Further Characterization of Human DNA Polymerase δ Interacting Protein 38*

Polymerase δ interacting protein 38 (PDIP38) was identified as a human DNA polymerase (pol) δ interacting protein through a direct interaction with p50, the small subunit of human pol δ. PDIP38 was also found to interact with proliferating cell nuclear antigen, which suggested that it might play a role in vivo in the processes of DNA replication and DNA repair in the nucleus. In order to characterize further this novel protein, we have examined its subcellular localization by the use of immunochemical and cellular fractionation techniques. These studies show that PDIP38 is a novel mitochondrial protein and is localized mainly to the mitochondria. PDIP38 was shown to possess a functional mitochondrial targeting sequence that is located within the first 35 N-terminal amino acid residues. The mature PDIP38 protein is about 50 amino acid residues smaller than the full-length precursor PDIP38 protein, consistent with it being processed by cleavage of the mitochondrial targeting sequence during entry into the mitochondria. His-tagged mature PDIP38 inhibited pol δ activity in vitro and interacted with human papillomavirus 16 E7 oncoprotein, suggesting that PDIP38 might play a role in the pol δ-mediated viral DNA replication. Although the localization of PDIP38 to the mitochondria suggests that it serves functions within the mitochondria, we cannot eliminate the possibility that it may be involved in pol δ-mediated DNA replication or DNA repair under certain conditions such as viral infection.

Chromosomal DNA replication is a tightly regulated process, which requires multiple protein complexes working in a highly coordinated manner. In eukaryotic cells, three major DNA polymerases, DNA polymerases α, δ, and ε, are involved in DNA replication (1–3). Although it is clear that the DNA polymerase α/primase is involved in initiating both leading and lagging strand synthesis, the precise roles of DNA polymerase (pol)3 δ and DNA polymerase ε remain to be fully defined. A mutant without the catalytic domain of the large subunit of pol ε in Schizosaccharomyces pombe was found to be viable (4), and pol δ and pol α were sufficient to replicate both the leading and lagging strands of double-stranded DNA in an SV40 DNA replication system (5). These earlier studies pointed to the fact that pol δ was likely to be the major polymerase for the elongation of both leading and lagging strands of chromosomal DNA in eukaryotic cells. This was supported by recent studies that showed that there was inefficient elongation of both leading and lagging DNA strands in Xenopus egg extracts depleted of pol δ (6). pol ε was suggested to play a role mainly in leading strand DNA synthesis (6). pol δ, working together with Fen1 and proliferating cell nuclear antigen (PCNA), is required for Okazaki fragment maturation, and the 3′–5′-exonuclease activity of pol δ is important for this process (7–9). pol δ is also involved in telomerase-mediated telomere addition in vivo (10) and participates in several DNA repair pathways (11, 12). The ability of pol δ to participate in multiple aspects of DNA replication/repair raises the question of how it is regulated. The association of pol δ with different proteins or protein complexes in vivo and post-translational modifications of the pol δ complex could be important mechanisms (13–16).

Mammalian pol δ was originally characterized as a tightly associated heterodimer of the catalytic subunit, p125, with a small subunit, p50 (17). More recently, we and others (17–21) have identified two additional subunits of mammalian pol δ, the p68 and p12 subunits, so that mammalian pol δ is now regarded as a heterotrimer of four subunits. Recently, we identified a novel protein, PDIP38, which interacted with the small subunit p50 of human pol δ, as well as with PCNA (22). PDIP38 joins a growing number of proteins that have been reported to have the ability to interact with the small p50 subunit of pol δ as well as with PCNA. These include p125 itself (18, 23, 24), p68 (25, 26), WRN (27, 28), and PDIP1 (29). PDIP1 could enhance PCNA-dependent pol δ activity in vitro (29), and WRN increased the rate of nucleotide incorporation by pol δ in the absence of PCNA (30). The function of PDIP38 in regard to its interaction with pol δ is unknown. To understand better the physiological function of PDIP38, we examined its cellular localization in detail. In this study, we demonstrate by both optical and biochemical methods that PDIP38 is a mitochondrial protein. We further identified a bona fide mitochondrial targeting sequence (MTS) located at the N-terminal region of PDIP38. The first N-terminal 35 amino acids of PDIP38 are necessary and sufficient for its targeting to the mitochondria. By using a series of N-terminal deleted PDIP38 fusion proteins, we also demonstrated that the cleavage site is most likely located 50 amino acid residues from the N terminus of the PDIP38 precursor protein. We found that PDIP38 could inhibit HPV, human papillomavirus; GST, glutathione S-transferase; Ni-NTA, nickel nitrilotriacetic acid; PBS, phosphate-buffered saline.

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‡ The abbreviations used are: pol, polymerase; PDIP, polymerase δ interacting protein; EGFP, enhanced green fluorescent protein; PCNA, proliferating cell nuclear antigen; MTS, mitochondrial targeting sequence; mhs70, mitochondrial heat shock protein 70; MPP, mitochondrial processing peptidase; MIP, mitochondrial intermediate peptidase;
the PCNA-dependent pol δ activity and could interact with HPV 16 E7 oncoprotein. These results indicated that PDIP38 might play a role in the pol δ-mediated viral DNA replication.

**EXPERIMENTAL PROCEDURES**

**Materials**—All chemicals were purchased from Sigma except as otherwise indicated. Oligonucleotides were synthesized by Qiagen (Stanford, CA). Cell culture media was purchased from Celgro (Herndon, VA). GST-HPV 16 E7 construct was provided by Dr. K. Munger, Harvard Medical School (31).

**Construction of PDIP38 Fusion Constructs and His-p21—** Standard low cycle PCR techniques were used to generate the C-terminal enhanced green fluorescent protein (EGFP)-tagged PDIP38 full-length and deletion mutants using PGFPN3 expression vector (Clontech). The PCR products were digested with HindIII and BamHI and cloned into the pQE32 (Qiagen, CA) expression vector between the SphI and BamHI sites. The PCR products were then washed four times with 0.3M ammonium formate, pH 7.8, at 37 °C and were terminated by spotting onto DE81 papers, which were then washed four times with 0.5 M ammonium formate, pH 7.8, once with 95% ethanol, and counted. For the inhibition assay using His-p21 or PDIP38-(51–368)-C-His was preincubated with either 100 ng of PCNA antibodies, Golden, CO). The following antibodies were obtained from Santa Cruz Biotechnology: mouse monoclonal antibody anti-PDIP38, mouse monoclonal antibody anti-EGFP (B-2); goat polyclonal antibody anti-lamin B (C-20); mouse monoclonal antibody anti-GFP (B-2); goat polyclonal antibody anti-IgG (Jackson ImmunoResearch) at room temperature for 1 h in the dark. For ectopically expressed EGFP fusion proteins, overnight incubation was performed with the indicated PGFP-N3 or pEGFP-C1 constructs, and 24 h after transfection, Hoechst 33258 (Sigma) was added at 10 μM and MitoTracker Red CMXRos (M-7512, Molecular Probes) at 20 nM for an additional hour, and the cells were fixed using 100% methanol. Fluorescence microscopy was performed on a Nikon microscope, and images were captured with a digital camera (Optronics) using Optronix MagFire, Image-ProPlus software.

**GST Pull Down Assay—** Mitochondrial fusion constructs were generated from HEK 293T cells using a mitochondria isolation kit from Sigma. The cells were also fractionated with the NE-PER kit (Pierce). The proteins were run on SDS-PAGE, transferred to nitrocellulose membranes, and blotted with the following antibodies: mouse monoclonal antibody anti-p50 (17D2 and 13D5) (26); rabbit polyclonal antibody anti-p68 (26); rabbit polyclonal antibody anti-p12 (26); rabbit polyclonal antibody anti-PDIP38 (22). The polyclonal rabbit anti-PCNA antibody was a gift from Dr. E. Mandrel (Northern Bioagents, Golden, CO). The following antibodies were obtained from Santa Cruz Biotechnology: mouse monoclonal antibody anti-PCNA (PC10); mouse monoclonal antibody anti-EGFP (B-2); goat polyclonal antibody anti-lamin B (C-20); goat polyclonal antibody anti-IgG (Jackson ImmunoResearch) at room temperature for 1 h in the dark. For ectopically expressed EGFP fusion proteins, overnight incubation was performed with the indicated PGFP-N3 or pEGFP-C1 constructs, and 24 h after transfection, Hoechst 33258 (Sigma) was added at 10 μM and MitoTracker Red CMXRos (M-7512, Molecular Probes) at 20 nM for an additional hour, and the cells were fixed using 100% methanol. Fluorescence microscopy was performed on a Nikon microscope, and images were captured with a digital camera (Optronics) using Optronix MagFire, Image-ProPlus software.

**RESULTS**

Human PDIP38 Laboratories Mainly to the Cytoplasm—We have identified previously a novel protein, PDIP38, that interacts with p50, the small subunit of human pol δ (22). The interaction of PDIP38 with pol δ was confirmed by a number of experimental approaches, including pull down and overlay experiments. This interaction suggested that PDIP38 might be a nuclear protein that participated in some aspect of DNA replication or repair. To understand better the likely physiological role(s) of PDIP38, we investigated its intracellular localization in cultured cells. The anti-PDIP38 antibody that was used (22) specifically recognized one major band migrating with appar-
PDIP38, Mitochondrial Localization, Viral DNA Replication

Presence of a Functional Mitochondrial Targeting Sequence at the N Termminus of PDIP38—Nuclear coded mitochondrial proteins contain an MTS at their N termini. Analysis of human PDIP38 by MITOPROTII (ihg.gsf.de/ihg/mitoprot.html, see Ref. 33) predicted an almost 100% (99.98%) probability of an MTS at the N terminus. A mitochondrial localization of human PDIP38 was also predicted by the Program TargetP (www.cbs.dtu.dk/services/TargetP/) (34, 35). The N-terminal region of human PDIP38 is rich in positively charged amino acids and is predicted to form an α-helix, characteristics that are typical of MTS sequences. The co-localization of PDIP38 with a mitochondrial marker, as well as the examination of the N terminus for the presence of a MTS, was therefore investigated by examining the localization of PDIP38-EGFP fusion proteins. We constructed plasmids expressing full-length PDIP38 fusion proteins with EGFP fused to its C terminus (PDIP38-C-EGFP) or to its N terminus (EGFP-N-PDIP38). HeLa cells were transfected with these plasmids as well as the parent pEGFP-N3 plasmid, and the live fluorescence images were captured by microscopy. In HeLa cells overexpressing PDIP38-C-EGFP (Fig. 2A, a), the PDIP38 fusion protein exhibited a punctated intracellular localization, whereas EGFP alone (Fig. 2A, b) as well as EGFP-N-PDIP38 (Fig. 2A, c) exhibited a diffused localization. HeLa cells were then transfected with PDIP38-C-EGFP or pEGFP-N3 plasmids; the nuclei were stained with Hoechst 33258 and the mitochondria with MitoTracker Red CMXRos. PDIP38 with EGFP fused to its C terminus was clearly co-localized with MitoTracker (Fig. 2C, a–d), whereas EGFP alone was not (Fig. 2D, a–d). The observation is consistent with the presence of an MTS at the N terminus, because an EGFP tag attached to the N terminus of PDIP38 would mask the MTS, which has to be localized at the N terminus in order to function. Examination of the sizes of the PDIP38 EGFP fusion proteins by Western blotting of cell lysates (Fig. 2B) showed that PDIP38 tagged at its C terminus is smaller than the N-terminally tagged PDIP38 on SDS-PAGE, a finding that is consistent with the expected cleavage of an N-terminal MTS after entry into the mitochondria (Fig. 2B).

The First N-terminal 35 Amino Acids Are Sufficient to Mediate the Mitochondrial Localization of PDIP38—In order to dissect the contribution of the N-terminal region of PDIP38 to mitochondrial localization, we constructed a series of N-terminal or C-terminal deletions of PDIP38 with C-terminal EGFP tags. The localization patterns of some of these EGFP fusion proteins are shown in Fig. 3 and summarized in Table I. Deletion of the first N-terminal 30 amino acids abolished the mitochondrial localization of the EGFP fusion protein (Fig. 3A), showing that the first 30 amino acids are necessary for mitochondrial localization of PDIP38. All C-terminal deletions of PDIP38 with a C-terminal EGFP tag exhibited a mitochondrial localization (Fig. 3, B and C, and Table I). The presence of a functional MTS in the N terminus of PDIP38 was confirmed by fusion of the first 35 amino acids of human PDIP38 to the N terminus of EGFP. This fusion resulted in the localization of EGFP to the mitochondria (Fig. 3B). Thus, the first N-terminal 35 amino acids of PDIP38 are unequivocally shown to function as an MTS.

PDIP38 Is Processed in Vivo by Proteolytic Removal of an ∼5-kDa N-terminal Region Containing the MTS—The MTS sequences of most nuclearly encoded mitochondrial proteins are processed by mitochondrial proteases during their import into the mitochondria, resulting in mature proteins that are smaller than their precursors. In our original studies, PDIP38 behaved as a protein of 38 kDa when Western-blotted in cell extracts, whereas the predicted molecular mass based on the sequence encoded by its cDNA was 42 kDa (22). These conflicting observations can now be explained as the likely consequence of mitochondrial processing. Analysis of the protein sequence by MITOPROTII (ihg.gsf.de/ihg/mitoprot.html, see Ref. 33) predicted a cleavage site between residues 58 and 59, whereas Program TargetP (www.cbs.dtu.dk/services/TargetP/) (34, 35) predicted a cleavage site between residues 50 and 51. Both predictions may be correct as many mitochondrial proteins are processed in a two-step process by MPP, followed by a second cleavage of eight residues by the MIP.

In order to obtain more information on the location of the cleavage site, we examined the sizes of full-length EGFP-tagged PDIP38 and a series of either N-terminal or C-terminal deletion mutants of PDIP38 with a C-terminally tagged EGFP when expressed in HeLa cells. As already shown (Fig. 2A), full-length PDIP38 tagged at its N terminus is blocked from

![Image](http://www.jbc.org/)
FIG. 2. Presence of a functional MTS at the N terminus of PDIP38. A, immunofluorescence of live images of HeLa cells transfected with the following: a, PDIP38-C-EGFP; b, pEGFP-N3; and c, EGFP-N-PDIP38. B, overexpressed C-terminally tagged EGFP-PDIP38-(PDIP38-C-EGFP) fusion protein migrates faster than N-terminally tagged EGFP-PDIP38-(EGFP-N-PDIP38) fusion protein on SDS-PAGE. Lane 1, total cell lysates of HeLa cells transfected with EGFP-N-PDIP38; lane 2, total cell lysates of HeLa cells transfected with PDIP38-C-EGFP. Cell lysates were run on 10% SDS-PAGE, transferred to nitrocellulose membranes, and Western-blotted with anti-GFP mouse monoclonal antibody. C, co-localization of PDIP38-C-EGFP with MitoTracker in mitochondria. a, PDIP38-C-EGFP; b, Mitotracker; c, merged image of PDIP38-C-EGFP and MitoTracker; and d, merged image of PDIP38-C-EGFP, MitoTracker, and nuclei. D, EGFP is not co-localized with MitoTracker in mitochondria. a, EGFP; b, MitoTracker; c, merged image of EGFP and MitoTracker; d, merged image of EGFP, MitoTracker, and nuclei.
entry into the mitochondria and exhibits a higher molecular weight than the corresponding C-terminally tagged full-length PDIP38, which is imported into the mitochondria (Fig. 2B). Examination of deletion mutants at the N terminus that were tagged with EGFP at the C terminus by expression in HeLa cells showed that these migrated at the same positions as their calculated molecular masses (data not shown), consistent with a failure to be imported into the mitochondria. The expression products of C-terminal PDIP38 deletions with a C-terminal EGFP tag, on the other hand, all migrated at positions smaller by ~5–6 kDa than their predicted molecular masses (data not shown).

We generated FLAG-tagged PDIP38 constructs to estimate the approximate cleavage site of PDIP38 in vivo. PDIP38 FLAG-tagged at its C terminus expressed in 293T cells migrated faster on SDS-PAGE than the PDIP38 that is FLAG-tagged at its N terminus as shown by immunoblotting with anti-FLAG (Fig. 4A, cf. lanes 2 and 3) and by immunoblotting with anti-PDIP38 (Fig. 4A, cf. lanes 5 and 6). Immunoblotting with anti-PDIP38 shows that the C-terminal FLAG-tagged PDIP38 migrates only slightly more slowly than the endogenous PDIP38 (Fig. 4A, lane 6, arrow c indicates the position of endogenous PDIP38), consistent with an expected increase of 1 kDa because of the addition of the FLAG tag. In addition, both N-terminal His6-tagged and C-terminal His6-tagged full-length PDIP38 overexpressed in E. coli migrated at the same size as their calculated molecular masses of 43 kDa (data not shown). The difference in migration between the N-terminal FLAG-tagged PDIP38 and the C-terminal FLAG-tagged PDIP38 is not because of faulty construction of the plasmids, because the in vitro translation products of these two constructs yielded 35S-labeled proteins of the same size on SDS-PAGE, which are both slightly larger than the nontagged precursor PDIP38 (Fig. 4B). This indicated that the N-terminal MTS of both endogenous PDIP38 and C-terminal FLAG-tagged PDIP38 are proteolytically excised in vivo. The difference of ~5–6 kDa indicates that the proteolytical cleavage of precursor PDIP38 removes between 50 and 60 residues of the N terminus.

The consensus sequence for the MPP and MIP cleavage sites is shown in Fig. 4C (36–38). The region between 50 and 60 amino acids contains an XRXRS (RRLHS, residues 48–52) motif that is one of the substrate recognition sites (R-2 motif) for MPP with an expected cleavage between amino acid residues 50 and 51 (36–38). The sequence also appears to be one that could be further cleaved by MIP between residues 58 and 59 (R-10 motif).

The cleavage site of PDIP38 in vivo was mapped by construction of a series of plasmids for the expression of N-terminal deletions of PDIP38 carrying a C-terminal FLAG tag. These deletions were then expressed in 293T cells. The sizes of these fusion proteins were compared with that of the full-length PDIP38 fusion protein by Western blotting with a mouse monoclonal anti-FLAG antibody (Fig. 4D). The overexpressed PDIP38-C-terminal FLAG-tagged deletion mutants lacking the first 30 N-terminal amino acids yielded a fusion protein that was larger than the processed FLAG-tagged PDIP38 (compare Fig. 4D, lanes 2–3). This is consistent with our immunofluorescence data that shows that the first 30 amino acids are required for the import of PDIP38 into mitochondria (Fig. 3A), so that this mutant would not enter the mitochondria and would not be processed. Thus, the cleavage site must be greater than 30 amino acid residues from the N terminus.

The migration of processed PDIP38 C-terminal FLAG-tagged fusion protein was compared with a series of stepwise deletions at the N terminus that would not be processed and whose sizes can be expected to reflect their amino acid sequence. The migration of processed PDIP38 is similar to that of the deletion mutant lacking the first 50 residues and is clearly bracketed between the deletion mutants lacking the first 40 and 60 amino acids, respectively (Fig. 4D, lanes 2 and 4–6). Thus, our data indicate that the cleavage site most likely is located at about 50 amino acids from the N terminus of the
PDIP38 precursor protein. Given the correspondence of the N-terminal region with the consensus for MPP cleavage, our data are consistent with the cleavage of PDIP38 by MPP between residues 50 and 51 (Fig. 4C) and also suggest that MIP is not involved in the processing of PDIP38.

**Localization of Endogenous PDIP38 to the Mitochondria**—The preceding data show that endogenous human PDIP38 displays an exclusive cytoplasmic localization with a punctated staining pattern. We also demonstrated that a functional MTS exists at the N terminus of PDIP38. In order to demonstrate that endogenous PDIP38 is localized in the mitochondria, HeLa cells were immunostained using both anti-PDIP38 antibody and an antibody (anti-mhsp70) against a known mitochondrial protein, mhsp70. The results show that almost all the endogenous PDIP38 is localized to the cytoplasm and, more importantly, is colocalized with the mitochondrial marker protein mhsp70 (Fig. 5A, a–d).

The localization of PDIP38 to the mitochondria was also demonstrated by cell fractionation. Mitochondria were isolated from HeLa cells by the use of a mitochondria isolation kit (Sigma). The presence of mhsp70 (Fig. 5B, a) and the absence of PCNA (Fig. 5B, c) indicated the purity of the isolated mitochondrial pellet (Fig. 5B, lane 5). PDIP38 (Fig. 5B, d) is clearly present in the mitochondrial pellet. Furthermore, both mhsp70 and PDIP38 are not subject to proteinase K degradation of the mitochondrial fraction (Fig. 5B, lane 7), as would be expected if PDIP38 was located within the intact mitochondria. When Triton X-100 was added to disrupt the mitochondrial membranes, treatment of the isolated mitochondrial pellet with proteinase K led to the degradation of both PDIP38 and mhsp70 (Fig. 5B, lane 8). Another mitochondria marker protein, Tom20, which binds to the outer membrane through its N-terminal domain, was readily digested by proteinase K even in the absence of Triton X-100 (Fig. 5B, b, lane 7). Taken together, these results show that endogenous PDIP38 is present inside the mitochondria. The large subunit p125 (Fig. 5B, e), small subunit p50 (Fig. 5B, f), third subunit p68, and fourth subunit p12 (data not shown) of pol δ were not present in the mitochondrial fraction as determined by Western blotting.

**Is PDIP38 Also Present in the Nucleus?**—Our fractionation results using the mitochondria isolation kit (Sigma) indicated that a significant amount of PDIP38 was also present in the nuclear fraction (Fig. 5B, lane 3, d). This is in contrast to our immunofluorescence results, which showed that both endogenous and overexpressed C-terminally tagged EGFP PDIP38 were almost exclusively present in the cytoplasm. Because we could also detect mhsp70 (Fig. 5B, lane 3, a) and Ldb-α (Fig. 5B, lane 3, h) in the same nuclear fraction, it is possible that the

**FIG. 4. Cleavage of the MTS signal peptide occurs around residue 50 in the N terminus of PDIP38.** A, N-terminal FLAG-tagged and C-terminal FLAG-tagged full-length PDIP38 migrate differently on SDS-PAGE. 293T cell lysates were run on 10% SDS-PAGE, transferred to nitrocellulose membranes, and Western-blotted either with anti-FLAG mouse monoclonal antibody (M2, Sigma, lanes 1–3) or with anti-PDIP38 polyclonal antibody (lanes 4–6). Lanes 1 and 4, 293T cells; lanes 2 and 5, 293T cells transfected with the N-terminal FLAG-tagged PDIP38 construct (pCDNA3.1(+)-N-FLAG-PDIP38); lanes 3 and 6, 293T cells transfected with the C-terminal FLAG-tagged PDIP38 construct (pCDNA3.1(+)-PDIP38-C-FLAG). The arrow a indicates the position of N-FLAG-PDIP38. The arrow b indicates the position of PDIP38-C-FLAG. The arrow c indicates the position of endogenous PDIP38. B, in vitro translation of PDIP38 constructs. Lane 1, control, full-length luciferase, 61 kDa; lane 2, pCDNA3.1(+)-N-FLAG-PDIP38; lane 3, pCDNA3.1(+)-PDIP38-C-FLAG; lane 4, pcDNA3.1(+)PDIP38 (* indicates the major in vitro translated product). C, two possible mitochondrial processing cleavage sites of PDIP38. The diagram shows the N-terminal sequence of PDIP38, with the MPP and MIP consensus cleavage sequence below it. The vertical lines indicate the expected cleavage sites of PDIP38 by MPP and MIP. D, the cleavage site of PDIP38 in vivo is located around amino acid residue 50. Lanes 1–9, the total cell lysates of 293T transfected with C-terminally FLAG-tagged PDIP38 (lanes 2 and 8, C-T); N-terminally FLAG-tagged PDIP38 (lane 9, N-T); and N-terminal deletions of C-terminally FLAG-tagged PDIP38 lacking the first 30, 40, 50, 60, and 72 residues respectively (lanes 3–7, Δ30-Δ72); lane 1, cells transfected with the empty vector. Cell lysates were run on 10% SDS-PAGE, transferred, and blotted with mouse monoclonal anti-FLAG antibody. A straight line indicates that PDIP38-C-FLAG migrates at a similar position to that of PDIP38-C-FLAG lacking the N-terminal 50 amino acid residues. * indicates the positions of fusion proteins.
nuclear fraction of HeLa cells isolated using this method was contaminated with unbroken cells or with mitochondrial proteins. We next fractionated the cells using the NE-PER kit from Pierce. We monitored our fractionation procedure using nuclear (lamin B) and cytoplasmic (1x-B-α, mhsp70) marker proteins. The exclusive presence of lamin B in the nuclear fraction (Fig. 6A) and 1x-B-α in the cytoplasm fraction (Fig. 6B) indicated that fractionation provided a clear separation of nuclear and cytoplasmic fractions. We observed that a small amount, about 5% of total PDIP38 (Fig. 6C), was nevertheless still present in the nuclear fraction. There was also a very minor fraction of mhs70 in the nuclear fraction. Thus, it is possible that the PDIP38 found in the nuclear fraction was because of incomplete removal of the mitochondria. Nevertheless, our data do not exclude the possibility that PDIP38 may be present in the nucleus, but if it were, it is only a minor fraction (~5%) of the total pool of PDIP38.

PDIP38 Interacts with HPV 16 E7—C-terminal His-tagged PDIP38 lacking the first N-terminal 50 amino acids (to mimic the endogenously mature protein) was overexpressed in E. coli and purified by Ni-NTA-agarose and Superdex 200 gel filtration column (Fig. 7A). PDIP38-(51–368)-C-His eluted at a position with a molecular mass of 34 kDa, as determined by comparison to protein standards on Superdex 200 using a semi-log plot of Mv versus elution volume. This is similar to the calculated molecular mass of PDIP38-C-His of ~34 kDa. We first determined if the N-terminally truncated PDIP38 was able to interact with the p50 subunit of pol δ. His-tagged p21 at the concentration of 800 nM inhibited up to 80% of the pol δ activity (40–42). We also assessed if PDIP38 has any effect on the pol δ activity in the absence of PCNA, and we found there was no appreciable effect observed (data not shown).

PDIP38 Interacts with HPV 16 E7—pol δ is well known to replicate several viral DNAs both in vitro and in vivo (40–42). It has been reported that HPV 18 E6/E7 expression significantly up-regulates the transcription of both p68 and p50 subunits of pol δ by 6.03- and 5.23-fold, respectively (43). HPV E6 and E7 proteins are two viral proteins expressed in HPV-associated human cancers (44). E7 binds to retinoblastoma protein and is involved in the transformation of cells by the
Fig. 7. **PDIP38 inhibits HeLa pol δ activity in vitro.** A, Superdex 200 chromatography was performed using 250 µl of PDIP38-(51–368)-C-His after initial purification using Ni-NTA-agarose chromatography. 10-µl aliquots were subjected to 12% SDS-PAGE, and gels were stained with Coomassie Blue. BC, 10 µl of PDIP38-(51–368)-C-His after initial purification using Ni-NTA-agarose chromatography. Arrow indicates the position of PDIP38-(51–368)-C-His with apparent molecular mass of 34 kDa on SDS-PAGE. B, GST-p50 (1 µg) was used to pull down 500 ng of purified PDIP38-(51–368)-C-His. The proteins were run on SDS-PAGE, transferred, and immunoblotted against anti-His mouse monoclonal antibody (Sigma). Lane 1, 150 ng of purified PDIP38-(51–368)-C-His as input; lane 2, control pull down with GST; lane 3, GST-HPV 16 E7 pull down of total HeLa cell lysate. C, PDIP38-(51–368)-C-His as input; lane 2, protein molecular weight marker; lane 3, control pull down with GST; lane 4, GST-p50 pull down of PDIP38-(51–368)-C-His; lane 5, GST-HPV 16 E7 pull down of PDIP38-(51–368)-C-His. Arrow indicates the position of PDIP38-(51–368)-C-His.

HPV. We determined that GST-tagged HPV 16 E7 was able to interact with pol δ as well as PDIP38 in total HeLa cell lysates. Our results indicated that GST-HPV 16 E7 oncoprotein could specifically pull down PDIP38 as well as the p125 subunit of pol δ from total HeLa cell lysates (Fig. 8A). Moreover, both GST-p50 and GST-HPV 16 E7 could specifically pull down purified mature PDIP38 (Fig. 8B), suggesting that PDIP38 could serve as a bridge protein connecting HPV E7 to the pol δ complex.

**DISCUSSION**

PDIP38 was originally identified in a yeast two-hybrid screen with the p50 subunit of human pol δ as the bait. Further investigation by a number of methods confirmed the interaction of PDIP38 and p50, and this interaction could take place when p50 was part of the pol δ complex (22). Furthermore, an interaction of PDIP38 and the pol δ complex in cell lysates was readily demonstrated in cell extracts. In addition, PDIP38 also interacted with PCNA, which functions as a DNA sliding clamp that binds pol δ (22). These strong interactions that PDIP38 might be a nuclear protein involved in some aspect of DNA metabolism involving pol δ. In this study, we show rigorously that PDIP38 is primarily a mitochondrial protein. We have unequivocally demonstrated that PDIP38 possesses a functional MTS at its N terminus. In addition, our studies show that the cleavage site for the mitochondrial processing of PDIP38 is located at ~50 residues from the N terminus. This agrees with the presence of a predicted cleavage site for mitochondrial processing protease between residues 50 and 51 (MPP, R-2 motif; see Refs. 36–38). MTSs with MPP cleavage sites conforming to a 10-residue consensus sequence are often cleaved by a second protease, MIP, by the removal of an octapeptide at the N terminus. Our data indicate that PDIP38 is not further processed by MIP. Although the sequence of PDIP38 conforms very well with the 10-residue consensus (Fig. 4C), a conserved Thr, Ser, or Gly at the +4 position from the MPP site is replaced by an Arg. This might account for the failure for PDIP38 to be cleaved by MIP. The exact cleavage site of mature PDIP38 in vivo needs to be determined by Edman N-terminal sequencing of purified endogenous PDIP38.

Our data show that PDIP38 is a previously unknown mitochondrial protein that is encoded by the chromosomal DNA and is imported into the mitochondria. The function of PDIP38 is still unknown, but its function is likely to be involved in some aspects of mitochondrial function. Because of the manner in which it was first identified, it would be expected to be present in the nucleus. In this regard, the possibility still exists that PDIP38 may be present in small amounts in the nucleus, where it serves a function that may or may not be related to its function in the mitochondria. Cell fractionation methods showed that a small amount of PDIP38 could be found in the nuclear fractions prepared by two different procedures, but in both cases we could not eliminate this as a contaminant from the mitochondrial fraction, because we could also detect small amounts of mhsps70. In addition, the size of the PDIP38 found in the nuclear fraction is similar to that of the processed mitochondrial PDIP38. If PDIP38 is present in the nucleus, then it must be transported there subsequent to its entry and processed in the mitochondria. This would only take place under conditions that would allow the nuclear import of PDIP38, which may be facilitated by the interaction of PDIP38 with nuclear export factors.
conditions that allow for the release of the mitochondrial proteins, i.e., under conditions leading to damage of the mitochondria. Thus, our findings do not completely eliminate a potential physiological role for the interaction between PDIP38 and the p50 subunit of pol δ that can be demonstrated in vitro, because this might be involved in signaling of mitochondrial damage to the nucleus. We could not detect any subunits (p125, p50, p68, or p12) of human pol δ in the mitochondrial pellet; therefore, the role of PDIP38 within the mitochondria is not likely mediated through an interaction with pol δ. Another possibility is that PDIP38 might be involved in mitochondrial DNA replication or DNA repair, through interaction with mitochondrial DNA replication proteins such as DNA polymerase γ. PCNA, which is generally considered to be a nuclear protein and whose functions are in the nucleus, has been reported to be able to stimulate mitochondrial polymerase γ activity (45). A more recent study (46) showed that monoclonal antibodies against PCNA specifically recognized a single band of 30 kDa in the mitochondria from the filamentous fungus and yeast. These studies suggest that PDIP38 might function in the mitochondria through its interaction with PCNA, even though the level of PCNA in mammalian mitochondria might be too low to be detected by Western blotting. Our results indicate that the association of PDIP38 with human pol δ might occur only when triggered by certain cellular events, the most likely of which are conditions that favor mitochondrial damage; this might include conditions that result in activation of DNA damage response or DNA repair.

There are several precedents for proteins that are involved in DNA replication and/or DNA repair to be localized to both the mitochondria and the nucleus. For example, human APE2 protein was shown to localize both to the mitochondria and the nucleus, and like PDIP38, the nuclear human APE2 protein also interacts with PCNA (47). Other proteins that were shown to be localized both in the mitochondria and the nucleus are human 8-oxoguanine DNA glycosylase OGG1 (48), Ku80 (49), human DNA topoisomerase IIIα (50), human uracil-DNA glycosylase (51, 52), and yeast DNA ligase I (53). In most cases, differential initiation sites or alternative splicing of pre-mRNA accounted for the differential intracellular localization of these proteins. In this context, it may be noted that Northern blots of total RNA from both HeLa and MCF7 cells indicated that there are two transcripts for PDIP38 with a major band of 2.0 kb and a minor band of 1.2 kb (22). Thus, there remains a possibility that other isoforms of PDIP38 that lack the N-terminal region of MTS may be localized to the nucleus. In addition, it is noted that the antibody we used was directed to the C-terminal 20 residues of PDIP38 and might not recognize all of the PDIP38 forms that may be expressed. Further investigation is needed to explore these possibilities.

The mitochondrial localization of human PDIP38 does raise the question of whether the interactions we reported between PDIP38 and p50 subunit of pol δ as well as PCNA occur under physiological conditions (22), given that both pol δ and PCNA mainly function in the nucleus. Although we cannot completely rule out the possibility that the interactions between PDIP38 and pol δ that are observed experimentally in cell extracts are due to the disruption of the mitochondria, we favor the idea that PDIP38 does contribute directly to certain DNA metabolic processes mediated by pol δ. Mature PDIP38 was capable of interacting with the p50 subunit of pol δ (Fig. 7B) and inhibited pol δ activity using a PCNA-dependent assay (Fig. 7C).

Another possibility is that PDIP38 might participate in viral DNA replication mediated by pol δ. Most viruses use cellular components to replicate their genomes in the nucleus (54). Initial studies in our laboratory show that GST-tagged HPV (human papillomavirus) 16 E7 oncoprotein can pull down PDIP38 as well as p125 of total HeLa cell lysates (Fig. 8A). In addition, in vitro experiments show that PDIP38 interacts with both p50 and HPV 16 E7 (Fig. 8B), suggesting that PDIP38 may be able to act as a bridge protein between HPV E7 and the pol δ complex. Raj et al. (55) reported that HPV E1-E4 protein could associate with mitochondria and lead to the perturbation of the cytokeratin network, disrupt the normal mitochondrial distribution, and reduce the mitochondrial membrane potential. It is possible that PDIP38 would move from the mitochondria to the nucleus after HPV infection, possibly through either structural links between mitochondrial and nuclear membranes or release from mitochondria after the reduction of the mitochondrial membrane potential (56). HPV 16 E7 was reported to interact with a number of cellular proteins including hTid1, a human homolog of the Drosophila tumor suppressor lethal (2) tumor imaginal discs, to allow the virus to replicate in differentiating epithelial cells (44). hTid1 is largely localized to the mitochondrial matrix and encodes two alternatively spliced isoforms that have opposing effects on apoptosis (57). The mouse mTid1 gene encodes three alternatively spliced isoforms that are localized to the cytosol, mitochondria, or nucleus depending on the cell type (58). hTid-1 was also reported to interact with herpes simplex virus, type 1 (HSV-1), UL9 protein and to play a role in HSV-1 DNA replication (59). It is not clear how hTid1 translocates from the mitochondria to the other cellular compartments during viral infection. Another finding that links PDIP38 with pol δ and viral replication is a report that PDIP38, p12, and p50 subunits of pol δ were also able to interact with the Epstein-Barr virus replication protein BBLF2/3 (60), which supports a possible role of PDIP38 in pol δ-mediated viral replication.

In summary, we have localized human PDIP38 to the mitochondria and demonstrated that it possesses a functional mitochondrial targeting sequence. We also demonstrate that mature PDIP38 is most likely processed by removal of the first N-terminal 50 amino acids during its entry into the mitochondria. Our findings suggest that PDIP38 may possess functions in the mitochondria that may not be related to its ability to associate with pol δ and PCNA. In this regard, P12, the fourth subunit of pol δ, was recently suggested to possess additional, cell proliferation-unrelated functions (61). Our results raise the possibility that PDIP38 may have functions that include interaction with the nuclear DNA replication system or in the linkage between pol δ and viral DNA replication.

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Further Characterization of Human DNA Polymerase δ Interacting Protein 38
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