alone can only be stimulated by PCNA by 2-fold at most, while the overexpressed p125/p50 heterodimer is stimulated much less than pol δ purified by immunoaffinity chromatography (15, 16). These findings suggest that additional factor(s) which may be removed during protein purification are required for a full PCNA response in our assay. This is consistent with the hypothesis that mammalian pol δ may also contain additional subunits.

Using the proteomics approach, by peptide sequencing of polypeptides associated with the core pol δ in highly purified preparations isolated by p125 immunoaffinity chromatography, we have previously identified a 68-kDa polypeptide that is encoded by KIAA0039 and which is associated with the pol δ core. The p68 polypeptide is the third subunit of mammalian pol δ (17). Using a combination of proteomic approaches and GenBank searches, we have identified a novel subunit of pol δ that is the mammalian homologue of Cdm1, which in S. pombe is the fourth subunit of pol δ. Mammalian pol δ may thus consist of at least four subunits.

EXPERIMENTAL PROCEDURES

MATERIALS—cDNA AA402118 was obtained from ATCC (Rockville, MD). Calf thymus tissue was obtained from Animal Technologies (Tyler, TX). Q-Sepharose, SP-Sepharose, heparin-Sepharose, Mono Q columns, and Mono S columns were obtained from Amersham Pharmacia Biotech (Piscataway, NJ).

Conventional Purification of Calf Thymus Pol δ—The immunoaffinity purification was performed as described previously by Jiang et al. (18).

Conventional Purification of Calf Thymus Pol δ—The following buffers were used: lysis buffer consisted of 50 mM Tris-HCl, pH 7.8, 1 mM MgCl2, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.25 mM sucrose, 5% glycerol, 0.2 mM phenylmethylsulfonyl fluoride, 0.1 mM/ml bacitracin, 10 mM benzamidine. TGEDD buffer consisted of 50 mM Tris-HCl, pH 7.8, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and 0.1% NP-40. PEG purification was performed as described previously by Jiang et al. (18).
5% glycerol. KGEEED buffer consisted of 20 mM potassium phosphate,
ph 7.0, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and 5% glycerol.
All steps were carried out at 0–4 °C. Pol δ activity was assayed using poly(dA)poly(dT) as a template (19). Eight hundred grams of frozen
calf thymus tissue in 4 liters of lysis buffer were homogenized in a
Waring blender. The suspension was centrifuged at 5,000 rpm at 4 °C
for 1 h and filtered through glass wool. The supernatant was mixed with
1.5 liters of DE52-cellulose equilibrated with TGEED buffer and stirred for
30 min. The mixture was then filtered through a Buchner funnel. The
DE52-cellulose was washed with TGEED and the pol δ activity was
striped off with 20% ammonium sulfate in TGEED buffer.

The 3.5 liters of DE52-cellulose fraction were precipitated by the
addition of 320 g/liter of ammonium sulfate. The suspension was stirred
for 30 min, kept on ice for an additional 30 min, and then centrifuged at
10,000 × g for 45 min. The precipitate was resuspended in TGEED and
dialyzed against TGEED buffer containing 50 mM NaCl with two
times and applied on a to 70-ml Q-Sepharose column. The bound proteins were eluted with a linear gradient of 50–750 mM NaCl in
TGEED. The peak fractions containing pol δ activity were pooled and
dialyzed against KGEEED buffer containing 25 mM KCl. The Q-Sepharose
fraction was loaded on a to 50-ml SP-Sepharose column. Pol δ was eluted with a linear gradient of 25–650 mM KCl in KGEEED. The fractions containing pol activity were pooled and applied to a 10-ml
Mono Q column, which was equilibrated with TGEED buffer containing
25 mM NaCl. The column was washed with 40 ml of TGEED buffer containing 25 mM NaCl. The activity was eluted with a gradient of
25–650 mM NaCl in 100 ml of TGEED at a flow rate 0.4 ml/min. The
Mono Q fractions were pooled and dialyzed against KGEEED buffer containing 25 mM NaCl and applied to a 5-ml heparin-Sepharose col-
umn. The column was washed with 2 column volume of TGEED conten-
ing 25 mM NaCl and eluted with a 50-ml gradient of 25–750 mM
NaCl in TGEED at a flow rate 0.5 ml/min.

The heparin-Sepharose fraction (17 ml) was dialyzed against two
changes of KGEEED buffer containing 50 mM KCl and loaded onto a 1-ml
Mono S column equilibrated with KGEEED buffer. The column was
washed with 5 ml of KGEEED buffer and then eluted with a 20-ml linear
gradient of KGEEED buffer from 50 to 700 mM KCl. The active fractions were combined and dialyzed against KGEEED buffer until the conduc-
tivity reached that of KGEEED containing 50 mM KCl. The fraction was
applied to a Source Q15 column. The enzyme was eluted with a linear
gradient of 50–650 mM NaCl in KGEEED. The fractions with enzyme
activity were pooled (3.0 ml) and concentrated to 270 µl using Centricon
30 (30,000 MW cutoff, Amicon). The concentrated enzyme (270 µl) was chromatographed on a FPLC Superdex 200 column equilibrated with
TGEED buffer containing 150 mM NaCl. Fractions above 30% of
the maximum peak of activity were pooled.

Protein Sequence Analysis—Polypeptide bands excised from a
Coomasie Blue-stained gel were used for protein sequence analysis by the
Harvard Microchemistry Facility using a microcapillary reverse-phase
high performance liquid chromatography nano-electrospray tandem
mass spectrometry (μLC-MS-MS) on a Finnigan LCQ quadrupole ion
trap mass spectrometer.

Antibodies—Peptide rabbit polyclonal antibodies against p125/hCdm1
and p68 were generated from a commercial source (SynPep, Dublin, CA)
and purified by a peptide affinity column made from the same peptide
antigen. For p12, the peptide contains amino acid residues 77 to 94 of
p12 (H_N-GLEPPEPVQVLYHGPDCOOH). For p68 (encoded by
KIAA0039) the 19-amino acid peptide from near the extreme N termi-
nus of p68 was used (H_N-TDQNKIVTYW-LSYTLGVCDOOH).

Western Blot Analysis—Proteins were transferred to 0.45 µm nitro-
cellulose membranes (Bio-Rad) after SDS-PAGE in transfer buffer (25
mM Tris-HCl, 192 mM glycine containing 10% v/v methanol) in a Genie
blotter (Idea Scientific, Minneapolis, MN) for 75 min for 0.8-mm thick
gels using a constant voltage of 12 volts. The membrane was incubated in
TBST buffer (20 mM Tris-HCl, pH 7.8, 150 mM NaCl, 0.05% Tween
20) containing 5% fat-free dry milk for 1 h at room temperature and
washed briefly with TBST. The membrane was incubated with primary
antibody for 1 h at room temperature or overnight at 4 °C. The mem-
brane was washed 3× with TBST and incubated with horseradish
peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG (Pierce,
Rockford, IL) for 1 h. The membrane was washed 3× with TBST. SuperSignal West Pico Chemiluminescent Substrate was used for sig-
nal detection (Pierce) and the signal was captured on a Blue Bio film
(Denville Scientific, Metuchen, NJ) after exposure for 15 s to 30 min and
developed.

RESULTS

Demonstration That p68 and p12 Are Subunits of Mammalian Pol δ—We have previously devised a conventional pro-
dure for the rigorous isolation of the pol δ core enzyme containing
p125 and p50 (3). In order to isolate a multisubunit form of
mammalian pol δ, a new purification scheme was devised, which
allowed the isolation of pol δ core that retained associ-
ated polypeptides. This involved successive chromatographies
on DE52, Q-Sepharose, SP-Sepharose, Mono Q, heparin-Sepha-
rose, Mono S, Source Q15, Superdex 200 supports, including
four FPLC chromatography steps (Mono Q, Mono S, Source Q15, and
Superdex 200). Table I shows the purification of pol δ by
this means. The specific activity of the preparation (about
9,000 units/mg) was comparable with that of pol δ purified by
immunoaffinity chromatography (18). Review of a number of
preparations isolated by the latter method gave an average
specific activity about 10,000 units/mg, with a PCNA stimula-
tion of 20–40-fold. The PCNA stimulation of 30-fold was found
for the preparation obtained by the new procedure. This is
similar to that of the immunoaffinity purified enzyme. The
average specific activity of the purified recombinant pol δ het-
rodimer in our hands is about 2000 units/mg, with maximum
PCNA stimulations of 6–10-fold. These results indicate that
rigorously purified pol δ p125/p50 heterodimer has lost a sig-
ificant fraction of its ability to respond to PCNA.

The final purification step used was FPLC gel filtration on
Superdex 200. Calibration of the column showed that the peak
of pol δ activity was eluted at a position indicating a much higher
molecular weight (280,000) than can be accounted for by
the two-subunit core. The Coomassie Blue staining of this pol δ
complex is shown in Fig. 1. There were six major bands in the
peak fractions of pol δ from fractions 48 to 50; these were of 125
kDa, 50 kDa, a doublet at about 25 kDa and a doublet at about
12 kDa. These polypeptide bands were excised from the Coom-
assie Blue-stained gel and sequenced at the Harvard Micro-
chemistry Facility using μLC-MS/MS methods. The sequencing
results are displayed in Table II. Both the 25-kDa polypeptides
were identified as the proteolytic products of KIAA0039, which
was recently found to be associated with pol δ by a PCNA
overlay assay (17). The KIAA0039 product was also eluted with
pol δ from a PCNA affinity column (20). Our data support the
view that p68 is the mammalian third subunit of pol δ but

| Fraction | Total volume | Protein | Total units | Specific activity |
|----------|--------------|---------|-------------|------------------|
| DE 52    | 3.615        | 18,075  | 397,000     | 22               |
| Q-Sepharose | 470   | 2,585   | 295,000     | 113              |
| SP-Sepharose | 176  | 722     | 468,000     | 648              |
| Mono Q   | 34           | 76.5    | 56,000      | 736              |
| Heparin-Sepharose | 17  | 12.2    | 39,000      | 3,211            |
| Mono S   | 3.3          | 2.1     | 9,400       | 4,500            |
| Source Q15 | 3.0   | 0.15    | 900         | 6,000            |
| Superdex 200 | 0.8   | 0.022   | 200         | 8,900            |
indicate that it is highly susceptible to proteolysis.

The sequence of the upper 12-kDa band showed that this was derived from keratin. The second 12-kDa polypeptide was found to be a novel protein. The partial sequence obtained from this protein was QFDLAWQYGPCTGITR (Table II). This sequence was searched against the known protein data bases, which did not provide a match. A tBlastn search of the EST data base showed a match with one human EST sequence, AA402118, which, however, did not have a well defined open reading frame. Concurrently, the S. pombe Cdm1 protein sequence, which represents the fourth subunit of pol δ, was used for a tBlastn search at NCBI. This also retrieved the human EST sequence, AA402118. The EST cDNA clone was obtained from ATCC and was resequenced and corrected. The corrected sequence contained an open reading frame that encoded a protein of 107 amino acid residues, with a predicted molecular mass of 12.4 kDa. This protein was designated as p12. The corrected DNA sequence has been deposited in GenBank with the accession number AJ179890 (Fig. 2).

The results indicate that p12 is a likely human homologue of the S. pombe Cdm1 protein, which has been reported to be the fourth subunit of S. pombe pol δ (10, 21). The S. pombe Cdm1 protein has a calculated molecular mass of 18.5 kDa and an apparent size of 22 kDa on SDS-PAGE, and is significantly larger than human p12. Sequence alignments were performed to assess the possible relationships between these two proteins. Protein sequence alignment indicates that the identity between p12 (107 residues) and S. pombe Cdm1 (160 residues) is 25% and the similarity is 39% (Fig. 3). It can be seen that the main region of identity of p12 is with the C-terminal half of S. pombe Cdm1.
residues 48 to 94 of p12 shows that there is a 44% identity. This degree of similarity is sufficient for p12 to be regarded as the mammalian homologue of \textit{S. pombe} Cdm1. Taken together with the sequence identification of the p12 and its co-purification with the calf thymus pol \textit{d} core through eight chromatography procedures, these findings provide strong evidence for the identification of p12 as a novel subunit of mammalian pol \textit{d}.

Western Blot Analysis of Immunoaffinity Purified Pol \textit{d}—We had previously shown that pol \textit{d} isolated by immunoaffinity chromatography contains the pol \textit{d} core in association with a number of other polypeptides (18), and also displayed a much higher molecular weight than could be accounted for by the core on gel filtration analysis (22). The failure to observe p12 in these studies could be due to its small size and the fact that it migrated close to the dye front under the conditions used. A preparation of pol \textit{d} was purified from calf thymus using immunoaffinity chromatography (18) and the preparation was assessed for the presence both of the p68 and p12 subunits. The presence of these two polypeptides on SDS-PAGE gels of the preparation are shown in Fig. 4. Polypeptides corresponding to 68 and 12 kDa were prominent components of the preparation, and their identity as the p68 and p12 polypeptides was confirmed by Western blotting (Fig. 4). Thus, the presence of all four subunits of pol \textit{d} (p125, p50, p68, and p12) were demonstrated in this preparation (Fig. 4). The KIAA0039 product in the Western blot was 68 kDa.

p68 Is the Third Subunit of Mammalian Pol \textit{d}, the Homologue of \textit{S. pombe} Cdc27 and \textit{S. cerevisiae} Pol32p—The p68 sequence has a conserved p21 Waf1-like PCNA binding motif at the extreme C terminus, as does \textit{S. pombe} Cdc27 and \textit{S. cerevisiae} Pol32p (13). The p68 sequence encoded by KIAA0039 was aligned with the sequences of Cdc27 and Pol32p (Fig. 5). Analysis of the alignments showed that p68 shares little sequence identity with Cdc27 and Pol32p. The only sequence conservation was the C-terminal PCNA binding motif in these three sequences. p68 and Pol32p both have nuclear localization motifs. p68 also has an unique proline-rich motif. Pairwise alignments using the Clustal W 1.8 program show that between Pol32p and Cdc27, Pol32p and p68, or Cdc27 and p68 there is only 15 to 16% sequence identity (not shown). However, evaluation of the significance of the alignment score for p68 with Cdc27 using the PRSS program provided a score of 0.4, i.e. the alignment score (% identity) would be attained by chance against the randomly shuffled Cdc27 sequence only 0.4 times in 100 attempts. This indicates that the similarity between these two proteins is significant.

### Table III

| DNA polymerase \textit{d} | Mammalian | \textit{S. pombe}\textsuperscript{a} | \textit{S. cerevisiae}\textsuperscript{b} |
|--------------------------|-----------|-----------------|-----------------|
| The catalytic subunit p125 | 125 kDa, Pol3 | 125 kDa, Pol3p | |
| The second subunit p50 | 55 kDa, Cdc1 | 58 kDa, Pol31p | |
| The third subunit p68\textsuperscript{c} | 54 kDa, Cdc27 | 55 kDa, Pol32p | |
| The fourth subunit p12\textsuperscript{d} | 22 kDa, Cdm1 | Not found | |

\textsuperscript{a} Isolated from \textit{S. pombe} (10).

\textsuperscript{b} Reconstituted as a three-subunit enzyme (13).

\textsuperscript{c} Identified as bovine p68 (encoded by KIAA0039) in p125 immunoaffinity purified pol \textit{d} (17). Isolated by PCNA affinity chromatography of mouse FM3A cell extracts (20).

\textsuperscript{d} Identified in this study.

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FIG. 4. Western blot analysis of pol \textit{d} subunits purified by p125-immunoaffinity chromatography from calf thymus. Calf thymus pol \textit{d} was purified through DE52, phenyl-agarose, and p125 immunoaffinity column chromatographies in the presence of protease inhibitors (18). Panel A, activity assay of the fractions eluted from the immunoaffinity column using poly(dA)/oligo(dT) as the template in the absence (closed triangles) and presence (closed circles) of PCNA. Panel B, Coomassie Blue staining of peak fraction number 28. Panel C, Western blotting of peak fraction number 28 using monoclonal antibody 78F5 against p125, 13D5 against p50, and rabbit peptide polyclonal antibodies against p68 and p12.

FIG. 5. Multiple sequence alignment of p68, Pol32p, and Cdc27. p68 (the protein product of KIAA0039, BAA05039) with \textit{S. pombe} Cdc27 (P30261) and \textit{S. cerevisiae} Pol32p (CAA89571) were analyzed using the Clustal 1.8 program. The PCNA binding motif is highlighted in dark gray. The putative nuclear localization signals of p68 and Pol32p are highlighted in light gray. The unique proline-rich motif in p68 is also highlighted.
The Fourth Subunit of Pol δ

**Table IV**
The third subunits and putative third subunits of pol δ

| Organism* | Accession No. | Protein | Length | PCNA binding motif |
|-----------|---------------|---------|--------|-------------------|
| 1         | BAA05039      | p68     | 466 amino acids | 355QVISITGGFORL466 |
| 2         | CAA99585.1    | p68     | 445 amino acids | 475NSMTSFHKKT485 |
| 3         | AAD35071.1    | p50     | 509 amino acids | 455QGNMSSFFKFC459 |
| 4         | AAD38629      | p125    | 431 amino acids | 425QAG1NFFSKCC431 |
| 5         | P30261        | Cdc27   | 372 amino acids | 365QKSI1SMSFGKR72 |
| 6         | CAA9571       | Pol32p  | 350 amino acids | 315QGTL2SFHRKKA55 |

*1. Homo sapiens; 2. C. elegans; 3. A. thaliana; 4. D. melanogaster; 5. S. pombe; 6. S. cerevisiae.*

**DISCUSSION**
The thrust of the earlier studies of pol δ was the rigorous isolation of the enzyme which culminated in the isolation of a two-subunit enzyme, containing a catalytic subunit of 125 kDa and a second subunit of 50 kDa (3, 23). The major difficulties in this process were the small amounts of protein available and the likelihood of proteolysis using extensive purification schemes due to the fragile nature of the mammalian system compared with other systems, i.e. prokaryotic or lower eukaryotes such as yeast. Recently, expression systems for the p125 (15, 24, 25), the p50 subunit (16), and the recombinant heterodimer have been developed (26). The p50 subunit has no known enzymatic functions, but has been shown to be required for the response of the p125 subunit to PCNA (16, 26). In this study we have shown for the first time the isolation of a four-subunit mammalian pol δ enzyme. This newly isolated pol δ contains the third subunit p68 and a previously unknown subunit, p12. The latter two are the mammalian homologues of S. pombe Cdc27 and Cdm1, respectively.

The association of p68 and p12 with pol δ was demonstrated by their isolation with p125 and p50 from calf thymus through extensive purification involving multiple conventional column chromatographies as well as by immunoaffinity chromatography. The conventional procedure included several FPLC steps including gel permeation chromatography. The strong association of the p68 and p12 polypeptides with the pol δ core provide very strong evidence for the proposal that these represent subunits of pol δ. p68 has also been isolated from mouse cell extracts using a PCNA affinity column in association with the pol δ core consisting of the p125 and p50 subunits (20). There are extensive technical problems associated with the identification of subunits of mammalian pol δ. As encountered in our studies, these include the susceptibility of the p68 polypeptide to proteolysis and the difficulties of isolation of pol δ from animal tissues to study stoichiometries of pol δ subunits in native enzyme preparations. Nevertheless, in these studies it is demonstrated that it is possible to rigorously isolate pol δ from calf thymus in a form which retains the p68 and p12 polypeptides. A key difference in the new method from the older procedure (18) was the avoidance of single-stranded DNA cellulose chromatography.

The identification of the fourth subunit of pol δ in mammalian systems now provides a parallel for the situation found in yeast. A comparison of the subunit structures of pol δ from the mammalian and the two yeast models is shown in Table III. The catalytic subunit of mammalian pol δ is strongly conserved in evolution, and shares a high degree of homology with the corresponding catalytic subunits in S. pombe and S. cerevisiae, the identity being greater than 48% (27). The p50 subunit is less conserved than the catalytic subunit, the identity between p50 and S. pombe being 33% (11). Furthermore, the finding that PCNA from human or yeast origin can activate the heterologous pol δ preparations strongly suggests that the pol δ complex is functionally conserved to a high degree (28). The functions of these subunits are still incompletely understood.

The third subunit of S. pombe pol δ was only recently identified (10) and is encoded by the cdc27+ gene, which is needed for the transition of G2/M in the cell cycle (11). The third subunit of S. cerevisiae pol δ is Pol32p, was isolated and identified in 1998. It was proposed as a candidate for dimerization factor of pol δ (13) based on the finding that the recombinant three-subunit enzyme could be shown to behave as a dimer on gel filtration (13). In addition, Pol32p was found to interact with the pol α catalytic subunit by the yeast two-hybrid method (29). These results suggest that Pol32p can (a) dimerize pol δ at the replication fork, and (b) provide a means for the proposed "polymerase" switch at the lagging strand through the interaction with pol α as suggested by Waga et al. (1).

p68, the mammalian homologue of S. pombe Cdc27, KIAA0039 was isolated from a PCNA affinity column (20) and from an immunoaffinity column of pol δ p125 (17). The third subunits of pol δ share a very low degree of similarity. In fact, Blast searches with Cdc27 failed to identify either p68 or Pol32p. tBlast searches using Pol32p only identified a Droso phila melanogaster third subunit of pol δ. Similarly, using p68 the putative Caenorhabditis elegans and Arabidopsis thaliana third subunit of pol δ were identified (Table IV). As already noted ("Results"), the third subunits of human, S. pombe, and S. cerevisiae are poorly conserved, although the relationships based on the alignments can be shown to be significant. The third subunits of pol δ from different species all contain a putative p21waf1-like PCNA binding motif (30, 31) at the extreme C terminus. An important aspect of the third subunit is that it interacts with PCNA, and also with the yeast p50 homologues (11, 13, 32). The ability of p68 to bind to PCNA (17, 20) may account for the loss of sensitivity to PCNA shown by pol δ p125/p50 heterodimer. In addition, all share in common a high content of charged amino acids which ranges from 29 to 35%. The calculated isoelectric points for these proteins are all basic, with the exception of the S. pombe Cdc27, which has an acidic isoelectric point. This common property suggests that p68 is likely to have an extended structure in solution, which is also consistent with its apparent liability to proteolysis. A third property of the third subunit may be an ability to interact with the p50 second subunit, which has been demonstrated in S. pombe and S. cerevisiae (11, 13) and also in mammalian pol δ. Two speculative functions of p68 may be to act as a linker protein between p50 and PCNA, which would provide additional stabilization of the pol δ-PCNA interaction. This possibility is consistent with the higher sensitivity to PCNA of the pol δ preparations which contain p68 compared with that of the heterodimer.

Thus far, the fourth subunit has only been identified in mammalian sources in this present work, and previously as Cdm1 in S. pombe. Interestingly, data base searches have failed to identify a homologue in S. cerevisiae, despite the fact that its entire genome has been cloned. This may be due to a lack of evolutionary conservation. The functions of this newly

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2. L. Liu and M. Y. W. T. Lee, unpublished observations.
described subunit also remain to be determined.

In summary, this work provides evidence for the identification of a novel subunit, p12, as a component of mammalian pol \( \delta \), as well as evidence for the isolation of pol \( \delta \) in a form that contains the core heterodimer in association with both p12 and the third subunit, p68.

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