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Phosphorylation of the p68 Subunit of Pol δ Acts as a Molecular Switch To Regulate Its Interaction with PCNA

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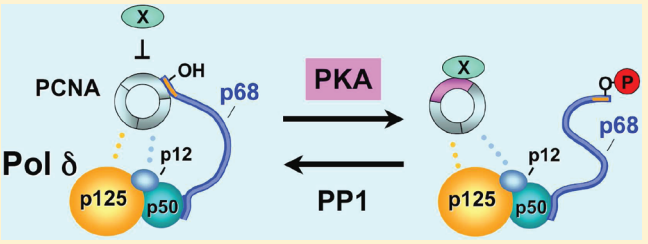
ABSTRACT: DNA polymerase delta (Pol δ) is a central enzyme for eukaryotic DNA replication and repair. Pol δ is a complex of four subunits p125, p68, p50, and p12. The functional properties of Pol δ are largely determined by its interaction with its DNA sliding clamp PCNA (proliferating cellular nuclear antigen). The regulatory mechanisms that govern the association of Pol δ with PCNA are largely unknown. In this study, we identified S458, located in the PCNA-interacting protein (PIP-Box) motif of p68, as a phosphorylation site for PKA. Phosphomimetic mutation of S458 resulted in a decrease in p68 affinity for PCNA as well as the processivity of Pol δ. Our results suggest a role of phosphorylation of the PIP-motif of p68 as a molecular switch that dynamically regulates the functional properties of Pol δ.



Eukaryotic DNA replication is a highly intricate cellular process where the concerted action of multiple protein assemblies is required to ensure duplication of the genome with exquisite efficiency and accuracy. Three DNA polymerases are central to this process: Pol α/primase, Pol δ, and Pol ε.[1] By virtue of its primase activity, Pol α/primase initiates *de novo* DNA synthesis on the leading strand as well as for each Okazaki fragment on the lagging strand. Subsequently, the task of replicating the bulk of the genome is shared by the primer-dependent DNA polymerases, Pol δ and Pol ε. Biochemical and genetic evidence support a model where Pol ε is largely responsible for leading strand synthesis, while Pol δ is largely responsible for lagging strand synthesis.[2] In addition, Pol δ plays essential roles in a variety of DNA damage repair pathways.[3−7]

At the center of the role of Pol δ in DNA synthesis and repair is its dynamic interaction with its cofactor, PCNA. PCNA is a homotrimer forming a toroidal molecule that allows it to function as a sliding clamp that tethers Pol δ to the DNA.[8] PCNA is thus essential for processive DNA synthesis by Pol δ and is loaded onto the primer/template by the clamp-loader, replication factor C (RFC).[9] In addition, PCNA plays a central role in coordinating the activity of Pol δ with other replication proteins such as Fen1 and DNA ligase. During lagging strand synthesis, the joining of Okazaki fragments requires the removal of the RNA−DNA primer by the process of Okazaki fragment maturation.[2,10,11] Pol δ performs limited strand displacement when it encounters the 5′ end of the previous Okazaki fragment, to create short 5′ flaps that are cleaved by Fen1, a flap endonuclease.[12] This generates a ligatable nick that is sealed by DNA ligase I. Longer flaps are processed by another endonuclease, Dna2.[2,10] Both Fen1 and DNA ligase I are PCNA binding proteins, so that current models envisage these two proteins engaging in a coordinated process during Okazaki fragment maturation. PCNA plays a key role as a platform for these proteins, which must act in a sequential and coordinated manner.[10,13] In addition to its roles in DNA replication, PCNA plays an important role as a platform that coordinates the dynamic recruitment of a plethora of protein factors to the replication fork or to sites of DNA damage to mediate various aspects of DNA repair.[14] The structures of a number of PCNA complexes have been solved; the association of many PCNA-binding partners with PCNA is mostly mediated by a conserved PCNA-interacting protein motif (PIP-box) with a consensus sequence Q-x-x-(M/L/I)-x-x-F-(Y/F).[8] The PIP-box plugs into a hydrophobic pocket that is the molecular recognition surface on PCNA.

Human Pol δ consists of four subunits: p125, p50, p12, and p68.[15,16] The subunit arrangement consists of a tightly associated core of p125 (the catalytic subunit) and p50, which is bridged by the smallest subunit, p12, and p68, which is attached to the p50 subunit.[17] In *S. cerevisiae*, Pol δ is a three-subunit protein, lacking the p12 subunit.[18] The nature of the interactions of Pol δ with PCNA have been extensively studied because of the importance of their association in regulating Pol δ functions. Our previous studies established that human p125,[19,20] p12,[17] and p68[16] subunits all interacted with PCNA. The finding that all three subunits interact with PCNA provides the basis for a multivalent interaction of Pol δ with PCNA, which is supported by experimental evidence that shows that loss of the p68 subunit in human Pol δ[17,21] or its yeast cognate, Pol32p,[22] does not completely remove the functional ability of Pol δ to perform processive DNA synthesis. Analysis of subassemblies of human Pol δ lacking either p68 or p12 showed that they retained PCNA binding, as well as some degree for processive DNA synthesis *in vitro*,[23,24] indicating that

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there may be multiple modes of interaction of Pol δ with PCNA. Human p68 is a 466-amino acid protein and is an extremely hydrophilic protein, with a high content of serine and threonine (S + T = 16.3%, S = 49, T = 27) as well as basic (K + R = 16.5%) and acidic (D + E = 12.7%) residues. In yeast, the cognate subunit Pol32 has hydrodynamic properties of an asymmetric rod-shaped molecule.[18] The location of the PIP-box at the remote C-terminus of p68 shows that Pol δ could be tethered by an extended linkage to PCNA, since the interaction domain for p50 is at the N-terminus. p68 also possesses an interaction site for Pol α[25] as well as a canonical peptide motif for the binding of the catalytic subunit of protein phosphatase-1.[26] Thus, p68 may function as a scaffold for protein−protein interactions (Figure 1).

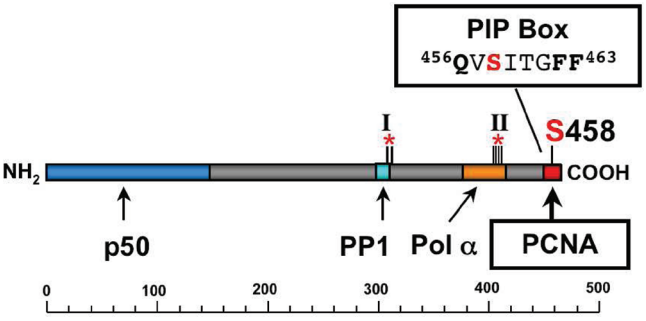

Figure 1. Map of protein interaction and phosphorylation sites in p68. The p68 sequence is shown as a bar, with the protein interaction domains in boxes as indicated below the bar. From the N-terminus these are the interaction sites for binding to the p50 subunit of Pol δ, the PP1 binding motif,[26] Pol α, and PCNA. The sequence of the PIP-box for PCNA interaction is shown above the bar with S458 shown in red. The positions of six *in vivo* phosphorylation sites[26,29] that lie adjacent or within the PP1 binding site and the Pol α interaction site are indicated by the asterisks: cluster I, S297 and T311; cluster II, Ser407, Ser409, Thr 411, and Ser413. The diagram is drawn to approximate scale.

The regulatory mechanisms that govern the functional properties of Pol δ are largely unknown. We have recently shown that one regulatory mechanism occurs at the level of protein turnover of p12. Genotoxic agents, such as UV and alkylating agents, induced the degradation of p12, resulting in increased discrimination against modified template bases and mismatched primers by Pol δ.[27,28] For p68, several lines of evidence point to phosphorylation as a potential regulatory mechanism of its functions. Global phosphoproteomic analysis has identified six phosphorylated S/T residues in human p68.[29] P68 was shown to be phosphorylated *in vitro* by CK2[26,30] and efficiently dephosphorylated by PP1.[26] The functional significance of these phosphorylation events is not yet clear; nevertheless, the location of the phosphorylation sites within or adjacent to motifs involved in protein−protein interactions (Figure 1) suggests a potential role of phosphorylation in the regulation of p68 functions.

Analysis of the human mitotic phosphoproteome has shown that S458 within the PIP-box of PCNA (Figure 1) is phosphorylated *in vivo*.[31] In this study we investigated the functional significance of phosphorylation of p68 on S458. We demonstrate that S458 is phosphorylated by PKA and provide evidence that introduction of a negative charge within the PIP-box decreases the PCNA binding capacity of p68. We also show that the Pol δ holoenzyme containing a phosphomimetic p68 mutant in which Ser458 is mutated to aspartate is defective in processive DNA synthesis *in vitro*. Together, these findings provide evidence for biochemical mechanisms that may modulate the multivalent association of Pol δ with PCNA and regulate its processivity.

MATERIALS AND METHODS

Recombinant Protein Expression and Purification. p68 was cloned with an 8x Histidine tag at its N-terminus between the Nde I and Hind III sites of the pTacTac vector using the KIAA0039 cDNA sequence as a template for PCR with the primers 5′ATGCTACA-TATGCATCACCATCAC-CATCACCATCACGGAGGAGCGGACCAGCTTTATC3′ (forward) and 5′ATGCAGGATCCAAGCTTGCTAGCT-TATTTCCTCTGGAAGAAG-CC3′ (reverse). The p68-S458D, p68-1-455, and p68-AAA mutants were similarly generated using the forward primers and the reverse primers 5′AGC-TAAAGCTTATTTCCTCTGGAAGAAGC-CAGTGATATC-CACCTGTCTGTTGGCTTTG3′, 5′AGCTAGAATT-CAAGCTTATCTGTTGGCTTTGCCCAG3′, and 5′AGC-TAAAGCTTATTTCCTCTGGGCGGCACCGGTAGCGGA-CACCTGTCTGTTGGC3′, respectively. The proteins were expressed in BL21 cells by induction with 1 mM IPTG for 5 h at 37 °C when the cultures reached an OD_{600} of 0.6.His-p68, and its mutants were purified using a standard purification protocol for Ni-NTA agarose (Qiagen). PCNA was expressed and purified as described previously[32] and labeled with digoxigenin according to the manufacturer's instructions (Roche Diagnostics). RPA was expressed and purified as described.[33] Protein concentrations were determined using the standard Bradford assay (Biorad).

Expression and Purification of the Recombinant Four-Subunit Pol δ Complexes. Hi-5 insect cells (100 mL of 2 × 10^6/mL) growing in suspension were coinfected with baculoviruses encoding p125, p50, p68, and p12 at a MOI of 5 for 48 h. The cell pellet was suspended in lysis buffer (20 mM Tris-HCl, pH 7.8, 10% glycerol, 0.5 mM EGTA, 1 mM EDTA, 1 mM $MgCl_2$, 50 mM NaCl, and 0.1% NP40) followed by disruption by passage through a French press and centrifugation at 15 000 rpm for 45 min. The supernatant was loaded onto a 78F5 anti-p125 immunoaffinity column.[16,34] The column was washed with TGEE buffer (40 mM Tris-HCl, pH 7.8, 10% glycerol, 0.5 mM EDTA, 0.1 mM EGTA) containing 0.4 M NaCl. Pol δ was eluted with TGEE containing 0.4 M NaCl/30% ethylene glycol. The elution of Pol δ was followed through the stimulation of [^{3}H]-dTMP incorporation into a poly(dA)/oligo(dT) template by PCNA. Fractions containing the peak activity of Pol δ were pooled, diluted four times in TGEED, and loaded onto a Mono-Q (5/5) column. The column was washed with 5 mL of TGEED/100 mM NaCl and eluted with TGEED/0.1−0.6 M NaCl gradient. Pol δ eluted at ca. 0.4 M NaCl.

Purification of the Native Pol δ Complex from HeLa Cells. The same protocol used for the purification of the reconstituted four-subunit Pol δ complex by immunoaffinity chromatography was used for purification of Pol δ from HeLa cells.The lysate from HeLa cells harvested from 10 L suspension culture were loaded onto a 10 mL p125 immunoaffinity column.[17] Eluted fractions across the peak activity of Pol δ were separated by 12% SDS-PAGE and stained with Coomassie blue or transferred to nitrocellulose membranes for Western blot analysis. CDK2, CDK4, CDC2, and PKA antibodies were purchased from Santa Cruz Biotechnology.

PKA Kinase Assay. His-p68-Wt or His-P68-S458D (1 μg, ca. 20 pmol) were incubated at 30 °C for 30 min with 10, 20, or 40 units of PKA (Promega) in 20 μL reaction mixtures containing 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 100 μM ATP (2000 pmol), and 10 μCi [γ-^{32}P]-ATP. The reactions were stopped by addition of SDS loading buffer and boiling. The products were run on SDS-PAGE gels which were subsequently stained with Coomassie blue, dried, and quantified by phosphorimaging.

Digoxigenin–PCNA Overlay Blot. His-p68 or its mutants (1 μg) were run on 12% SDS-PAGE and transferred to a nitrocellulose membrane. The buffers used in the overlay experiment were provided by the manufacturer (Roche Diagnostics). The membranes were blocked overnight at 4 °C with 1x blocking solution in 1x maleic acid buffer, washed for 10 min with 1× wash buffer, and incubated with digoxigenin-labeled PCNA (DIG-PCNA) in PBS for 1 h (1:10 000 dilution of a 0.15 mg/mL DIG-PCNA stock). The membranes were washed three times, 30 min/wash with wash buffer, and incubated with anti-DIG-HRP in 100 mM Tris-HCl pH 7.5/150 mM NaCl (1:10 000 dilution), followed by three washes, 20 min/wash with wash buffer. The signals were detected by ECL (Pierce) and analyzed using an AlphaImager. Relative signal densities were calculated as relative percentages of the highest reading.

ELISA Assays. His-p68, His-p68-S458D, or BSA (1 μg in 200 μL PBS/0.2% BSA buffer) were added to wells of a 96-well Ni-NTA-coated HisSorb plate (Qiagen) and incubated with shaking for 2 h. The wells were washed 4× with PBS/0.05% Tween 20. Increasing concentrations of PCNA in PBS/0.2% BSA/0.1% NP40 were added for 1 h. The wells were washed, and anti-PCNA antibody (1:10 000 in PBS/4% BSA) was added for 1 h and then washed and incubated with anti-mouse HRP for 45 min. The wells were washed and incubated with tetramethylbenzidine (TMB) substrate solution (BioFX Laboratories) and stopped after 30 min with the TMB stop solution. Absorbance readings were taken at 450 nm using the Multiskan Ascent plate reader, and the values were corrected for the background signal in the presence of BSA. The relative OD values were calculated as relative ratios to the highest reading.

Poly(dA)/Oligo(dT) Assay. The standard poly(dA)/oligo(dT) mixture contained 0.25 OD unit/mL of sparsely primed $poly(dA)_{4000}/oligo(dT)_{50}$ (Supertechs) (10% complex) in 50 mM Hepes pH 6.5, 5% glycerol, 0.1 mg/mL BSA, 5 mM $MgCl_2$, 5 μM dTTP, and 0.58 μM [^{3}H]-dTTP. The reactions were started by addition of PCNA and incubated at 37 °C. The reactions were terminated by spotting onto DE-81 papers, which were washed 3 times with 0.3 M ammonium formate, pH 7.8, and once with 95% ethanol, dried, and counted. One unit of DNA polymerase activity corresponds to the incorporation of 1 nmol of dTMP per 1 h at 37 °C. For analysis of the length of the reaction products, similar reactions were performed with [α-^{32}P]-dTTP. The reactions were terminated by the addition of an equal volume 20 mM EDTA in formamide and 0.1% xylene cyanol. The samples were denatured at 95 °C for 2 min, cooled on ice, and subjected to electrophoresis on a 12% polyacrylamide/8 M urea gel in 0.5× TBE. The products were visualized by phosphorimaging.

M13 Assay. Single-stranded M13mp18 (7250 bp, New England Biolabs) was primed by hybridization with a 20-mer oligonucleotide (5′-CTAGAGGATCCCCGGGTACC-3′) complementary to nucleotides 6262–6243 of the M13 genome. The standard M13 reaction contained 10 ng (42 fmol) of Pol δ, 40 mM Tris-HCl, pH 7.8, 1 mM DTT, 0.2 mg/mL BSA, 10 mM $MgCl_2$, 0.5 mM ATP, 50 mM NaCl, 250 μM each of dTTP, dCTP, dGTP, and 25 μM dATP, 3 μCi [γ-^{32}P]-dATP, 100 ng of primed M13 template, 80 ng of RFC, 200 ng of RPA, and the indicated amounts of PCNA in 30 μL reaction volume, at 37 °C for 30 min. The reactions were stopped by addition of 20 mM EDTA. Aliquots (5 μL) were spotted onto DE-81 papers which were washed 3 times with 0.3 M ammonium formate, pH 7.8, once with 95% ethanol, dried, and counted. The remainder of the reactions were run on 1.5% alkaline agarose gels at 50 V for 2.5 h. The products were visualized by phosphorimaging.

RESULTS

S458 is a Unique Site for Phosphorylation on p68 by PKA. p68 is rich in potential serine/threonine phosphorylation sites. The PIP-box (underlined) of p68 is harbored in the final 12 residues of the C-terminus (^{455}RQVSITGFFQRK^{466}). The PIP-box is immediately preceded by an arginine residue, so that S458 is in a context that conforms to the consensus PKA phosphorylation sequence (R-X_{1-2}-(S/T)-X). The putative PKA site in human p68 is well conserved in eukaryotic organisms from human to yeast (Table 1).

Table 1. PIP-Box Sequences of p68[a]

human	451 GKAN **RQVSITGFF** QRK*
mouse	447 GKAN **RQVSITGFF** QKK*
chicken	444 SRAN **KQISIMGFC** QKK*
Xenopus	441 SKGT **KQASIMGFF** QKK*
zebra fish	442 SKPT **KQPSIMGFF** QKK*
platypus	110 GKAN **RQASIMGFF** QKK*
S. cerevisiae	332 KRLK **KQGTLESFF** KRKAK*
S. pombe[b]	362 QSKP **QQKSIMSFF** GKK*

[a]Sequences were obtained from the UNIPROT database. The asterisks denote the preceding residues as the carboxy termini. Conserved residues of the PIP-box (QxxI/LxxxFF) as well as the preceding conserved basic residue are marked in bold. The sequence for the duck-billed platypus is from a partial sequence. [b]The basic residue for the PKA motif is not conserved; however, K364 may possibly allow phosphorylation of S369.

We first sought to examine whether p68 serves as a substrate for PKA, by performing an *in vitro* phosphorylation assay. The p68 subunit and its S458D mutant were individually expressed in *E. coli* as a 8x histidine-tagged protein and purified using Ni-NTA chromatography (Figure 2A). The purified proteins were subjected to phosphorylation by PKA in the presence of [γ-^{32}P]ATP. Recombinant p68 was efficiently phosphorylated by PKA *in vitro* (Figure 2B). Phosphorylation levels of p68-S458D by PKA were reduced to background levels, showing that that S458 is a unique site for phosphorylation of p68 by PKA. (Surprisingly, examination of the p68 sequence using the artificial neural network program Netphos2.0 predicted at least six additional sites). Taken together, these results show that S458 in the PIP motif of p68 is a good substrate for phosphorylation by PKA.

Our *in vitro* results suggested that PKA might be the endogenous kinase responsible for p68 phosphorylation *in vivo*. To obtain support for this possibility, we determined whether PKA can be found in association with the native Pol δ complex. The Pol δ complex was isolated using an immunoaffinity purification approach where HeLa cell lysates were separated

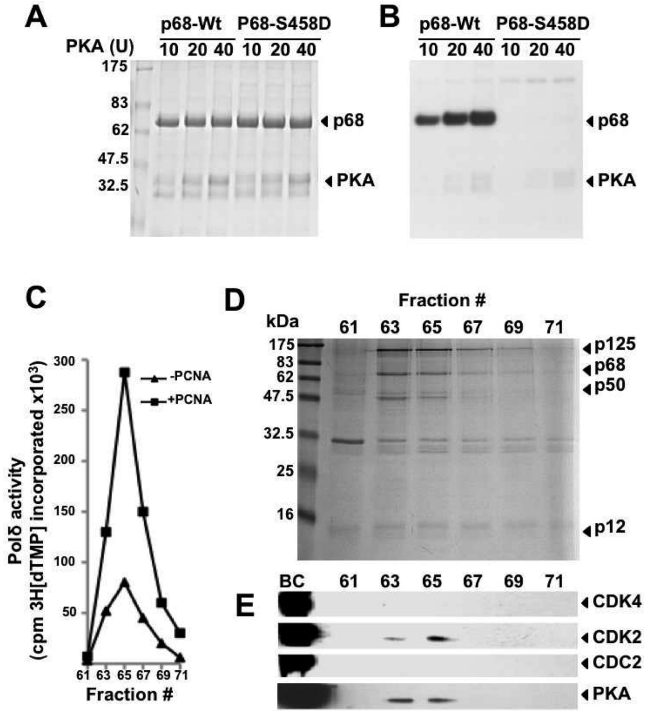

Figure 2. PKA phosphorylates S458 located in the PIP-box of p68. S458 is a unique site of phosphorylation on p68 by PKA. His-p68 Wt and S458D were expressed in *E. coli* and purified by Ni-NTA agarose. p68 Wt and p68-S458D (1 μg) were incubated with 10, 20, or 40 units of PKA in kinase buffer at 30 °C for 30 min. The reaction products were separated by 10% SDS-PAGE, stained with Coomassie blue and dried (panel A). Autoradiogram of the gel shown in panel A (panel B). PKA is associated with the native Pol δ complex. HeLa cell lysates were loaded onto an anti-p125 immunoaffinity column. Elution of Pol δ from the column was monitored by stimulation of the incorporation of ^{3}H[dTMP] by PCNA on a poly(dA)/oligo(dT) template (panel C). Eluted fractions in panel C were separated on 12% SDS PAGE and stained with Coomassie blue (panel D). Western blot of the eluted fractions of Pol δ in panel C. Fractions were subjected to Western blotting with antibodies against CDK4, CDK2, CDC2, and PKA (panel E).

on an anti-p125 column that we have previously used for isolation of Pol δ from cell lysates[24,28] as described in Materials and Methods. The elution of the Pol δ complex was monitored using the standard assay for the incorporation of ^{3}H-[dTMP] into a poly(dA)/oligo(dT) template/primer in the presence of PCNA (Figure 2C). SDS-PAGE and Coomassie blue staining of the four Pol δ subunits showed they were present in approximately stoichiometric amounts (Figure 2D). The eluted Pol δ would be expected to contain other proteins which associate with Pol δ and which are present in nonstoichiometric amounts, by analogy with immunoprecipitation experiments. We probed the eluted fractions of the Pol δ complex for PKA and cell cycle kinases using Western blot analysis (Figure 2E). The results show that PKA and the S-phase kinase CDK2 could be detected with the peak fraction of Pol δ, supporting an association of these kinases with Pol δ.

Phosphomimetic Mutation of Ser458 to Aspartate Decreases the Binding of p68 to PCNA. Since the interaction of the PIP-Box with PCNA is hydrophobic in nature, the introduction of a negative charge by phosphorylation of Ser458 would be expected to negatively affect the binding of p68 to PCNA. In order to circumvent the technical problems of obtaining fully phosphorylated p68 and the reconstitution of a Pol δ holoenzyme containing phosphorylated p68, we used a phosphomimetic mutation of Ser458 to aspartic acid. The binding of the p68-S458D mutant to PCNA was first assessed using an overlay blot assay by the use of digoxigenin-labeled PCNA to detect PCNA binding proteins on nitrocellulose membranes.[16,17] This method was used to probe for binding to equal amounts of p68-Wt and mutant proteins transferred onto a nitrocellulose membranes after SDS-PAGE. A deletion mutant of p68 lacking the last 11 residues that harbor the PIP-Box (p68 1-455) and a mutation (p68-AAA) of the conserved residues in the PIP-Box ($I_{459}XXF_{462}F_{463}$) to alanines ($A_{459}XXA_{462}A_{463}$) were expressed and purfied as well (Figure 3A). These were used as negative

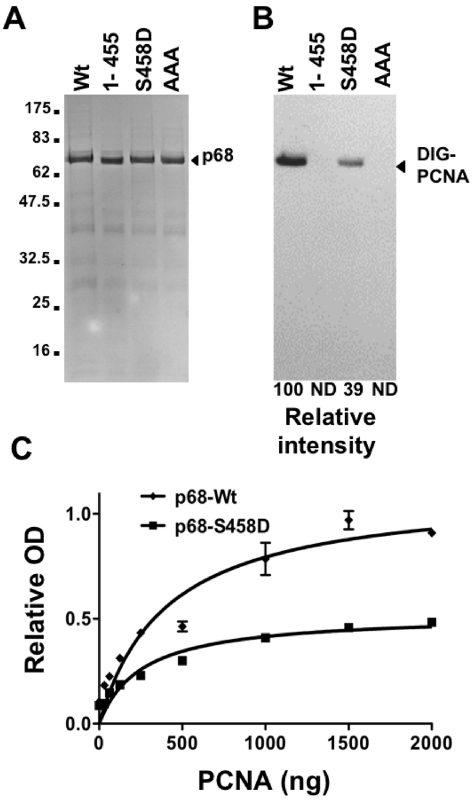

Figure 3. Effect of a phosphomimetic mutation of S458 to aspartic acid on the binding of p68 to PCNA. Overlay blot analysis of PCNA binding to PIP-motif mutants of p68. 1 μg of His-p68 Wt, 1-455, S458D, and AAA were loaded on 10% SDS-PAGE and stained with Coomassie blue (panel A) or transferred to a nitrocellulose membrane, overlaid with DIG-PCNA and probed with anti-DIG antibody (panel B). The chemiluminescence signal intensities were analyzed using an AlphaImager and calculated as relative percentages of the highest value. ND = nondetectable. Panel C: ELISA analysis of PCNA binding to the S458 D mutant of p68.1 μg of His-p68 wt, S458D, or BSA were bound to HisSorb Ni-NTA 96-well plates and washed, and increasing amounts of PCNA were added. The wells were washed and probed with anti-PCNA followed by addition of a chromogenic TMB substrate. Absorbance readings at 450 nm were taken and corrected for the background signal of BSA. The relative OD values were calculated as relative ratios to the highest reading. The results are shown graphically as the mean of three independent experiments.

controls for specific binding to PCNA mediated through the PIP-Box. The overlay assay showed nondetectable levels of binding of PCNA to the p68 1-455 and p68-AAA mutants and

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a 60% reduction in binding to the S458D mutant relative to p68-Wt (Figure 3B). To further evaluate the decrease in binding affinity of the S458D mutant to PCNA, an ELISA assay was used where p68-Wt and p68-S458D were adsorbed to Ni-NTA coated plates via their histidine tags and probed for binding to PCNA. The relative OD absorbance at 450 nm indicated a 50% decrease in the binding affinity of PCNA to p68-S458D relative to p68-Wt (Figure 3C). These results were consistent with those obtained with the overlay blot assay and together indicate an average of 50% reduction in the binding of PCNA to the S458D phosphomimetic mutant. The phosphomimetic mutant introduces a much weaker charge than would the phosphate moiety, so that the latter might be expected to have a much greater effect on PCNA binding.

The Pol δ Holoenzyme Containing the Ser458D Phosphomimetic Mutation of p68 Exhibits Functional Defects Associated with Loss of PCNA Binding. We reconstituted the four-subunit recombinant Pol δ complex harboring the p68-S458D mutation (Materials and Methods) to assess the functional consequences of the reduction of PCNA binding by p68. The four-subunit Pol δ complexes were purified by anti-p125 immunoaffinity followed by Mono-Q chromatography; representative protein stain and Western blot analysis of the purified enzymes showed that the four subunits were present in similar molar ratios in the reconstituted mutant as in the wild type (Figure 4A). This analysis was performed in order to ascertain that any changes observed were not due to incomplete reconstitution of the mutant Pol δ holoenzyme.

We examined the functional consequences of the decrease in PCNA binding of the Pol δ holoenzyme harboring the p68-S458D mutation. The properties of the Pol δ holoenzymes were first tested on linear, sparsely primed poly(dA)$_{4000}$ templates. The dose response curves of the activities of the Pol δ-p68-Wt and Pol δ-p68-S458D enzymes as a function of increasing concentrations of PCNA were indistinguishable, averaging around 17 500−20 000 units/mg at saturating concentrations of PCNA (Figure 4B). The distribution of lengths of the products generated by the two complexes were similar when examined on a 7% polyacrylamide/8 M urea gel (Figure 4C). This was not surprising, since we have previously observed that the Pol δ trimer in which p68 is absent exhibits a similar behavior.[17] The lack of observable changes establishes that, on a poly(dA)/oligo(dT) template which is relatively short, the multivalent interactions of Pol δ with PCNA are sufficient for processive synthesis and not compromised by the decrease in binding to PCNA via p68.

Therefore, we next investigated the properties of the Wt and mutant Pol δ holoenzymes in a more stringent assay[17,35] used to examine processive synthesis by Pol δ, viz., on a singly primed circular M13 template, which is 7.2 kb in length, thus challenging the enzyme with a longer template that may harbor secondary structures that could induce pausing. In addition, the assay requires PCNA to be loaded onto the primer template by RFC, which occurs at the primer terminus, as well as the addition of RPA single-stranded binding protein. Pol δ activity was first assayed in the presence of equimolar amounts of M13 template, saturating amounts of the single-stranded DNA binding protein (RPA) and clamp loader (RFC) and increasing amounts of PCNA in the presence of [α-^{32}P] dATP as described in Materials and Methods. At 30 min, in the presence of 100 ng PCNA (0.9 pmol of PCNA trimer), Pol δ-p68-Wt synthesized full-length (7 kb) M13 products, whereas Pol δ-p68-S458D synthesized products that reached only ca. 3 kb

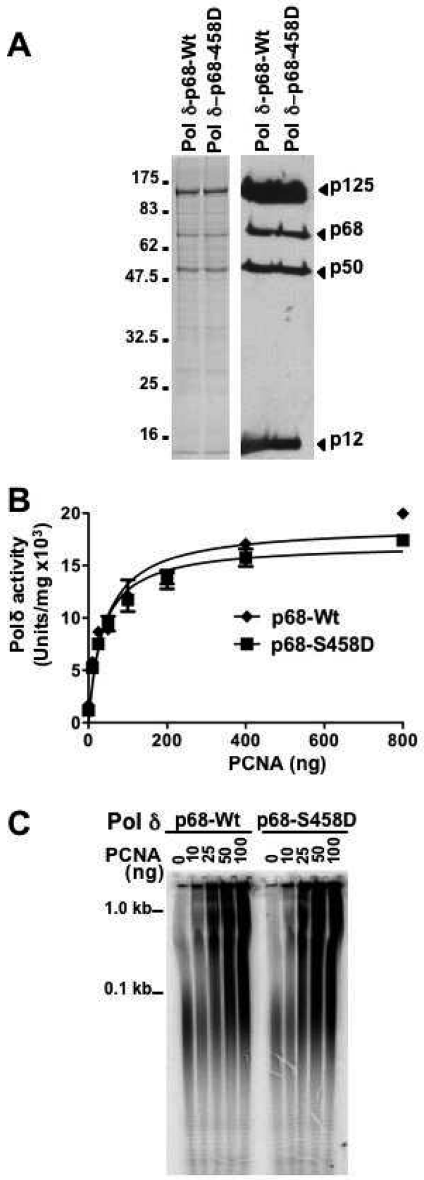

Figure 4. Isolation of a recombinant four-subunit Pol δ complex containing p68-S458D and functional analysis of its properties on a poly(dA)/oligo(dT) template. Panel A: Hi-5 insect cells were coinfected with baculoviruses encoding p125, p50, p12, and p68-Wt or p68-S458D. The four-subunit Pol δ complexes were purified by anti-p125 immunoaffinity followed by Mono-Q chromatography. Fractions containing the peak activity of Pol δ were separated by 12% SDS-PAGE and stained by Coomassie blue (left) and blotted with antibodies (right) against each one of the four subunits. The Coomassie blue band densities were quantified by densitometry and the ratios calculated relative to that of p125. The ratios of subunits for p125:p68-Wt:p50:p12 were 1: 0.96:1.57:0.988 and for p125:p68-S458D:p50:p12 were 1:0.98:1.66:1.08. Activities of recombinant Pol δ complexes containing p68-Wt or p68-S458D were assayed on poly(dA)/oligo(dT). Pol δ (10 ng) was assayed in 30 μL of reaction mixtures in the presence of increasing concentrations of PCNA for 20 min, at 37 °C (Materials and Methods). The activities were expressed as units/mg protein and were plotted against PCNA concentration (panel B). Similar reactions as in (B) were performed in the presence of [α-^{32}P] dTTP. The products were separated on a 6% polyacrylamide/8 M urea gel and detected by phosphoimaging. The results shown are representative of three independent experiments (panel C).

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(Figure 5A). With wild-type Pol δ, 100 ng of PCNA was required to achieve synthesis of full-length products compared

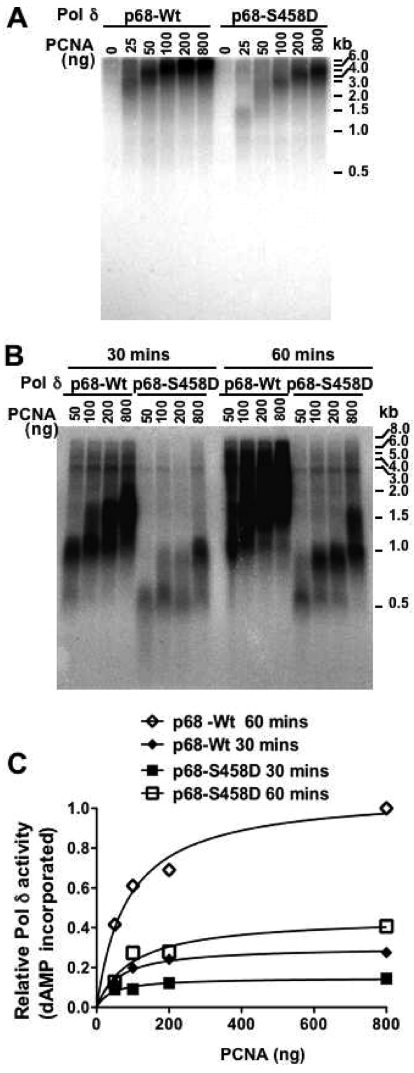

Figure 5. Functional analysis of the four-subunit Pol δ complex reconstituted with p68-S458D on a M13 template. 10 ng (42 fmol) of recombinant Pol δ complex containing p68-Wt or p68-S458D assayed in 30 μL reaction mixtures containing 100 ng (42 fmol) of singly primed M13 circular template in the presence of increasing concentrations of PCNA, RFC, and RPA, for 30 min at 37 °C (Materials and Methods). Aliquots of the reaction (10 μL) were separated on a 1.5% agarose alkaline gel and analyzed by phosphorimaging (panel A). Reactions similar to those in panel A were performed, using a 5-fold excess of M13 template (500 ng = 210 fmol) over enzyme for 30 or 60 min. The results shown are representative of three independent experiments (panel B). Relative dAMP incorporation was expressed as a ratio of incorporation by p68-Wt at 60 min and plotted against PCNA concentration. Data were fitted to a nonlinear regression for one site binding according to the formula Y = Vmax*.X/K_d + X using the GraphPad Prism software (panel C). The results shown are representative of three independent experiments.

to 800 ng of PCNA for Pol δ-p68-S458D (Figure 5A). These results suggested that p68-S458D decreased the affinity of Pol δ for PCNA as well as its processivity on the M13 template. To gain further insights into the mechanism for the decrease in processivity, similar reactions were performed with a 5-fold molar excess of the M13 template (Figure 5B). Under these conditions of excess template, Pol δ-p68-Wt synthesized products reaching only up to 2−3 kb in length at 800 ng of PCNA after 30 min, while full length products accumulated at 60 min (Figure 5B). Under the same conditions, the reduction in processivity of Pol δ-p68-S458D was much more pronounced; products reaching only up to 1.5−2 kb were synthesized at 800 ng of PCNA at 60 min (Figure 5B). At saturating concentrations of PCNA, the overall amounts of product synthesis by Pol δ-p68-S458D reached only 40% of that of Pol δ-p68-Wt (Figure 5C). These observations are similar to defects in the processivity of the Pol δ trimer lacking p68 that we have previously observed.[17] Taken together, these results suggest that p68-S458D mutation, which results in a decreased affinity of p68 to PCNA, leads to a loss of Pol δ processivity.

DISCUSSION

This study identifies S458 as a site for phosphorylation in the PIP-box of p68 by PKA. Analysis of PCNA binding to p68 containing a phosphomimetic S458D mutation demonstrated that this modification significantly reduces the interaction of p68 with PCNA. The structures of PIP-box peptides from human p21, p66, and Fen1 in complex with PCNA have been determined.[36] The common feature is the formation of a 3_{10} helix by the residues of the PIP-box that form a hydrophobic plug which inserts into a hydrophobic pocket on the surface of PCNA. Thus, the structural evidence is consistent with the view that introduction of the highly charged phosphate moiety on S458 would disrupt the hydrophobic interaction of the PIP-box with PCNA. Structural analysis shows that the p68 PIP-box interacts with a hydrophobic pocket on PCNA in common with other PIP-box motifs but differs from the classical p21 PCNA binding motif which has a C-terminal extension that interacts with the interdomain connecting loop of PCNA.[36] This is absent in the PIP-boxes of p68 and Fen1, which have lower affinities for PCNA.[36]

The question that arises is whether other PCNA binding proteins with a serine or threonine within the PIP-box could also be regulated by phosphorylation. Previous studies showed that phosphorylation of p21 on the cognate serine (S146) within its PIP-box significantly inhibited its binding to PCNA.[37] In the latter study, both PKA and by PKC were able to phosphorylate S146 *in vitro*. Phosphorylation of a fragment of RFC1 (the large subunit of RFC) containing the PCNA binding domain by Ca^{2+}/calmodulin-dependent protein kinase II was shown to result in the inhibition of its binding to PCNA.[38] Although the phosphorylation site on RFC1 was not determined, *in silico* modeling of the RFC1-PCNA interaction suggested T696 and S697 within the PCNA binding motif as the most likely targets mediating this effect.[39] These reports suggest a common mechanism of regulation of the interaction of other PCNA-binding proteins by phosphorylation of residues located in the PIP-Box. The location of phosphorylation sites within or adjacent to motifs involved in protein−protein interactions is a well-established regulatory mechanism, e.g., in the case of protein phosphate-1 (PP1) complexes, where phosphorylation of the PP1 binding motif RVxF[40,41] by PKA has been shown to disrupt PP1 binding.[42]

Analysis of the Pol δ holoenzyme containing the p68-S458D mutant confirmed that this resulted in a functional alteration that could be observed in a reduced processivity when tested on singly primed circular M13 templates. Overall, our studies

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provide support for the view that phosphorylation of S458 serves as a regulatory mechanism that fine-tunes the binding of Pol δ to PCNA as well as altering its processivity. The conservation of the S or T within the PIP-box of p68 and of a basic residue required for PKA substrate recognition over evolution from mammals to yeast (Table 1) is consistent with a functional role for phosphorylation of this site.

Functional analysis of the consequences of the modification of p68 at S458D on the properties of Pol δ showed no effect on Pol δ complex assembly or activity on a linear poly(dA)/oligo(dT) substrate (Figure 4) but a significant decrease in processivity upon encountering pause sites on an M13 template (Figure 5). These results are consistent with our previous studies with a reconstituted Pol δ complex lacking p68; the activity of a p125/p50/p12 complex was indistinguishable from the four-subunit Pol δ complex on a poly(dA)/oligo(dT) primer/template but showed defects in processivity on the M13 template. Removal of the p12 subunits also leads to diminished function on M13 templates, indicating that these subunits are essential for full Pol δ function.[17]

Our studies reveal a biochemical mechanism for the regulation of p68–PCNA interactions by protein phosphorylation. The question arises as to what functions could be served in a cellular context by this mechanism. There have been extensive advances in the elucidation of the structures of PCNA sliding clamps from a wide evolutionary range of organisms as well as of the complexes of PCNA with their binding partners. During Okazaki fragment maturation, Pol δ and Fen1 carry out cooperative, iterative cycles of strand displacement and flap cutting that functions as a nick translation process until the RNA–DNA primer is removed, following which the nick is sealed by DNA ligase I.[2,10] These three proteins all interact with the front face of PCNA and must alternately gain access to the primer terminus/template region of the DNA duplex. Recent studies support the concept that these PCNA binding proteins may adopt multiple conformations while bound to PCNA in a complex, which allows coordinated steric access to the DNA. Structural and functional studies of the PCNA complexes of the thermophilic archeon *Sulfolobus solfataricus* support a model of a lagging strand complex that contains the polymerase, Fen1, and DNA ligase bound to PCNA.[43,44] Structural studies show that Fen1 and DNA ligase both have hinge regions that allow large domains to swing away from the DNA, opening access to the primer/template. This flexibility is retained in eukaryotic Fen1[45] and DNA ligase I.[46]

These considerations suggest models for the function of p68 as a molecular switch that controls access to PCNA when Pol δ is engaged in DNA replication. The function of this switch is illustrated in Figure 6. The diagram shows Pol δ binding by all three of its possible interaction sites to PCNA. Phosphorylation of p68 at S458 would release its C-terminus from its binding to one of the PCNA monomers; as a consequence, that PCNA monomer would then be free to engage another PCNA binding protein. Moreover, the elongated and flexible shape of p68 would allow it to swing out of the way to permit another PCNA binding protein to share occupancy of PCNA with Pol δ (Figure 6). This could be a useful mechanism for controlling or initiating access of PCNA to other PCNA binding proteins. Also indicated in Figure 6 is that the release of p68 from PCNA could also act as a switch to allow changes in Pol δ conformation, a possibility that has not yet been tested. In addition, the occupancy of the vacated PCNA monomer by another replication protein could trigger further changes in the conformation of Pol δ in the Pol δ–PCNA complex that facilitates its access to the DNA.

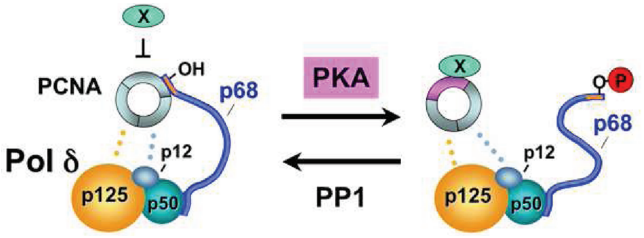

Figure 6. Phosphorylation of p68 as a molecular switch that regulates access of proteins to the PCNA–Pol δ complex. Left side: the model shows the interactions of the subunits of Pol δ with the PCNA trimer. p68 is represented as an extended molecule attached by its N-terminus to p50 and to a monomer of PCNA by its PIP-box (shown in pink with the hydroxyl group of S458). Pol δ is shown in a hypothetical conformation in which p125 and p12 interact with the other two monomers of PCNA. Accessory PCNA binding proteins shown as "X" will not be able to bind to PCNA. Right arrow: the phosphorylation of Ser458, shown as being mediated by PKA, leads to loss of p68 binding to PCNA, leaving the PCNA monomer shown in pink available for interaction with another PCNA binding protein, "X". This could also result in a change in the conformation of Pol δ within the PCNA–Pol δ complex, which might further facilitate the access of "X" to the DNA primer/template. (It is noted that DNA metabolizing proteins generally bind to the front face of PCNA, i.e., that facing the direction of the movement of the replication fork.) Left arrow: the left arrow shows the dephosphorylation of S458, which is performed by a PP1 molecule that is bound to the PP1 binding motif of p68 (not shown, see Figure 1). Release of the PIP-box from PCNA could facilitate the intermolecular dephosphorylation of S458 by PP1 because of the release of the constraints to its mobility. [The p50 subunit of Pol δ also interacts with PCNA[54] and is not shown in this diagram as we have found that this interaction is weaker than that of the other subunits (Zhou and Lee, unpublished data).]

A decrease in the processivity of Pol δ has been proposed to be required for proper Okazaki fragment maturation. In *S. pombe*, deletion of the PCNA-binding domain of the p68 homologue Cdc27 was shown to be sufficient to suppress cold sensitive mutants of the Cdc24 and the Dna2 endonuclease helicase, which are involved in the removal of the RNA–DNA primer during Okazaki fragments maturation. The model proposed to interpret these results posits that disruption of the Cdc27/PCNA interaction results in a less processive Pol δ, thus rendering the activity of the Cdc24–Dna2 complex dispensable since the Pol δ complex would be less proficient in strand displacement and as such generating less of the long flap structures which need Cdc24–Dna2 for their efficient removal.[47] A decrease in processivity of Pol δ resulting from phosphorylation of p68 might thus play a role in decreasing the strand displacement capacity of Pol δ during Okazaki fragment maturation.

In addition to providing a potential mechanism for events that require switching of Pol δ and its partners during Okazaki fragment synthesis and maturation, there are other possible events where a switching mechanism may be assisted by phosphorylation of p68. In the well-established DNA damage bypass pathway, the encounter of the replication complex with bulky lesions such as thymine–thymine dimers leads to ubiquitination of PCNA, followed by the recruitment of a translesion polymerase such as Pol η and a switch between the TLS polymerase and Pol δ.[48,49] The phosphorylation of p68 is a potential switch that could facilitate the exchange by weakening

Pol δ binding to PCNA. Interestingly, genetic studies of the *S. cerevisiae* POL32 gene that encodes the yeast homologue of p68 have placed it in the epistasis group of the RAD6 gene involved in the post replication pathway for translesion bypass synthesis in response to DNA damage.[50,51]

p68 has several important protein−protein interaction sites and has aspects of the function of a scaffolding protein. The protein−protein interaction sites include ones for the binding of Pol α and one for the binding of PP1 (Figure 1). The latter is important in relation to the control of the phosphorylation state of p68, since it is likely that phosphorylation of sites on p68 is reversed by an intramolecular reaction involving the bound PP1. Some parallels are known in the case of the PKA binding AKAP family of scaffolding proteins, some of which also bind PP1 or other protein phosphatases.[52,53] Such systems are obviously capable of performing extremely rapid cycling of the phosphorylation state of their target substrates. Thus, one might predict that this system provides for extremely rapid and repetitive modulation of p68 interaction with PCNA, such as might be encountered during Okazaki fragment maturation. Equally of interest is that our Western blotting data (Figure 2) support the possibility that PKA and/or CDK2 may interact with p68.

In summary, our studies reveal a mechanism that could modulate the interaction of the p68 subunit of Pol δ with PCNA by a protein kinase-mediated phosphorylation of ser 458. These studies add to the evidence that p68 may act as a nexus for protein−protein interactions for Pol δ that is regulated by protein phosphorylation−dephosphorylation (Figure 1). This study posits a model where the phosphorylation/dephosphorylation of the PIP motif of p68 provides a switch for regulation of the switching of replication proteins on the PCNA−Pol δ complex and opens a number of avenues for future investigation.

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Author Contributions

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ABBREVIATIONS

Pol δ, DNA polymerase δ; p68, the p68 subunit of Pol δ (also referred to as p66); PCNA, proliferating cell nuclear antigen; CK2, casein kinase 2; PKA, cAMP-dependent protein kinase; PP1, protein phosphatase-1; RPA, replication protein A.

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