Identification of a Novel Protein, PDIP38, That Interacts with the p50 Subunit of DNA Polymerase δ and Proliferating Cell Nuclear Antigen*

The yeast two-hybrid screening method was used to identify novel proteins that associate with human DNA polymerase δ (pol δ). Two baits were used in this study. These were the large (p125) and small (p50) subunits of the core pol δ heterodimer. p50 was the only positive isolated with p125 as the bait. Two novel protein partners, named PDIP38 and PDIP46, were identified from the p50 screen. In this study, the interaction of PDIP38 with pol δ was further characterized. PDIP38 encodes a protein of 368 amino acids whose C terminus is conserved with the bacterial APAG protein and with the F box A protein. It was found that PDIP38 also interacts with proliferating cell nuclear antigen (PCNA). The ability of PDIP38 to interact with both the p50 subunit of pol δ and with PCNA was confirmed by pull-down assays using glutathione S-transferase (GST)-PDIP38 fusion proteins. The PCNA-PDIP38 interaction was also demonstrated by PCNA overlay experiments. The association of PDIP38 with pol δ was shown to occur in calf thymus tissue and mammalian cell extracts by GST-PDIP38 pull-down and coimmunoprecipitation experiments. PDIP38 was associated with pol δ isolated by immunoaffinity chromatography. The association of PDIP38 with pol δ could also be demonstrated by native gel electrophoresis.

DNA replication is essential not only for duplication of the genome but also for maintenance of genomic integrity during DNA repair (1, 2). Chromosomal DNA replication in eukaryotic cells requires three distinct DNA polymerases-α, -δ, and -ε (1–6). polδ is required for replication of the leading strand and for completion of the lagging strand synthesis at the replication fork (7). The action of pol δ as a processive enzyme requires its interaction with proliferating cell nuclear antigen (PCNA), which functions as a molecular sliding clamp (1). The core mammalian pol δ enzyme consists of a tightly associated heterodimer of 125- and 50-kDa subunits. pol δ has been shown recently (8–14) to consist of at least four subunits, consisting of the core enzyme and two additional subunits in both yeast and mammalian systems. In the yeast Schizosaccharomyces pombe, Cdc27 and Cdm1 have been identified as the third and the fourth pol δ subunits, respectively, (8, 9). In Saccharomyces cerevisiae Pol32p has been identified as the homologue of the S. pombe third subunit (10). A human homologue of Cdc27, the KIAA0039 gene product (11–13), and p12, a human homologue of Cdm1 (14), have recently been identified and can be considered to be the third and fourth subunits of human pol δ. Our laboratory has been interested in the identification of additional protein components that are involved in the formation of the pol δ replication complex. In this study we report the identification of two novel proteins, PDIP38 and PDIP46, that interact with the p50 subunit of pol δ by the use of the yeast two-hybrid (15, 16) screening method. PDIP38 was shown to be a PCNA-binding protein, and its interaction with pol δ was established by additional experiments.

EXPERIMENTAL PROCEDURES
Yeast Two-hybrid Screening—The Matchmaker system 2 and human placental cDNA library (HL4025AH) were purchased from Clontech (Palo Alto, CA). The procedures for culture media and plates for yeast growth and selection were as described in the manufacturer’s protocols. Full-length cDNAs corresponding to the human pol δ catalytic subunit p125 and the small subunit p50 were ligated in-frame with GAL4 DNA-binding domain (amino acids 1–147) at the multiple cloning sites of the pAS2-1 vector. Positive clones were confirmed by transfection of the plasmid DNA into Y190 cells containing pAS2-1-p125 or pAS2-1-p50 for pairwise yeast two-hybrid assays for protein-protein interactions. After confirmation by pairwise yeast two-hybrid assays, the positive clones were sequenced. The primer 5′-AGA TTA CGC TAG CCT GGG TGG TC-3′, was used for automated sequencing. Blast searches were performed against the NRdb and ESTdb in the National Center for Biotechnology Information.

Pairwise Yeast Two-hybrid Interactions—Pairwise yeast two-hybrid interactions were performed as described previously (11).

PDIP38 Antibody—A synthetic peptide corresponding to the C-terminal 20 amino acid residues (H,N-PFP SelSNRDKEKTTPSGLHWC OOH) of the human PDIP38 sequence was used to generate rabbit polyclonal antibodies. These were purified by affinity chromatography using the immobilized peptide (SynPep, Dublin, CA).

Purification of pol δ from HeLa Cell Extracts by Immunoaffinity Chromatography—pol δ was purified by immunoaffinity chromatography on a column containing an immobilized antibody against the p125 subunit of pol δ (17). Frozen cells from 20 liters of mid log phase HeLa cells (Cell Culture Center, Cellnex Biosciences, Inc.) were lysed by sonication in 140 ml of TGE buffer (50 mM Tris-HCl, pH 7.8, 10% glycerol, 1 mM sodium bisulfite, 1 mM PMSF, 7 μg/ml pepstatin A, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. The lysate was centrifuged for 10 min at 27,000 × g. The supernatant was loaded onto a 20-ml pol δ p125 immunoaffinity column. The column was washed with 120 ml of TGE buffer containing 100 mM NaCl and eluted with TGE buffer containing 400 mM NaCl and 30% ethylene glycol. Fractions of 1.5 ml in volume were collected. The fractions were assayed for pol δ activity with poly(dA)•poly(dG) as the template and loaded onto a 5–15% gradient SDS-polyacrylamide gel for Western blot analysis (14).

Immunoprecipitation—Fresh HeLa cell pellet was extracted with...
high salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 10 mM KCl, 20 mM EDTA, 0.2 mM PMSF, 25 μM leupeptin, 25 μg/ml aprotinin, 10 μM benzamidine) for 20 min on ice, sonicated, and diluted with 2 volumes of wash buffer (50 mM Tris-HCl, pH 7.8, 20 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40). The diluted extract was precleared with protein A plus protein G beads and 5 μg of rabbit preimmune serum at 4 °C for 30 min. After centrifugation, the protein A and protein G beads were discarded. To the precleared Hela extract, 10 μg of PDIP38 rabbit polyclonal antibody or normal rabbit serum, protein A and protein G beads were added and incubated for 2 h at 4 °C. After incubation, protein A plus G beads were washed 5 times with wash buffer. Bound proteins were extracted with SDS-PAGE sample buffer by boiling and subjected to SDS-PAGE and immunoblotted with PDIP38 rabbit polyclonal antibody. PCNA mouse monoclonal antibody, β3-tubulin, and anti-PDIP38 antibody were used as controls.

**GST Pull-down Assays** — The GST-PDIP38 fusion protein expression vector was constructed by double digestion of pACT2-PDIP38 with EcoRI and XhoI and ligation of the isolated fragment into the pGEX-5X-3 vector (Amersham Biosciences). The fusion protein was expressed in *Escherichia coli* strain BL21(DE3, pLysS) (Strategene). The GST lysate or GST-PDIP38 fusion protein lysate (240 μl) in GST binding buffer (50 mM Tris-HCl, pH 7.8, 1 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 0.2 mM PMSF) was mixed with 200 μl of cell lysate containing recombinant pol δ p50 or PCNA. To this was added 100 μl of 50% suspension of glutathione-Sepharose 4B beads. The suspension was incubated with gentle rocking for 2 h at 4 °C. After collection by centrifugation, the beads were washed five times with GST binding buffer. Bound proteins were extracted with SDS-PAGE sample buffer by boiling and then were subjected to SDS-PAGE and immunoblotted as described above.

**Non-denaturing PAGE** — Log phase 293 cells from five 75-cm² flasks were washed with phosphate-buffered saline and resuspended in 1 ml of buffer containing 10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 1 mM PMSF, 5 mM sodium bisulfite, 1 mM dithiothreitol, 5 μg/ml leupeptin, and 2.5 μg/ml aprotinin. The cells were sonicated and centrifuged at 14,000 × g for 30 min at 4 °C. Aliquots of the supernatant (150 μl, 10 mg/ml protein) were run on a 3–15% gradient gel with a 3.5% stacking gel at 4 °C. The extracted proteins were subjected to SDS-PAGE, transferred onto a nitrocellulose membrane, and immunoblotted with antibody against PDIP38.

**RESULTS**

**Yeast Two-hybrid Screening** — A yeast two-hybrid screen of a human placental cDNA library was performed (see “Experimental Procedures”) using human p125 and p50 as the baits. With pol δ p125 as bait, all of the positive clones that were studied were identified as the pol δ p50 subunit. This is consistent with previous data on the strong interaction between these two pol δ subunits which still coelute on glycerol gradient ultracentrifugation after treatment with 3.4 M urea (17).

When p50 was used as the bait, 22 positives were obtained. These were sorted into three groups by restriction enzyme digestion and examination of the products on agarose gel electrophoresis. DNA sequencing of the inserts was used to identify the positives. Two of these were found to be novel genes of previously unknown function. These will be referred to as polynemase δ interacting protein 38 (PDIP38) and polynemase δ interacting protein 46 (PDIP46). Twelve identical PDIP46 and six identical PDIP38 clones were isolated. The third group, consisting of four isolates, was identified as p21Nφat, a known PCNA-binding protein (18–21). Further tests of the interactions of p50 with p21Nφat, PDIP38, and PDIP46 were performed by the use of a pairwise yeast two-hybrid analysis, followed by a liquid assay for β-galactosidase activity (11). The results confirmed the ability of p50 to interact with p21Nφat, PDIP38, and PDIP46 (Fig. 1).

An important additional result was obtained in this experiment. The interaction of PDIP38 with PCNA was tested and was shown to be positive (Fig. 1). The finding that PDIP38 interacts with PCNA is highly significant, because the latter is a crucial accessory protein that functions as a molecular sliding clamp and endows pol δ with the processivity required for chromosomal DNA replication (1, 7). Because PDIP38 also interacts with the p50 subunit, it suggests that it could interact both with pol δ and with PCNA. Only very weak activities for a p50-p50 interaction or a p50-PCNA interaction were found, and the strength of the interactions was similar to those of negative controls when only p50-pAS2-1 (the p50-GAL4 DNA-binding domain fusion construct) or p50-ACT2 (the p50-GAL4 activation domain fusion construct) was present in the yeast cells which grew in Trp⁻ or Leu⁻ media, respectively.

**Data Base Analysis of PDIP38 and PDIP46** — The PDIP46 isolate from the two-hybrid screening was sequenced. A sequence of 1950 bp was obtained. The sequence matched the following three genomic sequences: AC002094, from human chromosome 17; AC002324 from mouse Chromosome 11; and L144429 from *Caenorhabditis elegans* cosmid ZK6529. It also matched over 100 EST sequences from the ESTdb. The human genomic sequence AC002094 was found to have one error (Fig. 2A), in that it is missing a cytosine at position 43699, which results in an incorrect open reading frame (ORF). The corrected AC002094 sequence (Fig. 2A) was used for ORF prediction and allowed the construction of an exon-intron map of the PDIP38 gene (Fig. 2B). The predicted ORF matched the cDNA sequence derived from the clone isolated from the yeast two-hybrid screen and was also consistent with the sequences of a number of the EST clones found by BLAST searches. The derived human PDIP38 cDNA (gi:15213478) encodes a protein of 368 a.a. (GenBank™ accession number CAB77058). The function of this gene is unknown, and its interaction with pol δ is currently under investigation.

The entire insert of PDIP38 in the pACT2 vector was sequenced and found to consist of 1967 bp. BLAST searches of the NRdb revealed that the PDIP38 sequence matched the following three genomic sequences: AC002094, from human chromosome 17; AC002324 from mouse Chromosome 11; and L144429 from *Caenorhabditis elegans* cosmid ZK6529. It also matched over 100 EST sequences from the ESTdb. The human genomic sequence AC002094 was found to have one error (Fig. 2A), in that it is missing a cytosine at position 43699, which results in an incorrect open reading frame (ORF). The corrected AC002094 sequence (Fig. 2A) was used for ORF prediction and allowed the construction of an exon-intron map of the PDIP38 gene (Fig. 2B). The predicted ORF matched the cDNA sequence derived from the clone isolated from the yeast two-hybrid screen and was also consistent with the sequences of a number of the EST clones found by BLAST searches. The derived human PDIP38 cDNA (gi:15213478) encodes a protein of 368 a.a. (GenBank™ accession number AF179891) The PDIP38 insert in the pACT2 vector contained the entire coding sequence except for the first three N-terminal amino acids. The cognate mouse PDIP38 ORF was derived from the mouse genomic clone.
The missing cytosine at position 43620 of the AC002094 genomic clone, is shown below that of the human gene.

(A002324). The predicted ORF also consists of 368 amino acid residues and has an identity of 95% with human PDIP38 at the amino acid level. The exon-intron map of the mouse PDIP38 gene is shown in Fig. 2B.

Analysis of the PDIP38 Amino Acid Sequence—Data base searches were performed with the BLASTp program using the National Center for Biotechnology Information Blast2 service against the SWISS-PROT + TREMBL + TREMBL new data bases. In addition to the mouse PDIP38 cDNA, sequences for the Drosophila and C. elegans proteins (accession numbers Q9VNC0 and Q95PW5) were found; these had 57 and 35% identity, respectively, to the human PDIP38 sequence. The C-terminal region of PDIP38 (residues 233–353) was found to be related to the bacterial APAG proteins and to the F box A protein (22). The APAG gene was found in E. coli (23) and has been identified in a number of other bacteria. The E. coli APAG gene encodes a protein of 125 a.a. whose function is unknown. The human and mouse FBA proteins (371 a.a. and 367 a.a., respectively) contain the APAG domain at their C termini.

Alignment of the human and mouse genomic sequences of PDIP38. A, the 5′ end of the PDIP38 transcript was aligned with the sequences available in the current ESTdb (C06428, U46408, and AL079399) and NRdb (AC002094). The missing cytosine at position 43699 of AC002094 is highlighted. B, organization of the human and mouse PDIP38 genes. The diagram shows the organization of the human PDIP38 gene, starting at nucleotide 43620 of the AC002094 genomic clone and ending at nucleotide 53879. The organization of the mouse PDIP38 gene, starting at the beginning of the open reading frame at nucleotide 33275 and ending at nucleotide 23952 of the mouse AC002324 genomic clone, is shown below that of the human gene.

Northern Blot Analysis of PDIP38 Expression—Northern blot analysis revealed a single major transcript of ~2.0 kb of PDIP38 in both HeLa and MCF7 total RNA extracts using a 323-bp probe (436–759 from the human PDIP38 cDNA sequence). The size of the transcript is in good agreement with the sequence information. A minor transcript (1 kb) was also detected. At this point it cannot be ascertained if the minor transcript (1 kb) represents a second transcript of the same gene, the existence of a related gene, or a degradation product of the 2-kb transcript (data not shown).

Demonstration of Protein-Protein Interactions between PDIP38 with PCNA or p50 by Pull-down Assays—The existence of protein-protein interactions between human PDIP38 and PCNA and PDIP38 and p50 were further investigated by GST pull-down experiments. The coding sequence of PDIP38 was inserted into the pGEX-5X-3 vector, and the recombinant GST-PDIP38 was expressed (see "Experimental Procedures"). The GST-PDIP38 was used to demonstrate the ability to bind to PCNA and p50 by pull-down assays using E. coli lysates containing the recombinant proteins (Figs. 4, A and B). These experiments in which bacterially expressed proteins were used indicate that PDIP38 directly interacts with PCNA and also with the pol δ p50 subunit, and the observed interactions are not due to the presence of a bridging protein.

Purified GST and GST-PDIP38 adsorbed onto glutathione-Sepharose beads were then used in a variation of the pull-down assay to determine whether pol δ p125 could be bound from partially purified calf thymus pol δ preparations. The GST-PDIP38 pull-down assay was positive (Fig. 4C). This ability of GST-PDIP38 to bring down p125 is presumably because of the tight association of p125 with p50, because PDIP38 does not interact with p125, at least in the yeast two-hybrid assay (data not shown), and PCNA is absent in the pol δ preparation.

The interaction between human PCNA and PDIP38 that was identified by the yeast two-hybrid pairwise assay (Fig. 1) was confirmed by the use of PCNA overlay assays using either biotinylated or digoxigenin-labeled PCNA. By this assay, PCNA binds to GST-PDIP38 (Fig. 5, B and C, lanes 2). The specificity of the interaction was shown by a negative reaction for GST alone (Fig. 5, B and C, lanes 1). Inspection of the PDIP38 sequence shows the presence of three putative PCNA-binding motifs (19–21) between residues 81–88, 151–158, and 193–200 (Table I).

Demonstration of Interactions between PDIP38 and PCNA or pol δ in Cell Extracts by Coimmunoprecipitation—The association of PDIP38 with p50, p125, and PCNA in HeLa cell extracts was demonstrated by coimmunoprecipitation experiments. A polyclonal antibody to a PDIP38 peptide derived from the C terminus (see "Experimental Procedures") was used. The PDIP38 antibody was able to immunoprecipitate PDIP38 from HeLa lysates and also recognized a polypeptide of 38 kDa on SDS-PAGE by Western blotting. (The PDIP38 polypeptide migrates on SDS-PAGE with an apparent size of 38 kDa as determined by Western blotting, although its amino acid sequence indicates a calculated molecular mass of 42 kDa. The reason for this difference is unknown.) The immunoprecipitates were then Western blotted with antibodies against PCNA and p125 (Fig. 6). The results for the Western blot with PCNA showed strong signals. This is consistent with evidence for a direct interaction of PDIP38 and PCNA obtained by the overlay and GST pull-down experiments (Figs. 4 and 5). p125 and p50 were also immunoprecipitated. The most likely interpretation for the coimmunoprecipitation of p125 with PDIP38 is that PDIP38 is bound to the pol δ heterodimer via p50. These results show that the interaction of PDIP38 with p50 is not restricted to two proteins in isolation but that the interaction can be demonstrated in cell extracts with pol δ.

PDIP38 Is Associated with Pol δ Purified by Immunaffinity Chromatography—pol δ was purified from HeLa cells by a modification of the immunaffinity chromatography method used for the isolation of pol δ from calf thymus tissues (17) and HeLa cell lysates. A HeLa cell lysate from 20 liters of cell culture was directly chromatographed on a p125 immunaffinity column without passage through DE52 and phenyl-agarose...
steps, and a lower salt buffer (0.1 M NaCl) was used to wash the immunofluorescence column. The column was eluted with TGEE buffer containing 0.4 M NaCl and 30% ethylene glycol (see “Experimental Procedures”). Western blotting for PDIP38 showed that it is present with all four subunits (p125, p50, p68, and p12) in the peak fraction of pol δ activity as judged by pull-down assays. The results are shown in Fig. 7. The p125 antibody recognizes a single band that elutes close to thyroglobulin (M, 669,000). It is seen that the major band recognized by the PDIP38 antibody coincides with the same band that is recognized by the p125 antibody (Fig. 8A). Two other smaller bands were also observed. The high molecular weight band was excised from the gel and run in a second dimension on SDS-PAGE. Western blotting with antibody against PDIP38 showed the presence of a band corresponding to PDIP38 (Fig. 8B). Both p125 and p50 could also be shown to be present in the high molecular band by a similar analysis (data not shown). These experiments show that PDIP38 is associated with the pol δ complex in cell extracts.

### DISCUSSION

The motivation for these studies was to identify novel proteins that interact with pol δ using the yeast two-hybrid system. Pol δ, rigorously isolated from mammalian tissues, has been shown to consist of a tightly associated heterodimer of the p125 and p50 subunits. Two less tightly associated subunits, p68 and p12, have recently been identified as homologues of the p125 and p50 subunits. Two less tightly associated subunits, p68 and p12, have recently been identified as homologues of the p125 and p50 subunits.

A yeast two-hybrid screen of a human cDNA library using the p125 and p50 subunits was obtained, but so far a homologue of the S. pombe fourth subunit (10) has not been identified. A yeast two-hybrid screen of a human cDNA library using the p125 and p50 subunits was carried out. Our yeast two-hybrid screening using p50 as the bait revealed three positives. Two of these were proteins of unknown function, and the third was identified as p21.

Obviously, protein-protein interactions revealed by the yeast two-hybrid system do not necessarily indicate that the native (non-fusion) proteins are capable of interaction, nor do they provide any evidence that such interactions take place in a cellular context. In these studies, we have further characterized the interaction of PDIP38 with the p50 subunit of pol δ.

The ability of PDIP38 to interact with p50 and also with PCNA was demonstrated by coimmunoprecipitation and GST pull-down assays. Pull-down experiments using bacterially expressed p50 and PCNA established that the interactions were direct. These experiments were important for the validation of the coimmunoprecipitation experiments from cell extracts. The coimmunoprecipitation experiments revealed that PDIP38 interacts with the pol δ complex in cell extracts, in that we could show the coimmunoprecipitation of p125 as well as p50. The results indicate that PDIP38 is able to bind to p50 when the latter is in association with p125. More significantly, an association of PDIP38 with pol δ in a cellular context. In these studies, we have further characterized the interaction of PDIP38 with the p50 subunit of pol δ.

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PDIP38, a Novel Pol δ Associated Protein

Whereas the functions of PDIP38 are still unknown, our studies have shown that it possesses one property that is of relevance to the functions of pol δ, viz. the ability to bind to PCNA. PCNA has been shown to bind a number of proteins through a short peptide motif (18–21), exemplified by that present in p21Waf1, which binds to the interdomain connector loop of PCNA (18). Inspection of the PDIP38 sequence shows that there are three putative PCNA-binding motifs. These are shown in alignment (Table I) with those of examples of the known PCNA-binding proteins, p21 (18–21, 28), DNA (cytosine-5)methyltransferase (29), the pol δ p68 subunit (11–14, 30), FEN1 (19), and DNA ligase I (32). The PCNA-binding motif is QXXXXXF(F/Y), where Z is generally an aliphatic residue (20). The three candidate sequences (PDIP38) all possess only a single aromatic residue, but there exists at least one known PCNA-binding motif with a single aromatic residue, that for DNA (cytosine-5)methyltransferase (29).

In yeast, the third subunit of pol δ (10, 31) binds to both the homologue of p50 and PCNA. p68, the mammalian third sub-

| Protein | Sequence |
|---------|----------|
| PDIP38 (a.a. 81–88) | QLFHSLIF |
| PDIP38 (a.a. 151–158) | QTEAVTFL |
| PDIP38 (a.a. 193–200) | QHELFERF |
| p21WAP1 (a.a. 144–151) | QTSMTFY |
| MCM7 (a.a. 164–171) | QTITSHF |
| Pol δ p68 (a.a. 456–463) | QVISITGFF |
| FEN1 (a.a. 337–344) | QGRLLDFF |
| DNA ligase I (a.a. 2–9) | QRISMSFF |

Table I: Putative PCNA binding motifs in PDIP38

![Figure 4](image_url) Demonstration of the interaction of human PDIP38 with PCNA, pol δ p50, and pol δ 125 by GST pull-down assays. A and B, lanes 1 and 2, cell lysates of E. coli cells expressing GST or GST-PDIP38 were mixed with lysates of E. coli cells containing over-expressed PCNA (A) or pol δ p50 (B) and pulled down with glutathione-Sepharose 4B beads (see “Experimental Procedures”). Western blots of the bound proteins were performed using monoclonal antibody 74B1 against PCNA (A) or with monoclonal antibody 17D2 against pol δ p50. Lane 3 shows the input recombinant PCNA or recombinant pol δ p50. C, lane 1, calf thymus pol δ purified to the DE52 column chromatography step (14); lanes 2 and 3, the partially purified calf thymus pol δ preparation was mixed with E. coli lysates containing GST and PDIP38-GST fusion proteins, respectively, and pulled down with glutathione-Sepharose. Western blot analysis was performed with monoclonal antibody 78F5 against pol δ p125.

![Figure 5](image_url) Demonstration of the interaction of human PDIP38 with PCNA by overlay assay. A, GST (lane 1) and GST-PDIP38 fusion protein (lane 2) were purified by affinity chromatography on glutathione-Sepharose and stained with Coomassie Blue after SDS-PAGE. B, overlay using biotin-labeled PCNA. C, overlay using digoxigenin-labeled PCNA.

![Figure 6](image_url) Demonstration of the interaction of human PDIP38 with PCNA and pol δ 125 by coimmunoprecipitation experiments. HeLa cell extracts were immunoprecipitated (IP) with PDIP38 peptide polyclonal antibody and protein A and protein G beads (see “Experimental Procedures”). The bound proteins were analyzed by immunoblotting (WB) with PDIP38 rabbit polyclonal antibody (lanes 1–3), PCNA monoclonal antibody 74B1 (lanes 4 and 5), or pol δ p125 monoclonal antibody 78F5 (lanes 6 and 7), respectively. Lane 1, mock immunoprecipitation with rabbit preimmune serum; lanes 2, 4, and 6, HeLa input; lanes 3, 5, and 7, immunoprecipitate with PDIP38 antibody.

![Table I](image_url) Putative PCNA binding motifs in PDIP38

unit of pol δ, binds to PCNA (11–13, 30) and interacts with the p50 subunit in the yeast two-hybrid assay.2 In this respect, the properties of PDIP38 parallel those of the third subunit of pol

* L. Liu and M. Y. W. T. Lee, unpublished observations.
than did the four-subunit pol. Cdc27 is absent was found to bind much less strongly to PCNA in S. pombe noted for p68 (14). This possibility is consistent with studies of chromatography-purified pol/H9254 for the presence of p68, p50, and p12 (31). Further studies using the isolated PDIP38 and Western blotted with a monoclonal antibody against the p125 subunit of pol δ (left lane) on a rabbit polyclonal antibody against PDIP38 (right lane). The proteins were transferred to nitrocellulose membranes which were then Western blotted with a monoclonal antibody against the p125 subunit (A) was excised and subjected to SDS-PAGE as described under “Experimental Procedures.” After electrophoresis, the proteins were transferred to a nitrocellulose membrane and Western blotted with PDIP38 antibody (right lane). The left lane is the 293 crude cell extract.

![Figure 7](image1.png)

**A**. nondenaturing gel electrophoresis of 293 cell extracts was performed as described under “Experimental Procedures.” The proteins were transferred to nitrocellulose membranes which were then Western blotted with a monoclonal antibody against the p125 subunit of pol δ (left lane) or a rabbit polyclonal antibody against PDIP38 (right lane). The protein standards used as molecular weight markers were thyroglobulin (669,000), ferritin (440,000), and catalase (232,000). B, the band corresponding to that immunoblotted by the antibody against the p125 subunit (A) was excised and subjected to SDS-PAGE as described under “Experimental Procedures.” After electrophoresis, the proteins were transferred to a nitrocellulose membrane and Western blotted with PDIP38 antibody (right lane). The left lane is the 293 crude cell extract.

![Figure 8](image2.png)

**Fig. 8.** Association of PDIP38 with pol δ during native gel electrophoresis. A, non-denaturing gel electrophoresis of 293 cell extracts was performed as described under “Experimental Procedures.” The proteins were transferred to nitrocellulose membranes which were then Western blotted with a monoclonal antibody against the p125 subunit of pol δ (left lane) or a rabbit polyclonal antibody against PDIP38 (right lane). The protein standards used as molecular weight markers were thyroglobulin (669,000), ferritin (440,000), and catalase (232,000). B, the band corresponding to that immunoblotted by the antibody against the p125 subunit (A) was excised and subjected to SDS-PAGE as described under “Experimental Procedures.” After electrophoresis, the proteins were transferred to a nitrocellulose membrane and Western blotted with PDIP38 antibody (right lane). The left lane is the 293 crude cell extract.

PDIP38 is present in pol δ isolated by immunoaffinity chromatography-purified pol δ from HeLa cells. A cell lysate from 20 liters of HeLa cell culture was fractionated on a pol δ immunoaffinity column (see “Experimental Procedures”). A, assay of the fractions eluted from the column for pol δ activity on a poly(dA)-oligo(dT) template in the presence (solid circles) and absence (open circles) of PCNA. The peak fractions (numbers 43, 46, and 49) of pol δ activity were analyzed by Western blot for the presence of p125 and PDIP38 (B) and for the presence of p68, p50, and p12 (C).

δ. This similarity raises a possibility that PDIP38 might be able to substitute p68 to form a variant pol δ holoenzyme. This is an intriguing consideration, bearing in mind that pol δ can be regarded as taking part in a number of cellular functions that include (a) synthesis at the leading strand, (b) synthesis at the lagging strand, and (c) DNA repair and recombination processes. Each of these processes may require a specific and likely different assembly of proteins surrounding the pol δ core heterodimer. This issue is also important in considerations of attempts to isolate a pol δ replication complex from tissues or cells, because there may exist different multiprotein complexes involving pol δ. Further studies using the isolated PDIP38 protein are currently underway to determine its ability to interact with the purified pol δ complex and to assess its functional effects on pol δ activity.

Although the functions of PDIP38 are at this point unknown, a protein that binds to both p50 and PCNA could serve as a structural role to strengthen the interaction of the pol δ heterodimer with PCNA by binding to pol δ and PCNA, as already noted for p68 (14). This possibility is consistent with studies of S. pombe Cdc27, where the three-subunit enzyme in which Cdc27 is absent was found to bind much less strongly to PCNA than did the four-subunit pol δ (31). Given the trivalent nature of PCNA, there is also the possibility that PDIP38 could serve as a bridging protein between two pol δ-PCNA complexes. This could be envisioned as a linking of two pol δ-PCNA assemblies via PDIP38 in which it is attached to one assembly via PCNA and to the other via p50.

Data base analysis showed that PDIP38 has a striking conservation between the C-terminal 111 residues with the bacterial APAG proteins. A similar conservation has been found for one other mammalian protein, the F box A protein (22). We suggest this conserved region in PDIP38 be called the APAG domain. The conservation of the APAG domain across a wide evolutionary range argues that it is likely to have some significant biological function. The functions of the APAG protein or of the members of the family (Fig. 3) that includes PDIP38 are unknown. The presence of the highly conserved GXXGXXG motif suggests the presence of a pyrophosphate-binding domain, i.e. that they may be able to bind either pyrophosphate or nucleotide triphosphates (27). Thus, exploration of the possible binding of these compounds to PDIP38 may provide clues to its function. The FBA protein contains an F box at its N terminus (residues 9–58). The latter motif provides the family of F box proteins with the ability to bind to the core of the ubiquitin-protein ligase complex, Skp1-cullin-F box protein (33). The F box proteins bind to specific substrates, which are thereby targeted for intracellular degradation via the proteosome. The presence of an APAG domain in the F box A protein would suggest that it may be a protein interaction domain; however, the target of the F box A protein is currently unknown.

The current work establishes that p50 interacts with PDIP38, and it is noteworthy that p50 has also been reported to interact with a protein termed p36 also known as PDIP1 (polynucleotide δ interacting protein 1) and the Werner helicase (34, 35). This suggests that p50 may serve as a nexus or scaffold for the interaction of other subunits or accessory proteins with pol δ.

In summary, we have identified two novel proteins, PDIP38 and PDIP46, that interact with the p50 subunit of pol δ. The interaction of PDIP38 with p50 was extensively characterized,
and although its functions are currently unknown, these findings indicate that the number of proteins that may be involved in the formation of the pol δ enzyme complex may involve additional proteins besides the four known subunits.

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Identification of a Novel Protein, PDIP38, That Interacts with the p50 Subunit of DNA Polymerase δ and Proliferating Cell Nuclear Antigen

Li Liu, Esther M. Rodriguez-Belmonte, Nayef Mazloum, Bin Xie and Marietta Y. W. T. Lee

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