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Two Fundamentally Distinct PCNA Interaction Peptides Contribute to Chromatin Assembly Factor 1 Function▽

Tom Rolef Ben-Shahar,[1] Araceli G. Castillo,[2] Michael J. Osborne,[4] Katherine L. B. Borden,[4] Jack Kornblatt,[3] and Alain Verreault[4]*

Cancer Research UK, London Research Institute, 44 Lincoln's Inn Fields, London WC2A 3PX, United Kingdom[1]; Departamento de Genética, Facultad de Biologia, Universitad de Malaga, 29010 Malaga, Spain[2]; Department of Biology, Concordia University, Montréal, Québec H4B 1R6, Canada[3]; and Institute for Research in Immunology and Cancer, Département de Pathologie et Biologie Cellulaire, Université de Montréal, CP 6128, Succursale Centre-Ville, Montréal, Québec H3C 3J7, Canada[4]

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Chromatin assembly factor 1 (CAF-1) deposits histones H3 and H4 rapidly behind replication forks through an interaction with the proliferating cell nuclear antigen (PCNA), a DNA polymerase processivity factor that also binds to a number of replication enzymes and other proteins that act on nascent DNA. The mechanisms that enable CAF-1 and other PCNA-binding proteins to function harmoniously at the replication fork are poorly understood. Here we report that the large subunit of human CAF-1 (p150) contains two distinct PCNA interaction peptides (PIPs). The N-terminal PIP binds strongly to PCNA in vitro but, surprisingly, is dispensable for nucleosome assembly and only makes a modest contribution to targeting p150 to DNA replication foci in vivo. In contrast, the internal PIP (PIP2) lacks one of the highly conserved residues of canonical PIPs and binds weakly to PCNA. Surprisingly, PIP2 is essential for nucleosome assembly during DNA replication in vitro and plays a major role in targeting p150 to sites of DNA replication. Unlike canonical PIPs, such as that of p21, the two p150 PIPs are capable of preferentially inhibiting nucleosome assembly, rather than DNA synthesis, suggesting that intrinsic features of these peptides are part of the mechanism that enables CAF-1 to function behind replication forks without interfering with other PCNA-mediated processes.

Eukaryotic cells in S phase not only have to replicate their entire genome but also faithfully reproduce preexisting chromatin structures onto the two nascent chromatids. The duplication of chromatin structures during DNA replication is a challenging task for eukaryotic cells. Newly synthesized histones are deposited very rapidly behind replication forks (150 to 300 bp), almost as soon as enough DNA has emerged from the replisome to allow the formation of nucleosome core particles (52). A key protein involved in coupling nucleosome assembly to DNA replication is chromatin assembly factor 1 (CAF-1). CAF-1 is a complex of three polypeptide subunits, known as p150, p60, and RbAp48 in vertebrates, that mediates the first step in nucleosome formation by depositing newly synthesized histone H3/H4 onto DNA (25, 50).

In mouse and human cells, CAF-1 localizes to virtually all DNA replication foci throughout the S phase (28, 38, 49, 54). This strongly argues that CAF-1 is a physiologically relevant histone H3/H4 nucleosome assembly factor. In addition, disruption of CAF-1 function in human cells results in a severe loss of viability that is accompanied by spontaneous DNA damage and a block in S-phase progression (20, 40, 60). Thus, unlike in *Saccharomyces cerevisiae*, the function of CAF-1 in vertebrates cannot be replaced by that of other nucleosome factors, such as members of the Hir protein family or Rtt106 (24, 27, 29). This may be because, unlike CAF-1, HIRA (a human homologue of yeast Hir1 and Hir2) does not associate with core histones that are synthesized during S phase (55). In human cells, the ability to promote nucleosome assembly preferentially onto replicating DNA is thus far unique to CAF-1.

This distinctive property of CAF-1 is mediated through proliferating cell nuclear antigen (PCNA), a homotrimeric ring that encircles double-stranded DNA (4) and acts as a sliding clamp to tether DNA polymerases to their DNA substrate and thereby enhance their processivity. Several lines of biochemical and genetic evidence support the role of PCNA in CAF-1-mediated nucleosome assembly. First, CAF-1 colocalizes with PCNA in vivo and binds directly to PCNA in vitro (27, 35, 49, 61). Second, even in the presence of excess unreplicated DNA, CAF-1 can select fully replicated plasmid DNA molecules as preferential substrates for histone deposition, but only when those molecules are associated with PCNA (49). Third, PCNA-driven DNA synthesis can also attract CAF-1 to sites of DNA repair events, such as nucleotide excision repair (12, 15, 32, 35). Fourth, a specific PCNA mutation impairs the role of CAF-1 in telomeric silencing in *S. cerevisiae* (48, 61). Interestingly, a number of PCNA mutations that reduce its interaction with other PCNA-binding proteins have apparently no effect on CAF-1 function in vivo (48, 61). This implies that the interaction of CAF-1 with PCNA is substantially different from that of other PCNA-binding proteins.

Enhancing DNA polymerase processivity is not the only function of PCNA in DNA replication. The sliding clamp also directly binds to other replication enzymes, such as DNA ligase 1, DNA polymerase δ, and FEN1 (14, 21, 37). In addition to its roles in DNA synthesis and nucleosome assembly, PCNA also directly binds to a number of enzymes that continuously mon-

* Corresponding author. Mailing address: Institute for Research in Immunology and Cancer, Département de Pathologie et Biologie Cellulaire, Université de Montréal, CP 6128, Succursale Centre-Ville, Montréal, Québec H3C 3J7, Canada. Phone: (514) 343-6816. Fax: (514) 343-7383. E-mail: alain.verreault@umontreal.ca.

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itor and correct the quality of nascent DNA. These include enzymes involved in epigenetic inheritance, such as the maintenance DNA methyltransferase DNMT1 (8), base excision repair (UNG2) (42), mismatch repair (MSH3 and MSH6) (9), DNA lesion bypass (23), and many other processes (31, 36). Even subtle defects in many of these processes, including CAF-1-dependent nucleosome assembly (39), lead to either chromosome rearrangements or mutator phenotypes, which are common features of many human cancers. Surprisingly, many of these enzymes interact with PCNA via canonical PCNA interaction peptides (PIPs) that conform to the consensus sequence QXXhXXaa, where Q is a glutamine, h is a hydrophobic residue (valine, methionine, leucine, or isoleucine), a is an aromatic residue (phenylalanine, tyrosine, tryptophan, or occasionally histidine), and X represents any amino acid. Therefore, regulatory mechanisms must exist to ensure that these fundamentally distinct PCNA-dependent processes occur in a carefully orchestrated manner without mutually interfering with each other.

In order to understand how the action of CAF-1 is coordinated with that of other PCNA-binding proteins at replication forks, we carried out a thorough study of CAF-1 PIPs by analyzing their functions using a number of assays. We found that the p150 subunit of CAF-1 contains two fundamentally distinct PIPs. The N-terminal motif (PIP1) binds strongly to PCNA in vitro but is dispensable for nucleosome assembly during simian virus 40 (SV40) DNA replication. In contrast, despite the lack of a key conserved residue, the second PIP (PIP2) of CAF-1 is crucial for replication-dependent nucleosome assembly in vitro and for targeting CAF-1 to DNA replication foci in vivo. Remarkably, although PIP2 exhibits some features of canonical PIPs, it binds only weakly to PCNA in vitro. We suggest that regulated PCNA binding via this peptide may play an important role in ensuring that CAF-1 can efficiently deposit histones behind replication forks without competing with the numerous other enzymes that require continuous access to PCNA during DNA replication. Consistent with this, we show that CAF-1 PIPs possess the ability to preferentially interfere with nucleosome assembly rather than with DNA synthesis.

MATERIALS AND METHODS

Construction of expression plasmids. pCITE1 plasmids encoding the wild type and PIP mutants of p150 were used to generate constructs for expression of either full-length or truncated forms of p150 in *Escherichia coli*. PCR products encoding the full length or fragments of human p150 that lacked a stop codon were inserted in-frame with the C-terminal His tag into the NcoI and XhoI sites of the pET28a$^+$ vector (Novagen). To create constructs for cytomegalovirus promoter-driven expression of a green fluorescent protein (GFP)-p150 fusion in mammalian cells, full-length cDNAs encoding mouse p150 PIP mutants were excised from pCITE4a vectors as BamHI-NotI fragments and inserted downstream of the GFP open reading frame into compatible sites (BglII and Bsp120I) of the EGFP-C1 vector (Clontech). To delete the dimerization region of mouse p150, a C-terminal fragment of p150 was excised from the GFP expression vectors as a SacI-BamHI fragment (the SacI site encompasses codons 649 to 650 within the dimerization region of the mouse p150 cDNA, while the BamHI site is in the polylinker of the EGFP-C1 vector, downstream of the p150 open reading frame). The original fragment was replaced by a SacI-BamHI PCR product amplified from the wild-type p150 cDNA that spans the region from residue 676 to the stop codon. The ΔD mutations therefore removed residues 651 to 675 of p150 in our mammalian cell expression vectors for GFP-p150.

DNA constructs. Mutations in human PCNA, human p150, and mouse p150 were generated by site-directed mutagenesis of the pET23/C-His-PCNA (22), pCITE1 hp150wt (25), or pCITE4a mp150wt (38) plasmids using a QuikChange kit (Stratagene). All constructs were verified by sequencing the entire cDNAs.

Protein expression and purification. Buffers contained 1× EDTA-free protease inhibitor cocktail (Roche). Human PCNA with a C-terminal hexahistidine tag (PCNA-His_6) was expressed in Codon Plus BL21(DE3) pLysS *E. coli* cells (Stratagene) transformed with the pET23/C-His-PCNA plasmid. Cells were grown to mid-exponential phase in Luria-Bertani medium containing 100 μg/ml ampicillin at 37°C, and expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 h. PCNA mutants were expressed similarly. Cells were resuspended in 20 ml wash buffer (50 mM sodium phosphate, pH 8.0, 150 mM NaCl, 10% glycerol, 20 mM imidazole, 0.02% Nonidet P-40, 5 mM 2-mercaptoethanol) per liter of original culture. After sonication, the lysate was clarified by centrifugation for 30 min at 20,000 × *g* and 4°C. The supernatant was incubated overnight at 4°C with nickel-nitrilotriacetic acid agarose beads (Qiagen) equilibrated in wash buffer. Beads were washed six times with wash buffer, and PCNA-His_6 was eluted in two steps with 250 mM and 500 mM imidazole in wash buffer. To isolate homotrimeric PCNA, the eluate was further purified over a Superdex 200 gel filtration column (GE Healthcare) in buffer A (50 mM HEPES-KOH, pH 7.6, 100 mM NaCl, 10% glycerol, 0.02% Nonidet P-40, 5 mM 2-mercaptoethanol).

Wild-type and PIP mutants of human p150 (both truncations and full length) with C-terminal hexahistidine tags were expressed and purified similarly to PCNA-His_6, with the following modifications. After induction with 0.5 mM IPTG, the *E. coli* cultures were incubated at 24°C for 4 h. Buffers were similar, except for the NaCl concentration, which was 300 mM. For nucleosome assembly assays, wild-type p150 and PIP mutants were expressed in rabbit reticulocyte lysates using the TNT quick coupled transcription/translation system (Promega) according to the manufacturer's instructions.

Protein interaction assays. Full-length p150 and fragments of p150 purified from *E. coli* were bound to 20 μl of beads of p150 monoclonal antibody (SS1) (51) cross-linked to protein G-Sepharose (GE Healthcare) using dimethylpimelimidate (17). After equilibration in buffer A, these beads were incubated with either purified PCNA-His_6 or human 293 cell S100 extract (53) in 200 μl of buffer A for 2 h at 4°C. Following washes in buffer A, bound PCNA was detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and either Coomassie blue staining for recombinant PCNA or immunoblotting with the PC10 monoclonal antibody (59) to detect PCNA from the S100 extract. To avoid detection of dimethylpimelimidate-modified SS1 antibody chains, the PC10 monoclonal antibody was directly conjugated to horseradish peroxidase using the EZ-Link Plus activated peroxidase kit (Thermo). Other protein binding assays were performed as described above but in buffer S100 (50 mM HEPES-NaOH, pH 7.6, 10% glycerol, 0.02% Nonidet P-40, 5 mM 2-mercaptoethanol, 1× EDTA-free Roche protease inhibitor cocktail) containing either 150 or 500 mM NaCl.

Peptides were synthesized with a biotin moiety attached to the N-terminal amino group via a 6-carbon methylene spacer and included an N-terminal cysteine residue to quantify the amounts of peptides by the Ellman assay. The peptides were purified by reverse-phase high-performance liquid chromatography and dissolved in water, and their concentration was determined using the Ellman assay (11). The peptides were mixed with either purified PCNA-His_6 or S100 extract in 200 μl of buffer A for 1 h at 4°C. Complexes of biotinylated peptides and PCNA were isolated using 10 μl of magnetic beads coated with streptavidin (Dynal). After washes in buffer A, the samples were analyzed as described above to detect the bound PCNA.

ITC. Recombinant PCNA-His_6 was purified as described above and dialyzed against either 10 mM sodium phosphate (pH 7.0)–10 mM NaCl or 20 mM HEPES-NaOH (pH 7.6)–100 mM NaCl. The concentrations of PCNA-His_6 and PIPs were determined by measuring their absorbances at 205 nm (47). We verified that the presence of biotin did not influence PIP binding to PCNA or the absorbance at 205 nm. The concentration of PIPs in the injection syringe was 1 mM, and the concentration of PCNA-His_6 was 50 μM, except for the low-affinity PIP of DNA polymerase κ, which was injected at 2 mM into a solution containing 100 μM PCNA-His_6. Isothermal titration calorimetry (ITC) was performed with a Microcal VP-ITC instrument maintained at 23°C.

STD NMR experiments. ^{1}H-nuclear magnetic resonance (NMR) and saturation transfer difference (STD) spectra were acquired at 25°C on a Varian Inova 600 MHz spectrometer equipped with a 5-mm pulsed-field gradient HCN cold probe. The one-dimensional STD spectra were acquired using the Biopack pulse sequence. Samples were prepared in 10 mM sodium phosphate (pH 7.0)–10 mM NaCl–50 μM Tris(2-carboxyethyl) phosphine in 90% H_2O–10% D_2O. The ratio of PCNA to PIP2 was ~1:10 (50 μM:0.5 mM). Saturation of PCNA was achieved by Gaussian-shaped pulses of 50-ms duration for a total time of 2 s with a 1-ms delay between the pulses. The on-resonance irradiation of PCNA was set at −1.2 ppm, and the off-resonance irradiation was applied at 35 ppm. Subtraction of on-

and off-resonance spectra was achieved via phase cycling. A 25-ms spin-lock pulse with a field strength of 5,000 Hz was applied to remove background protein signals. Control STD experiments were performed with free PIP2 to ensure that peptide signals were not detected in the absence of PCNA-His_6. A total of 256 scans were acquired for each sample. STD spectra were processed with exponential window functions (line broadening, 5 Hz) and zero filled twice.

Supercoiling assays for nucleosome assembly during SV40 DNA replication. Supercoiling assays were carried out as described previously (25, 50, 53).

Cell culture and immunofluorescence. Similar results were obtained with either mouse NIH 3T3 or L cells plated on poly-L-lysine-coated glass coverslips and grown in Dulbecco's modified Eagle medium with 10% fetal bovine serum. Cell transfection was essentially as described previously (38), with the following changes. Cells were left for 24 to 48 h posttransfection prior to fixation with 4% paraformaldehyde in phosphate-buffered saline for 5 min at room temperature. Detection of endogenous PCNA with the mouse monoclonal antibody PC10 and heterochromatin staining with Hoechst 33258 were as described previously (38).

RESULTS

PCNA interaction peptides of CAF-1. Given that the vast majority of PCNA-binding proteins contain canonical PIPs, we searched for these motifs in the three subunits of CAF-1 (p150, p60, and RbAp48). We found three putative PIPs in CAF-1 which are, at least to some degree, conserved from yeast to humans (Fig. 1A). Two of them reside in the p150 subunit, one is located near the N terminus (PIP1), and the second (PIP2) is within the so-called KER domain (25). The third putative PIP was found in p60. Because we failed to detect any direct interaction between a p60/RbAp48 complex and PCNA (data not shown), investigation of this motif was not pursued. The PIP2 region studied here was also identified in Cac1, the yeast orthologue of human p150, through an unbiased genetic screen for CAF-1 mutants defective in telomeric silencing (27). However, a significant difference was the substitution of a highly conserved glutamine that is present in yeast CAF-1 and other canonical PIPs. In vertebrates, this glutamine is replaced by a lysine residue in p150 PIP2 (Fig. 1A). This is a potentially important difference, because the side chain of this conserved glutamine plays a key structural role in the interaction of canonical PIPs with PCNA (4, 5, 7, 16, 33, 46, 57). In human p150, PCNA binding in vitro was ascribed to the N-terminal region of p150 (residues 1 to 31) (35). This was puzzling, given that the entire N-terminal region of human p150 (residues 1 to 296) was found to be dispensable for nucleosome assembly during DNA replication (25).

Site-directed mutations in human and mouse p150 cDNAs were designed to delete groups of 5 amino acids that constitute PIP1, PIP2, or both motifs simultaneously (Fig. 1B). We also created mutations that targeted surface-exposed residues in human PCNA (5, 16). Three pairs of amino acids were individually changed into alanine residues (Fig. 1C) to generate mutations in human PCNA which are equivalent to the *pol30-8*, *pol30-79*, and *pol30-90* mutations that have been studied in *S. cerevisiae* PCNA (1, 10). Despite the fact that it does not reside in a PCNA region that contacts canonical PIPs, the *pol30-8* mutation impairs CAF-1-dependent telomeric silencing in yeast (48, 61). In contrast, the *pol30-79* mutation substantially decreases the affinity of other proteins for PCNA (10, 14) but, surprisingly, does not affect CAF-1 function in telomere-mediated silencing (48, 61). The *pol30-79* mutation disrupts the interdomain connector loop (IDCL) that links the two halves of each PCNA monomer (Fig. 1C). The IDCL and the underlying β-sheet form a hydrophobic pocket in PCNA that accommodates the aliphatic and aromatic residues of canonical PIPs (4, 5, 7, 16, 46), while the *pol30-90* mutation lies near the C terminus of PCNA (Fig. 1C), a region that interacts with the side chain of the conserved glutamine in some proteins with canonical PIPs (7, 14, 46, 57).

In vitro, p150 binds directly to PCNA via PIP1. Human p150 and PCNA were expressed and purified from *E. coli*. Equivalent amounts of wild-type human p150 and PIP mutants were immobilized onto protein A-Sepharose beads coated with p150 monoclonal antibody. PCNA bound directly to the p150 beads (Fig. 2A), showing that the interaction did not require posttranslational modification of either p150 or PCNA. The PIP1 mutation severely compromised PCNA binding in this assay (Fig. 2A). In contrast, the PIP2 mutation had essentially no effect (Fig. 2A). This was unexpected, because a much larger deletion that includes the PIP2 region identified here was previously shown to abolish CAF-1 binding to PCNA in human cell extracts (60). In addition, a mutation of the aromatic residues in the region of yeast CAF-1 corresponding to PIP2 resulted in a partial loss of telomeric silencing and crippled the ability of yeast CAF-1 to promote replication-dependent nucleosome assembly in vitro (27). Two fragments of human p150 also bound to PCNA in a PIP1-dependent manner, and deletion of PIP2 in the longer p150 fragment (residues 1 to 462) did not impair PCNA binding (Fig. 2C). Thus, at least in this assay, the lack of PIP2-mediated PCNA binding was not due to the presence of another inhibitory domain C-terminal of PIP2. The *PCNA-79* mutation that affects the IDCL abolished PIP1-mediated PCNA binding in vitro, while the other two mutations (*PCNA-8* and *PCNA-90*) had no effect on PCNA binding in this assay (Fig. 2C). This was not expected because, in the yeast system, the *pol30-8* mutation cripples CAF-1 function in vivo, whereas the *pol30-79* mutation has no effect on CAF-1-mediated silencing (48, 61). Thus, the binding of human p150 to PCNA in vitro is dominated by PIP1, rather than PIP2, and PIP1 binding to PCNA requires the interdomain connector loop, as is the case for many other proteins with canonical PIPs.

Nucleosome assembly during DNA replication is mediated by PIP2. Next, we sought to determine whether PIP1 and/or PIP2 were required for CAF-1 function. We first assessed the relative contribution of the two PIPs to replication-dependent nucleosome assembly in the SV40 system. Equivalent amounts of wild-type p150 and PIP mutants (Fig. 3A) were expressed in the rabbit reticulocyte lysate and added to SV40 DNA replication extracts which contained the two other subunits of CAF-1 (p60 and RbAp48) (25, 50). In this assay, each nucleosome deposited onto plasmid DNA constrains one negative superhelical turn from relaxation by DNA topoisomerases present in the extract. Efficient nucleosome assembly results in the formation of replicated plasmid DNA that is extensively supercoiled. Surprisingly, the PIP1 mutation had no discernible effect on the ability of p150 to mediate nucleosome assembly. Both wild-type p150 and the PIP1 mutant promoted supercoiling of replicated DNA at the same input concentrations (Fig. 3B). In contrast, mutants lacking only PIP2 or both PIPs were completely inactive in replication-dependent nucleosome assembly, even when added in concentrations much higher than the minimal amount of wild-type p150 that was sufficient to assemble all the replicated DNA into nucleosomes

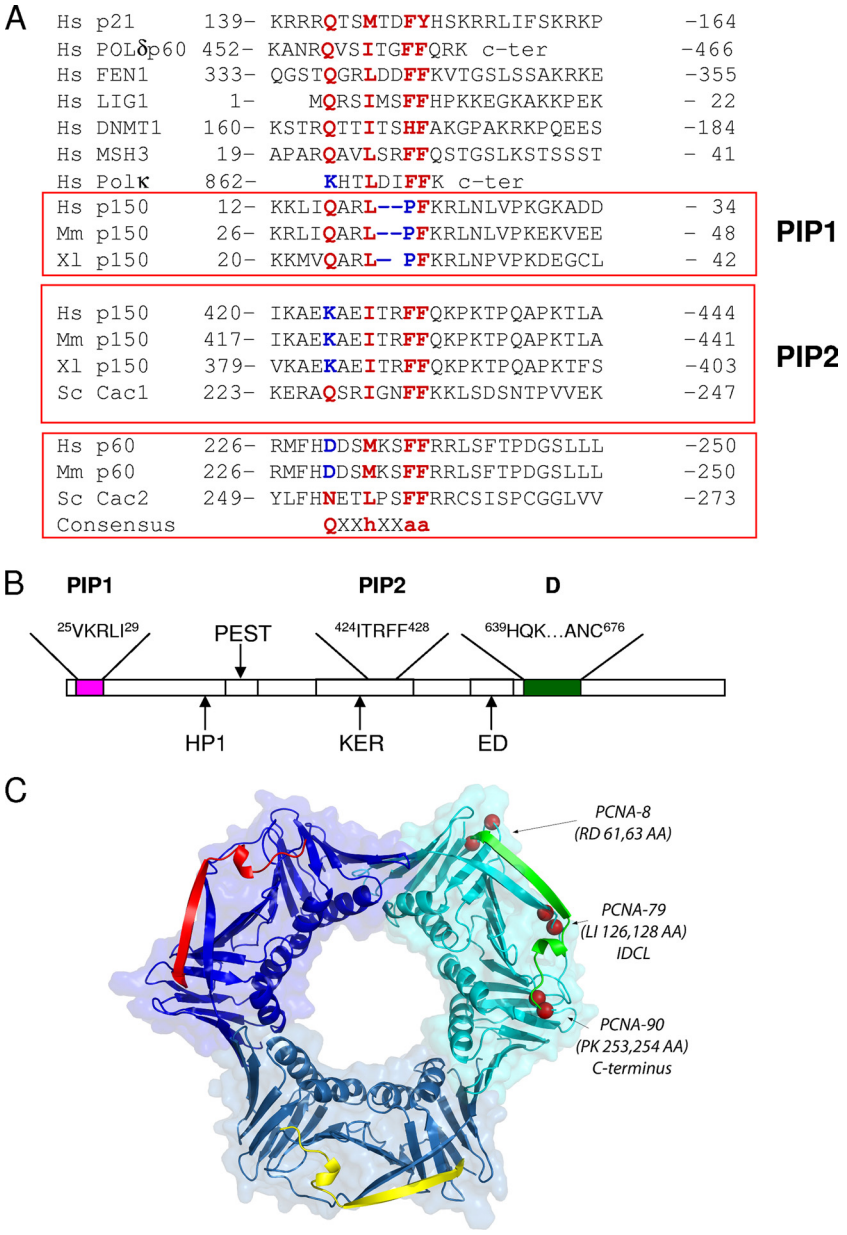


FIG. 1. CAF-1 p150 PIP and PCNA mutations. (A) Alignment of PIPs from human proteins to the motifs found in CAF-1 subunits derived from a number of distinct species: Hs, *Homo sapiens*; Mm, *Mus musculus*; Xl, *Xenopus laevis*; Sc, *Saccharomyces cerevisiae*. The conserved residues of canonical PIPs are highlighted in red, whereas nonconservative changes in the corresponding residues of CAF-1 p150 and DNA polymerase κ PIPs are shown in blue. (B) Location of PIPs in the primary structure of mouse CAF-1 p150 relative to previously described domains (25, 38, 44). (C) Front side view of a PCNA homotrimer (PDB 1AX8) (16) bound by three p21 PIPs (indicated in green, yellow, and red). The front side is the face of the PCNA ring that points in the direction of DNA synthesis. The mutations analyzed are present in the three PCNA monomers but, for clarity, are shown in the cyan subunit only.

(Fig. 3B). In the presence of high concentrations of complexes of Asf1 and H3/H4, CAF-1 can promote histone deposition onto nonreplicating DNA (27). However, at the low concentrations used here, none of the PIP mutants had any detectable ability to promote replication-independent nucleosome assembly, as judged by the absence of supercoiling in nonreplicated DNA (Fig. 3B, total DNA). The PIP2 mutation therefore did not interfere with nucleosome assembly onto replicating DNA by forcing CAF-1 to deposit histones onto nonreplicating DNA, which is present in excess in this system. The three p150 PIP mutants that we studied were wild type for binding to other known p150-associated proteins, such as a complex of p60 and RbAp48, histone H3/H4, and HP1 proteins (data available upon request). Thus, the defects in nucleosome assembly activity reported here most likely arise from alterations in PCNA binding, rather than large-scale disruption of the p150 structure. In summary, although stable PCNA binding in solution occurs mainly through PIP1, the replication-depen-

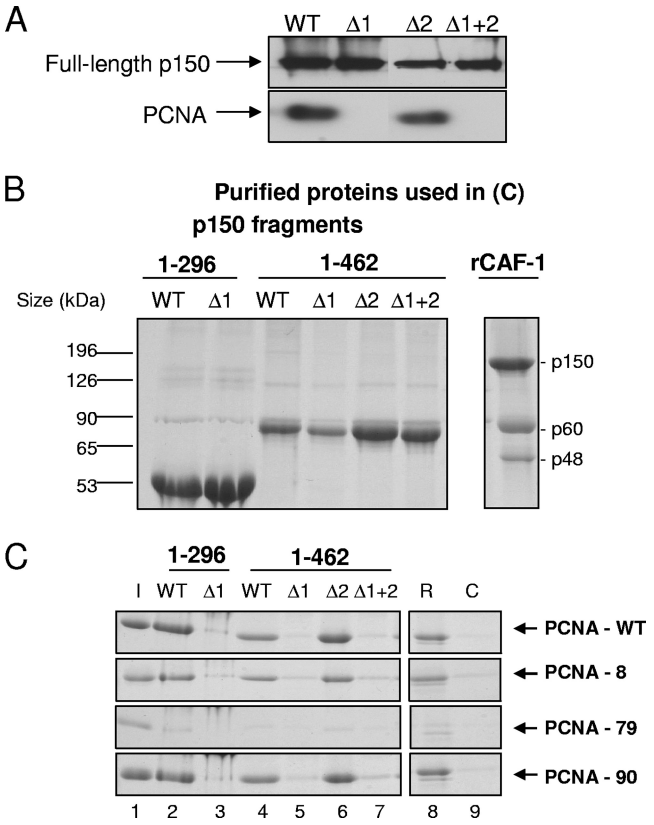


FIG. 2. Binding of CAF-1 p150 to the interdomain connector loop of PCNA is mediated by PIP1. (A) Wild-type human p150 and PIP mutants (~9.5 pmol) were bound to p150 monoclonal antibody beads. Recombinant PCNA-His_6 (~100 pmol of trimer) was incubated with the p150 beads for 2 h at 4°C. After washes in buffer A, bound proteins were detected by immunoblotting. Results shown are for bound p150 (top panel) and bound PCNA (bottom panel). (B) Coomassie blue-stained gel of human p150 fragments purified from *E. coli* and bound to p150 monoclonal antibody beads. Recombinant CAF-1 purified from insect cells is also shown. (C) Only the *PCNA-79* mutation in the interdomain connector loop affects PCNA binding via PIP1 in vitro. Wild-type and mutant forms of PCNA-His_6 were incubated with beads coated with the p150 fragments shown in panel B for 1 h at 4°C. After washes in buffer A, bound PCNA-His_6 was detected by Coomassie blue staining. Lane I, 40% of input PCNA; lane R, positive control with recombinant CAF-1 bound to beads; lane C, Negative control without p150 bound to beads.

dent nucleosome assembly activity of CAF-1 is mediated by PIP2 (Fig. 3B).

p150 targeting to DNA replication foci is mainly dependent upon PIP2. Consistent with their ability to bind PCNA in vitro, canonical PIPs have been implicated in targeting a number of enzymes to DNA replication foci (6, 30, 37, 42). Given that PIP1 was dispensable for nucleosome assembly but nonetheless bound strongly to PCNA in vitro, it seemed possible that PIP1 may serve to target CAF-1 to DNA replication foci in vivo. In order to address this issue, we expressed wild-type p150 and the PIP mutants as GFP fusion proteins and determined their localization by fluorescence microscopy. Consistent with previous reports (28, 38, 49, 54), the localization of wild-type GFP-p150 coincided with that of DNA replication foci, as assessed either by PCNA staining or by detecting sites of bromodeoxyuridine incorporation. The colocalization of wild-type GFP-p150 with PCNA was observed in different stages of S phase that could be readily distinguished by their distinct patterns of PCNA staining (Fig. 4). In addition to PIP1, the N-terminal region of p150 also contains a PXVXL motif that binds to HP1 proteins (38, 56). Through this motif, endogenous p150 and HP1 proteins form a complex that localizes near sites of pericentric heterochromatin replication during late S phase (43, 45). In our experiments, all the PIP mutants colocalized with HP1 not only during heterochromatin replication but also outside of S phase. This is likely because PCNA is not needed for p150 and HP1 to interact with each other (38, 45). However, this PCNA-independent interaction between CAF-1 and HP1 prevented us from determining whether the PIPs were necessary for CAF-1 to bind PCNA during heterochromatin replication. Therefore, we only studied the effects of the PIP mutations on p150 targeting to early- and mid-S-phase DNA replication foci.

The p150 PIP mutants exhibited an unexpected localization pattern. In a fraction of the transfected cells, the mutants colocalized relatively well with PCNA (Fig. 4B and C and K and L). However, in other transfected cells, the GFP-p150 PIP mutants localized to both replication foci and heterochromatic foci, even when heterochromatin was not undergoing replication, as revealed by the absence of PCNA staining in the large heterochromatic foci that were readily detectable by Hoechst staining (Fig. 4D, E, and F and M, N, and O). In a small fraction of the transfected cells, the GFP-p150 PIP mutants localized exclusively to heterochromatin, even though the cells were clearly in early or mid-S phase as judged by their patterns of PCNA staining (Fig. 4G, H, and I and P, Q, and R). PIP mutations therefore resulted in an aberrant localization of GFP-p150 to heterochromatin in early or mid-S phase, a phenotype that was never observed with wild-type GFP-p150. This was not an artifact of overexpression. The localization of wild-type and mutant proteins was essentially the same in individual cells that exhibited a broad range of GFP fluorescence. To determine the frequency of these anomalous localization patterns, a large number of transfected cells were scored for each localization pattern. This analysis revealed that mistargeting of GFP-p150 to heterochromatin was a relatively rare event in PIP1 and PIP2 single mutants but occurred more frequently when both PIPs were mutated simultaneously (Fig. 5). Thus, the two PIPs contribute to prevent abnormal localization of p150 to heterochromatin in early and mid-S phase. However, even when both PIPs were mutated, 43% of cells still showed colocalization of GFP-p150 with PCNA with no aberrant localization to heterochromatin during early stages of S phase (Fig. 5, ΔPIP1 + 2). In addition, 55% of cells contained p150 PIP mutants in both nonreplicating heterochromatin and DNA replication foci (Fig. 4M, N, and O and 5). Deletion of both PIPs was therefore not sufficient to abolish the targeting of GFP-p150 to DNA replication foci.

It has been demonstrated that p150 forms homodimers (44). Therefore, we hypothesized that the retention of GFP-p150 PIP mutants in DNA replication foci was due to their dimerization with endogenous p150 molecules carrying wild-type PCNA interaction peptides. To test this possibility, we created an additional series of GFP-p150 expression constructs where the PIP mutations were combined with a deletion of the dimer-

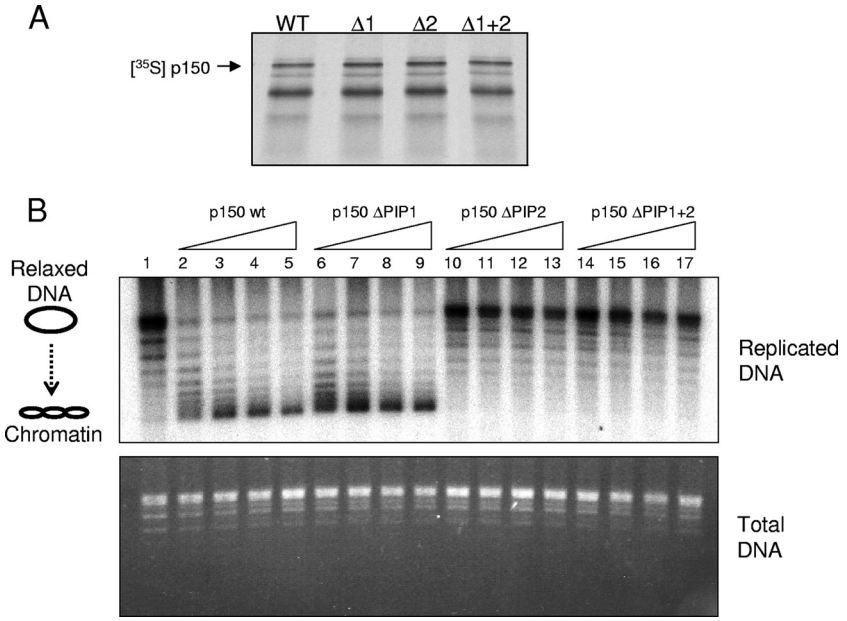


FIG. 3. Nucleosome assembly during SV40 DNA replication is dependent upon PIP2. (A) Wild-type and PIP mutants of human p150 were expressed in the rabbit reticulocyte lysate and detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. (B) Nucleosome assembly reactions performed in the SV40 DNA replication system (without T antigen) in the presence of increasing amounts of wild-type p150 or PIP mutants. Each p150 protein was titrated over an eightfold range of protein concentrations in twofold increments. Replicated DNA was detected by incorporation of [α-^{32}P]dAMP and autoradiography. Total DNA was stained with ethidium bromide. Lane 1, negative control reaction lacking p150.

ization domain. The dimerization domain mutation, either by itself or in combination with a PIP1 mutation, only led to a modest increase in the frequency of S-phase cells in which GFP-p150 was inappropriately localized to heterochromatin (Fig. 5, ΔD and ΔD ΔPIP1). However, when combined with a PIP2 mutation, the absence of dimerization domain caused a pronounced defect in targeting of GFP-p150 to DNA replication foci (Fig. 5, ΔD ΔPIP2), which was almost equivalent to

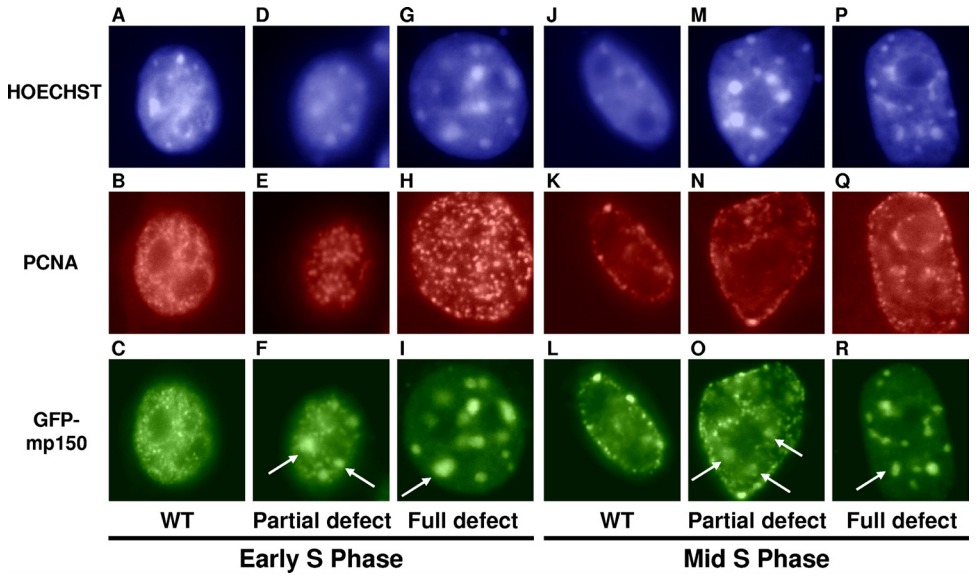
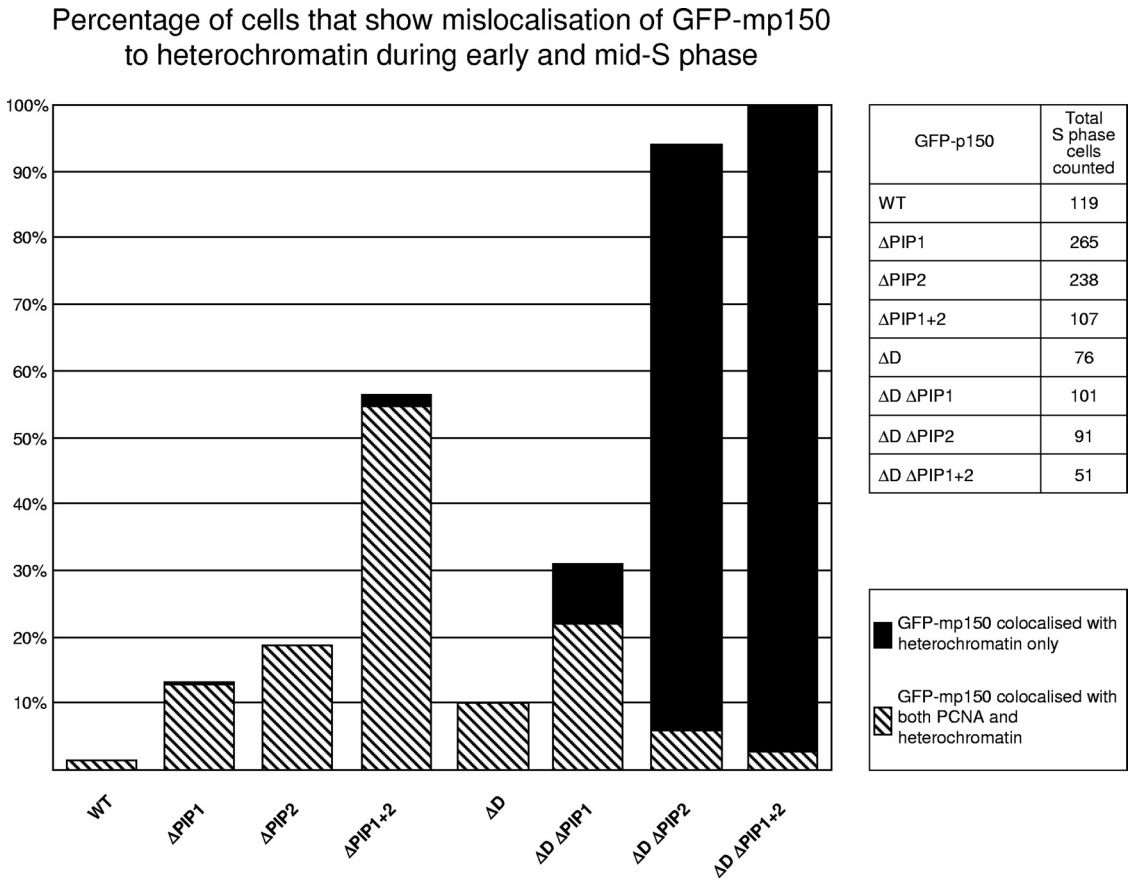


FIG. 4. PIP mutations result in mistargeting of mouse p150 to heterochromatin in early- and mid-S-phase cells. Mouse NIH 3T3 cells were transfected with constructs for cytomegalovirus promoter-driven expression of GFP-p150 PIP mutants and stained with a PCNA monoclonal antibody to detect DNA replication foci and Hoechst 33258 to reveal foci of pericentric heterochromatin. White arrows indicate examples of p150 PIP mutants that were mistargeted to heterochromatin in either early- or mid-S-phase cells. The images in panels A to F and J to O were obtained with the ΔPIP1 + 2 mutant, which rarely showed a full defect in GFP-p150 localization to DNA replication foci (Fig. 5). The images in panels G to I and P to R were derived from observations of the ΔD ΔPIP2 mutant, for which a complete lack of localization of GFP-p150 to DNA replication foci was very frequent (Fig. 5).



GFP-p150	Total S phase cells counted
WT	119
ΔPIP1	265
ΔPIP2	238
ΔPIP1+2	107
ΔD	76
ΔD ΔPIP1	101
ΔD ΔPIP2	91
ΔD ΔPIP1+2	51

FIG. 5. The two PIPs of mouse p150 function contribute to prevent mistargeting of CAF-1 p150 to heterochromatin. Severe defects in localization of p150 to DNA replication foci were observed in GFP-p150 mutants lacking both PIP2 and the dimerization region. Cells transfected with each PIP mutant were examined to assess the percentage of transfected cells showing mistargeting of GFP-p150 to heterochromatin in either early or mid-S phase. The fraction of cells with a partial defect in targeting (GFP-p150 observed in both replication foci and heterochromatin) is indicated by the stippled columns. The fraction of cells with a complete defect in targeting (GFP-p150 absent from replication foci and present in heterochromatin only) is represented by the black columns. ΔD, deletion of the dimerization motif.

that seen when both PIPs and the dimerization domain were deleted simultaneously (Fig. 5, ΔD ΔPIP2 and ΔD ΔPIP1 + 2). In at least 95% of cells expressing either of these two mutants, the GFP-p150 signals in early- and mid-S-phase cells were largely restricted to heterochromatin with little or no colocalization with PCNA (Fig. 4G, H, and I and P, Q, and R). Thus, although both PIPs contribute to p150 targeting to DNA replication foci, PIP2 is more crucial. However, the requirement for PIP2 is much more apparent when the dimerization domain is absent. This is likely due to dimerization of the GFP-p150 PIP mutants with endogenous p150 carrying wild-type PIPs.

p150 PIPs are distinct from canonical PIPs. A number of crystal structures of complexes between PIPs and PCNA have been solved. The canonical PIPs adopt very similar conformations when bound to PCNA and, as stated earlier, the side chain of a conserved glutamine residue (Fig. 1A) is involved in numerous interactions with PCNA (4, 5, 7, 16, 33, 46, 57). Interestingly, PIP2 contains a lysine, rather than a glutamine in this position (Fig. 1A). PIP1 also exhibits a number of differences from canonical PIPs (Fig. 1A). Because of these differences, both p150 PIPs likely adopt a conformation which is different from that of canonical PIPs when bound to PCNA. We addressed whether the p150 motifs are biochemically distinct from canonical PIPs by using the synthetic peptides described in Fig. 6A. A peptide derived from the cyclin-dependent kinase inhibitor p21, whose binding to PCNA has been extensively studied (5, 16, 62), was used as a canonical PIP. We first tested whether the synthetic peptides derived from p150 bound directly to PCNA in a manner analogous to that of canonical PIPs. The p21 PIP and p150 PIP1 both bound directly to PCNA, and their interaction was strongly reduced by replacing pairs of hydrophobic residues with alanines (Fig. 6B). Interestingly, even the LI hydrophobic residues of PIP1 that are immediately before the conserved glutamine (Fig. 6A) make a substantial contribution to the interaction of p150 PIP1 with PCNA (Fig. 6B). In canonical PIPs, the identity of residues preceding the conserved glutamine is not important for PCNA binding because their side chains do not interact with PCNA (46, 57). This result underscores the fact that PIP1 is distinct from canonical PIPs. The p21 PIP was able to bind PCNA from the S100 extract used for nucleosome assembly, and this binding was not affected by the addition of an equimolar amount of PIP1 as competitor (Fig. 6C, lanes 1 to 4). In

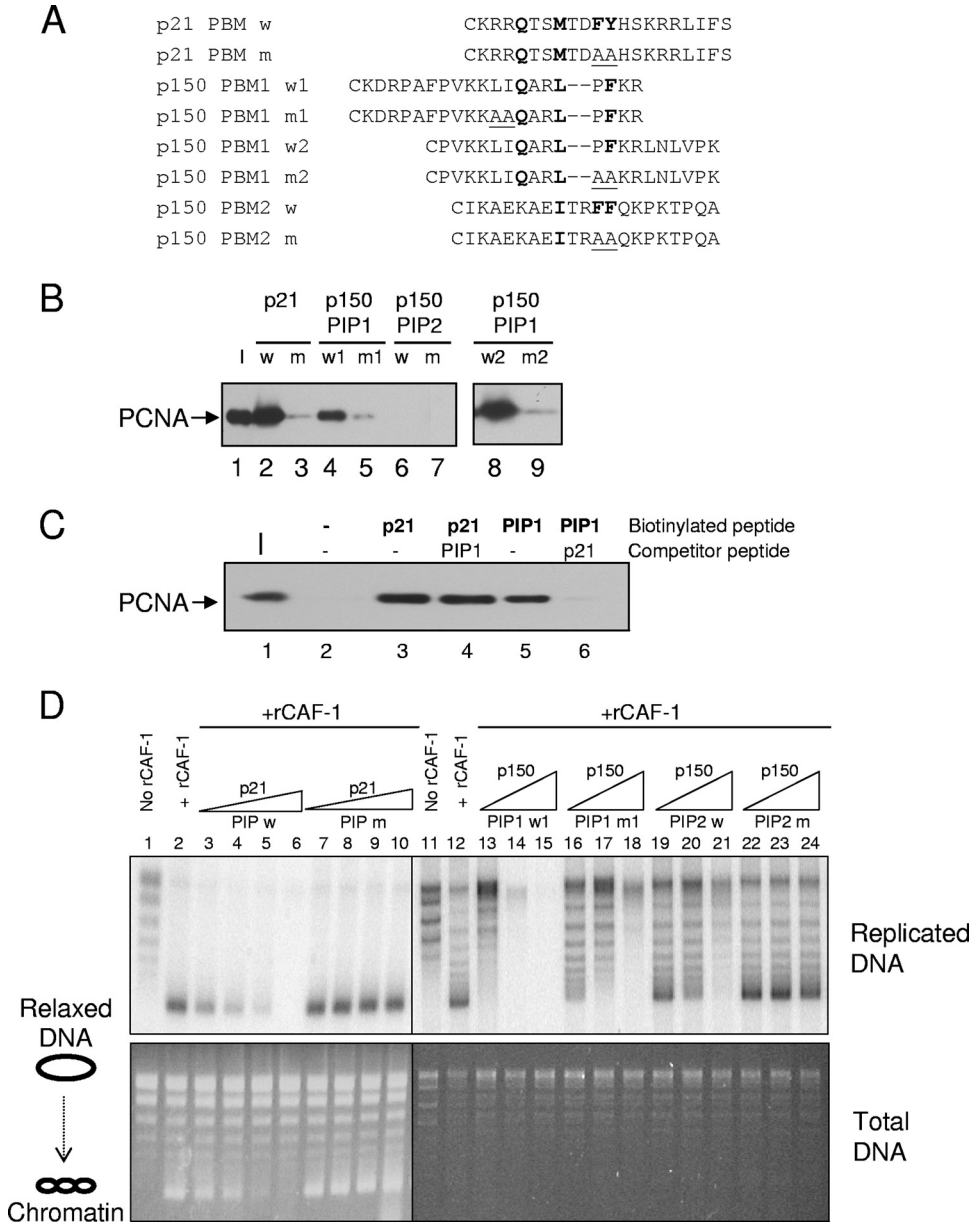


FIG. 6. Both PIPs of human p150 preferentially interfere with nucleosome assembly, rather than DNA synthesis, even though they exhibit striking differences in their respective abilities to bind to PCNA. (A) Synthetic peptides used in panels B to D. Residues characteristic of canonical PIPs are shown in bold. Wild-type and mutant peptides are denoted as w and m, respectively, and the mutated residues are underlined. (B) Biotinylated synthetic PIPs and PCNA-His_6 were incubated in an equimolar ratio (ratio of peptide to PCNA monomer) in buffer A for 1 h at 4°C. The biotinylated peptides were isolated using streptavidin-coated magnetic beads and, after washes, bound PCNA-His_6 was detected by immunoblotting. Lane I, 10% of input PCNA. (C) The p21 PIP has a higher affinity for PCNA than p150 PIP1. The same experiment as described for panel B was performed using S100 extract as a source of PCNA, either in the absence or presence of nonbiotinylated peptides that competed for PCNA binding to biotinylated PIPs. The molar ratio of biotinylated and competitor peptide in lanes 4 and 6 was 1:1. Lane I, 25% of input S100 extract. (D) T antigen-independent DNA synthesis reactions were performed in the S100 extract in the absence (negative control) or presence of 0.45 pmol of recombinant CAF-1 (rCAF-1; positive control), which was sufficient to promote supercoiling of all the replicated DNA (lanes 1 and 2 and 11 and 12). Increasing amounts of wild-type and mutant PIPs were titrated into reaction mixtures that contained fixed amounts of rCAF-1 to determine whether the peptides had any preference to inhibit nucleosome assembly rather than DNA synthesis. The amounts of peptide used were 0.2, 0.4, 0.8, and 1.6 nmol for the p21 PIPs (lanes 3 to 6 and 7 to 10) and 1.6, 3.2, and 6.4 nmol for all the p150 PIPs (lanes 13 to 24). Replicated DNA was detected by incorporation of [α-^{32}P]dAMP and autoradiography. Total DNA was stained with ethidium bromide.

contrast, the binding of PIP1 to PCNA derived from the S100 extract was eliminated by the addition of an equimolar amount of p21 PIP competitor (Fig. 6C, lanes 5 and 6), suggesting that PIP1 has a lower affinity for PCNA than the p21 PIP. Consistent with the results shown in Fig. 2, PIP2 did not bind at all to PCNA in this pull-down assay (Fig. 6B). In addition, unlike wild-type PIP2, a peptide with a lysine-to-glutamine substitution in PIP2 was able to deplete PCNA from S100 extracts

(data available upon request). In contrast, a PIP2 modified with acetyl lysine did not allow PCNA binding in the S100 extract (data available upon request), arguing that acetylation of the lysine in wild-type p150 PIP2 would not be sufficient to provide stable PCNA binding.

The lack of p150 PIP2 binding to PCNA in pull-down assays was puzzling, because PIP2 was more important than PIP1 for replication-coupled nucleosome assembly (Fig. 3) and targeting of CAF-1 to DNA replication foci (Fig. 5). We therefore decided to assay the binding of PIP2 to PCNA by ITC, a technique more suitable to detect and quantify weak interactions that might be disrupted during the extensive washing steps performed in pull-down assays. When ITC experiments were conducted in 10 mM sodium phosphate, pH 7.0, and 10 mM NaCl, PIP2 did bind to PCNA with a K_d of 5.44 μM (Fig. 7A and Table 1), and binding required the aromatic residues of p150 PIP2, as is the case for canonical PBMs (Table 1). Under these conditions, PIP2 bound PCNA more strongly than the PIP of DNA polymerase κ (Fig. 7 and Table 1) which, like CAF-1 PIP2, also contains a lysine rather than a canonical glutamine (Fig. 1A). Moreover, at low salt concentrations, wild-type PIP2 and PIP2 with a lysine-to-glutamine mutation had similar affinities for PCNA-His_6 (Fig. 7B and Table 1). However, at the higher ionic strength under which the pull-down assays were performed, binding of wild-type PIP2 was much weaker, whereas the lysine-to-glutamine substitution in PIP2 still showed robust binding to PCNA (Fig. 7D and E and Table 1). This was apparent from the fact that the heat exchanges which resulted from injection of wild-type PIP2 were much smaller than those obtained with PIP2 in which the lysine was replaced by a canonical glutamine (Fig. 7D and E).

We also monitored binding of PIP2 to PCNA by STD ^{1}H-NMR spectroscopy (34). In this experiment, the PCNA protein is selectively irradiated to saturation under conditions where PIP2 signals are not directly perturbed. Magnetization is transferred to protons of PIP2 that are in close proximity to PCNA, and this can only be observed when PIP2 binds to and dissociates rapidly from PCNA (34). Thus, in the STD experiment, signals appear only when PIP2 is bound transiently to PCNA, allowing for easy identification of relatively weak binding. Moreover, the strongest signals in the STD spectrum correspond to protons of PIP2 in intimate contact with PCNA. Signals in the ^{1}H-STD spectra of the wild-type and the lysine-to-glutamine PIP2 were clearly detected in the presence of PCNA (Fig. 8A and B), indicating an interaction. The largest peaks in these two spectra (Fig. 8A and B), hence those that arise from close contact with PCNA, originate from the same residues and were assigned, based on two-dimensional homonuclear total correlation spectroscopy and nuclear Overhauser effect spectroscopy NMR experiments (data not shown), to the Phe and the Ile residues of PIP2. Consistent with the ITC data, mutation of the Phe residues abolished the signals obtained in the STD NMR experiment (Fig. 8C), demonstrating the importance of the PIP2 Phe residues for PCNA binding.

We compared the respective abilities of our peptides to compete with CAF-1 and replication enzymes to inhibit either nucleosome assembly or DNA synthesis. These experiments were conducted in either the presence or absence of T antigen in the S100 extract for DNA synthesis-coupled nucleosome assembly. In the absence of T antigen, DNA synthesis is primed by nicks created in the template DNA and is much less vigorous than in the complete system. In spite of this, the synthesis is relatively processive and requires PCNA loading which, in turn, elicits CAF-1-mediated nucleosome assembly (35). Recombinant CAF-1 purified from insect cells served as a source of nucleosome assembly activity. In reactions lacking T antigen, titration of the p21 PIP inhibited DNA synthesis (Fig. 6D), as previously reported for T antigen-driven SV40 DNA replication (58). Inhibition of DNA synthesis was also observed with the CAF-1 p150 PIPs, but this required larger amounts of peptide than in the case of the p21 PIP (16- and 32-fold for p150 PIP1 and PIP2, respectively) (Fig. 6D). The ability of CAF-1 p150 PIP1 to inhibit DNA synthesis was reduced, but not completely abolished, upon mutation of the LI hydrophobic amino acids to alanine residues (LI in PIP1) (Fig. 6A and D). This suggests that the residual affinity for PCNA of this mutant form of PIP1 (Fig. 6B) was sufficient to interfere with DNA synthesis. The fact that the p21 PIP inhibited DNA synthesis at substantially lower concentration than PIP1 (Fig. 6D) was consistent with the lower affinity of PIP1 for PCNA revealed by competitive binding assays (Fig. 6C). In addition, even CAF-1 p150 PIP2, which exhibits the lowest affinity for PCNA at moderate ionic strength, based on pull-down assays and ITC, was able to interfere with DNA synthesis (Fig. 6D). This effect was dependent upon the aromatic residues of PIP2 (Fig. 6C), suggesting that PIP2 inhibits DNA synthesis by competing with other PCNA-binding proteins in the S100 extract.

Careful titration of PIPs revealed an interesting qualitative difference between the p21 and CAF-1 p150 peptides. DNA synthesis was progressively inhibited by increasing concentrations of the p21 peptide without any discernible appearance of partially supercoiled replicated DNA molecules (Fig. 6D, lanes 2 to 6). In contrast, the addition of CAF-1 PIP1, PIP2, or a 296-residue fragment derived from the N-terminal domain of p150 all led to the formation of replicated DNA molecules with intermediate degrees of supercoiling (Fig. 6D, lanes 11 to 24; additional data available upon request). These correspond to plasmid DNA molecules that are incompletely assembled into nucleosomes (26). Thus, over a narrow range of peptide concentrations, the p150 PIPs inhibited nucleosome assembly but had little effect on total DNA synthesis as determined by autoradiography of the replication products. This was not observed with the p21 PIP (Fig. 6D, lanes 2 to 6) and may therefore reflect structural features of the noncanonical CAF-1 p150 PIPs that allow them to target PCNA in a manner that preferentially competes with CAF-1 without interfering with DNA synthesis. However, two lines of evidence argue that this discrimination is not absolute. First, when present at high concentrations, the two p150 PIPs inhibited DNA synthesis (Fig. 6D, lanes 14, 15, and 21). Second, in the complete system for T antigen-driven SV40 replication, the ability of the p150 PIPs to preferentially inhibit nucleosome assembly was lost (data not shown).

DISCUSSION

Human CAF-1 contains two noncanonical PIPs. In this report, we showed that the large subunit of human CAF-1 (p150) contains two fundamentally distinct PIPs. PIP1, which is located near the N terminus of p150, binds directly to the inter-

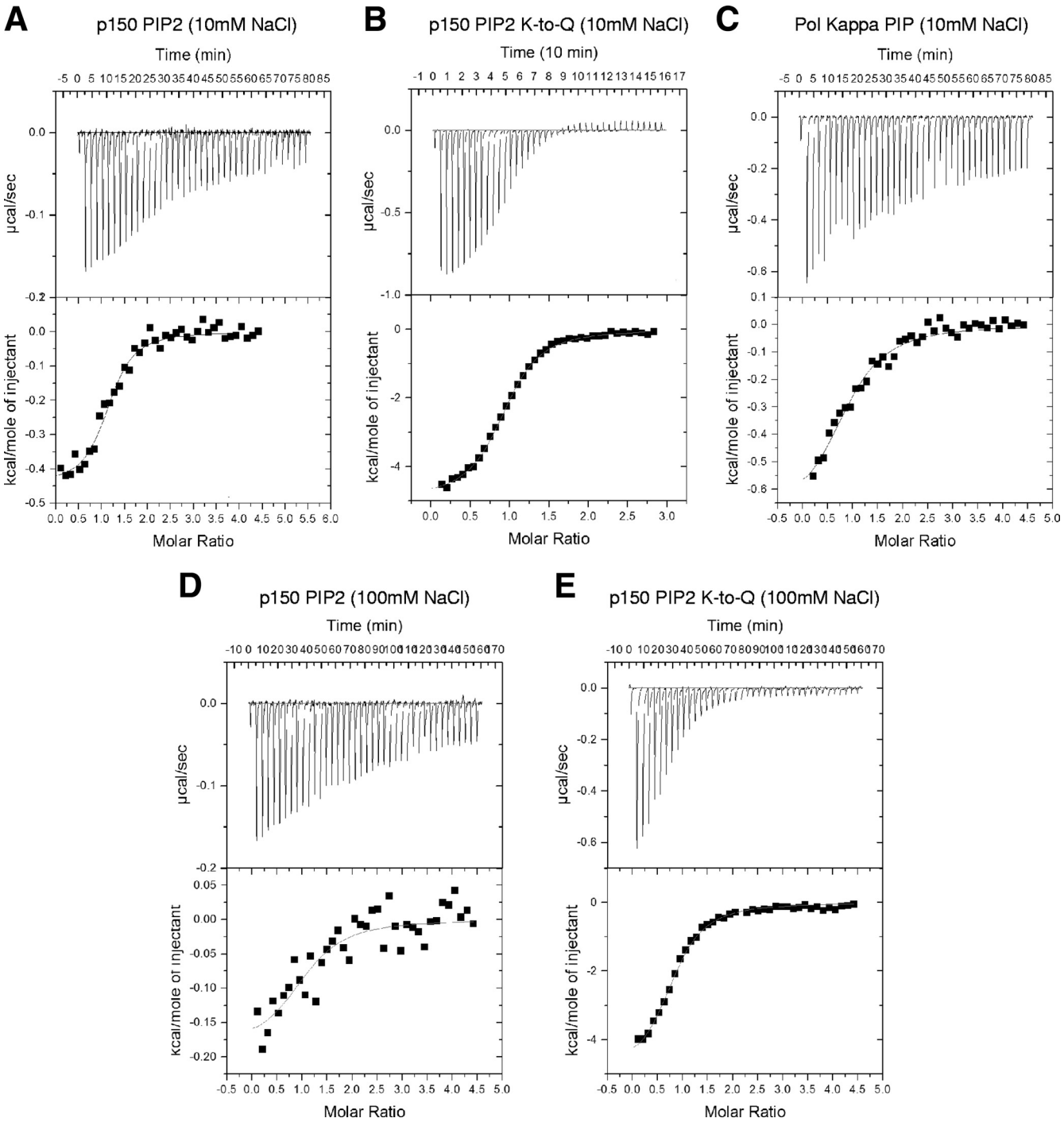


FIG. 7. The noncanonical CAF-1 p150 PIP2 and DNA polymerase κ PIP bind directly to PCNA at low salt concentration based on isothermal titration calorimetry. Synthetic peptides were progressively titrated into a sample cell containing PCNA-His_6. In each panel, the upper half shows the measured heat exchanges during each peptide injection. The lower half of each panel shows the enthalpic changes as a function of the molar ratio of peptide to PCNA monomer. The black squares correspond to the experimental data points. (A to C) Titrations performed in 10 mM sodium phosphate, pH 7.0, 10 mM NaCl. (D and E) Titrations performed in 20 mM HEPES-NaOH, pH 7.6, 100 mM NaCl.

domain connector loop of PCNA (Fig. 2). Surprisingly, despite its robust interaction with PCNA in vitro, PIP1 is not required for CAF-1-dependent nucleosome assembly during SV40 DNA replication and only makes a modest contribution to targeting CAF-1 to DNA replication foci (Fig. 3 and 5). In contrast, PIP2 binds weakly to recombinant PCNA at moderate ionic strength (Fig. 2, 6, and 7; additional data available upon request) but is nonetheless crucial for replication-dependent nucleosome assembly in the SV40 system (Fig. 3). PIP2 also plays an important role in directing CAF-1 p150 to DNA

TABLE 1. Thermodynamic parameters of PCNA binding to PIPs[a]

Exptl buffer and peptide	K_d (μM)	N[b]	ΔG (kcal mol^{-1})	ΔH (kcal mol^{-1})	ΔS (cal K^{-1} mol^{-1})
10 mM Na-phosphate (pH 7.0), 10 mM NaCl					
p150 PIP2 wild type	5.44	0.94	−7.12	−0.680	21.8
p150 PIP2 K-to-Q	4.74	0.95	−7.21	−4.93	7.70
p150 PIP2 FF-to-AA	No binding				
Pol[c] κ PIP	23.9	0.95	−6.25	−0.706	18.7
20 mM HEPES-NaOH (pH 7.6), 100 mM NaCl					
p150 PIP2 wild type	ND[d]				
p150 PIP2 K-to-Q	7.42	0.85	−6.94	−4.97	6.66
p150 PIP2 FF-to-AA	No binding				

[a] Determined by isothermal titration calorimetry at 296 K. All data are derived from Fig. 7, and the binding curves were fit to one binding site per PCNA monomer, as observed in several crystal structures of PIPs bound to PCNA.
[b] Stoichiometry of peptide binding per PCNA monomer.
[c] Pol, polymerase.
[d] ND, not determined. The very small measured heat exchanges led to data scattering (Fig. 7D), making it difficult to determine accurately the inflection point of the binding curve and therefore the K_d.

replication foci during early and mid-S phase by preventing untimely association of p150 with pericentric heterochromatin (Fig. 4 and 5). Although the CAF-1 motifs share some features with canonical PIPs, such as the presence of conserved hydrophobic and aromatic residues that are important for function, CAF-1 PIPs also exhibit unique properties. Under specific in vitro conditions, CAF-1 PIPs possess an intrinsic ability to preferentially inhibit nucleosome assembly mediated by CAF-1 and PCNA, rather than simply block PCNA-dependent DNA synthesis (Fig. 6D). These results suggest that CAF-1 PIPs bind to PCNA in a manner that is related to, but somewhat distinct from, canonical PIPs. Although the structure of CAF-1 p150 PIP2 bound to PCNA will be necessary to reach a definite conclusion, this model is supported by the fact that the binding of canonical PIPs to PCNA is enthalpy driven (5), whereas the binding of noncanonical PIPs, such as CAF-1 PIP2 and the DNA polymerase κ PIP, is not (Table 1).

Low-affinity PIPs are physiologically important. Unexpectedly, we found that p150 PIP2 is critical for CAF-1 function, even though it binds to PCNA more weakly than PIP1 in vitro. The weak binding of PIP2 to PCNA can be readily explained based on the available crystal structures of canonical PIPs (QXXhXXaa) complexed with PCNA (4, 5, 7, 16, 33, 46, 57). In these structures, the carbonyl and the amino groups of the highly conserved glutamine side chain are involved in several interactions with PCNA. In addition, two positively charged residues of PCNA (Arg210 and Lys254 in human PCNA) are in close proximity to the glutamine side chain. In human p150 PIP2 and DNA polymerase κ, the conserved glutamine residue of canonical PIPs is replaced by a lysine (Fig. 1A). Due to the loss of the multiple interactions mediated by the glutamine side chain and the proximity of positively charged PCNA residues, a lysine at that position would considerably weaken PCNA binding. Interestingly, a recent crystal structure showed that a lysine at that position of the DNA polymerase κ PIP is incompatible with the multiple interactions made by the glutamine present in canonical PIPs (19). Two of our results also strongly support this conclusion. First, we observed that a substitution of the lysine by a glutamine enhances direct binding of CAF-1 p150 PIP2 to PCNA at moderate ionic strength (Fig. 7 and Table 1). Second, unlike wild-type PIP2, a synthetic p150 peptide containing the lysine-to-glutamine substitution was able to deplete PCNA from S100 extracts (data available upon request). We therefore conclude that the noncanonical PIP2 of CAF-1 p150 and DNA polymerase κ have a low intrinsic affinity for PCNA at physiological ionic strength, at least in part because the conserved glutamine residue is replaced by a lysine.

Interestingly, DNA lesion bypass polymerases η and ι also bind to PCNA in a manner that is structurally distinct from enzymes that contain canonical PIPs (19). It is tempting to speculate that this is a general mechanism to ensure that neither CAF-1 nor the error-prone DNA polymerases interfere with other PCNA-binding enzymes that are part of the replisome (e.g., replicative DNA polymerases, RFC, FEN1, and DNA ligase 1) or enzymes that act quickly on nascent DNA immediately behind replication forks (e.g., the maintenance DNA methyltransferase, uracil DNA glycosylase, and mismatch repair enzymes). This hypothesis is consistent with our

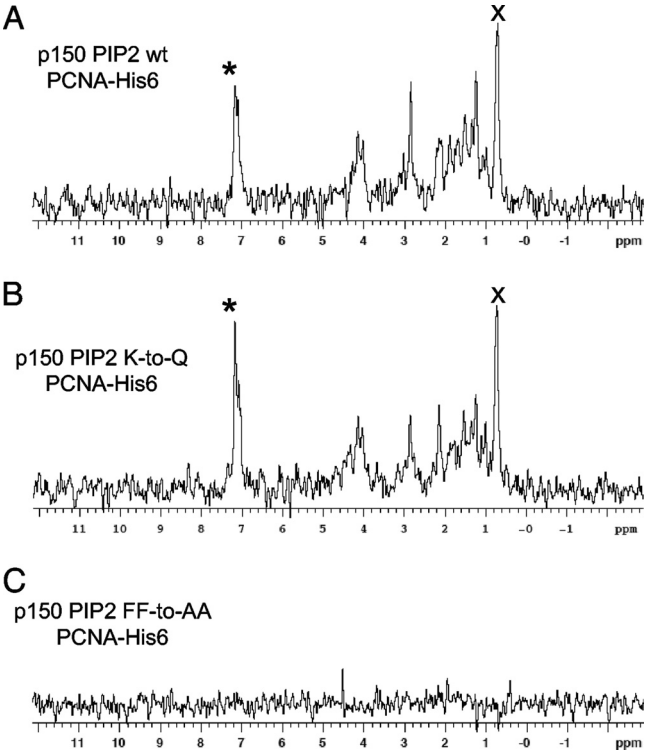


FIG. 8. Hydrophobic residues of CAF-1 p150 PIP2 are critical for its binding to PCNA-His_6 as measured by STD NMR in 10 mM sodium phosphate, pH 7.0, 10 mM NaCl. ^{1}H STD NMR spectra of wild-type PIP2 (A) and lysine-to-glutamine PIP2 (B) in the presence of PCNA-His_6 (see Materials and Methods for details) are shown. Given their sequence similarities, many of the same peptide resonances were observed in the STD NMR spectra. In particular, the intense peaks originating from the ring protons of the Phe residues (~7.25 ppm; labeled with an asterisk) and the methyl protons of Ile (~0.85 ppm; labeled with an X) suggest they are in close proximity to PCNA-His_6. (C) The ^{1}H STD NMR spectrum of the the PIP2 FF-to-AA mutant in the presence of PCNA-His_6 shows no signal, indicating that they do not interact under these conditions.

data showing that p150 PIP2 can interfere with DNA synthesis in vitro when present at sufficiently high concentrations (Fig. 6D). In vivo, the concentration of CAF-1 may be accurately controlled such that the low affinity of PIP2 for PCNA ensures that CAF-1 can bind PCNA without impeding replication, maintenance DNA methylation, or quality control of nascent DNA. However, several lines of evidence suggest that this is not the full story and that additional mechanisms act to prevent CAF-1 from interfering with DNA replication while enabling it to function through PCNA when replication is under way. It is paradoxical that CAF-1 p150 PIP2 is required for PCNA-dependent nucleosome assembly in vitro (Fig. 3) but, in the absence of DNA synthesis, CAF-1 does not bind stably to PCNA in the same S100 extract used for nucleosome assembly (Fig. 2; additional data available upon request). A possible explanation for this is that CAF-1 binding to PCNA is only enhanced during ongoing DNA replication. For instance, the S-phase protein kinase CDC7-DBF4 directly phosphorylates CAF-1 p150, which increases its interaction with PCNA and replication-coupled nucleosome assembly in vitro (13). In addition, as originally shown for FEN1 (14) and as more recently suggested by studies of Cdt1 (18), certain proteins interact differently with PCNA, depending on whether PCNA is free in solution or encircles DNA during replication (14, 18). This may well be the case for CAF-1. Thus, by analogy with DNA polymerase κ, which requires both its PIP and DNA damage-induced PCNA monoubiquitylation for function (2, 3, 19, 41), it is possible that several features of CAF-1 contribute to ensure that other PCNA-binding enzymes have been assembled into a functional replisome before CAF-1 can bind stably to PCNA loaded onto DNA. Further studies are needed to fully understand how CAF-1 access to PCNA is coordinated with that of other PCNA-binding proteins in vivo. The answer to this question will have profound implications not only for replication-coupled nucleosome assembly but also for the faithful propagation of genetic and epigenetic information in proliferating cells.

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