Protein Phosphatase-1 Is Targeted to DNA Polymerase δ via an Interaction with the p68 Subunit†

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ABSTRACT: Protein phosphatase-1 (PP1) is a Ser/Thr protein phosphatase that participates in the phosphorylation/dephosphorylation regulation of a diverse range of cellular processes. The PP1 catalytic subunit (PP1) achieves this by its ability to interact with many targeting subunits such that PP1 activity is thereby specified against phosphoprotein substrates in the microvicinity of its targeting subunit. DNA polymerase δ (Pol δ) is a key enzyme in mammalian chromosomal replication. It consists of four subunits, p125, p50, p68, and p12. We identify p68 as a novel PP1 targeting subunit. PP1 was shown to associate with human DNA polymerase δ by affinity chromatography and coimmunoprecipitation assays from mammalian cell lysates and *in vitro* by pull-down assays. The binding domain for PP1 was identified as the sequence KRVAL, a variant of the canonical RVxF PP1 binding motif. These studies provide the first evidence for the targeting of PP1 to DNA polymerase δ. We also show that CK2 phosphorylates the Pol δ p125, p68, and p12 subunits and that these phosphorylated subunits are substrates for PP1. These findings identify a new role for p68 as a PP1 targeting subunit that implicates PP1 in the dephosphorylation of Pol δ. Our findings also show that CK2 is a strong candidate for the protein kinase involved in the *in vivo* phosphorylation of p68.

Protein phosphatase-1 is one of the major Ser/Thr protein phosphatases and is ubiquitous in eukaryotic cells. Its activity plays crucial roles in many cellular processes, including those of cell division and mitosis (*1*). The catalytic subunit of protein phosphatase-1 (PP1)[1] exists as three isoforms, PP1α, PP1β, and PP1γ1, with a fourth isoform (PP1γ2) that is expressed in testis by alternative splicing of the PP1γ gene (*2*). The PP1 catalytic subunits are small proteins of ca. 37 kDa, which are highly conserved, with their main differences falling in the C-termini (*3*). The PP1 catalytic subunit exists in the cell in complex with a large number of targeting/regulatory subunits, thereby generating different enzyme forms that allow it to perform a diverse number of cellular functions. The targeting subunits bring PP1 to the vicinity of its substrates, thereby providing specificity, such that dephosphorylation of a given substrate by PP1 is dependent on a targeting subunit (*1, 4*). Central to this paradigm is the requirement of a targeting subunit for the *in vivo* dephosphorylation of a given phosphoprotein substrate. This has been demonstrated in yeast where deletion of the PP1 targeting subunits produces functional PP1-null phenotypes for the substrates associated with the targeting subunit (*5*). The structural basis for the ability of PP1 to interact with a large number of proteins with diverse structures has been determined and is dependent on a hydrophobic pocket distal to the catalytic site that binds peptides with the "RV*X*F" motif in which the Val and Phe are highly conserved (*6*). Variants of this motif are present in the majority of PP1 binding proteins, and the sequence specificity of the PP1 binding site has been analyzed by peptide display library panning (*7*). Thus far, as many as 100 PP1 interacting proteins have been identified (*1*), and their identification can provide significant information as to the potential *in situ* targets of the PP1-targeting protein complex. In addition to these targeting subunits, there are inhibitor proteins that also interact with PP1 with very high affinity; these include inhibitor-1 (*1*), inhibitor-2 (*8*), and inhibitor-3 (*9*).

In this study we report the discovery of a novel human PP1 binding protein, p68. p68 is one of the four subunits that constitute human DNA polymerase δ (Pol δ). Pol δ, together with Pol α and Pol ε, is involved in eukaryotic chromosomal DNA replication. Pol δ and Pol ε are responsible for the synthesis of the leading and lagging strands, while Pol α/primase is involved in primer synthesis (*10, 11*). In addition to its role in DNA replication, Pol δ also functions as a gap-filling enzyme in DNA repair processes (*11*). Human Pol δ consists of a tightly associated heterodimer of the p125 catalytic subunit and the p50 subunit (*12*), which forms the holoenzyme with a third subunit, p68[2] (*13, 14*), and a fourth subunit, p12 (*14*). The activity of Pol δ as a processive DNA polymerase is dependent on PCNA, which acts as a DNA sliding clamp (*10, 11*). The human p12 subunit has been

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[1] Abbreviations: PP1, catalytic subunit of protein phosphatase-1; PCNA, proliferating cell nuclear antigen; Pol δ, DNA polymerase δ; CK2, casein kinase 2; PKA, cAMP-dependent protein kinase; cdk, cyclin-dependent kinase; Rb, retinoblastoma protein.

[2] Also referred to as p66.

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shown to bind to both the p125 and p50 subunits, so that it may serve a structural function; in addition, it also binds to PCNA and may play a role in the interaction of Pol δ with PCNA (*15*). The p12 subunit is not found in *Saccharomyces cerevisiae* Pol δ (*16*), although it is present in *Schizosaccharomyces pombe* Pol δ (*17*). Homologues of the human p68 subunit are present in yeast Pol δ as Pol32 in *S. cerevisiae* and as Cdc27 in *S. pombe* (*11*). p68 has very little sequence homology to its yeast counterparts, but they all possess a PCNA binding motif at the extreme C-terminus (*13, 14, 18, 19*). Two other conserved regions are a N-terminal region that attaches them to the Pol δ core via the p50 subunits and a Pol α binding region at the C-terminus (*19*−*21*). p68 is likely to be a highly extended and flexible polypeptide in solution, like its yeast homologues (*11, 18, 22*), as it has a high content of hydrophilic amino acids. Extensive genetic studies have implicated Pol32 in other functions in yeast: these include error-prone postreplication repair and translesion synthesis (*19*), break-induced replication and telomere maintenance (*23*), and gene silencing (*24*). While genetic studies in yeast point to several important roles for Pol32, the biochemical basis for the involvement of Pol32 is still not fully understood, and much less is known of the functions of human p68. There is also evidence that p68 is posttranslationally modified by ubiquitination and sumoylation (*25*) and by phosphorylation at serine and threonine residues *in vivo* (*26, 27*), although the roles of these modifications in Pol δ functions are unknown.

The role of phosphorylation in the regulation of the DNA polymerases is not well understood. Pol α is the best studied of the mammalian DNA polymerases in this regard, and its subunits are phosphorylated at different stages of the cell cycle (reviewed in ref *28*). Earlier studies showed that the p125 subunit of Pol δ is phosphorylated *in vivo* by metabolic labeling with $^{32}P_i$ (*29*) and by cyclin/cdks when they are coexpressed in Sf9 cells (*30*). Cyclin-dependent kinases have also been shown to be associated with Pol δ isolated by immunoaffinity chromatography on immobilized PCNA (*31*). There have been no detailed studies of the phosphorylation of purified Pol δ *in vitro* by protein kinases. Human p68 has been shown to be phosphorylated *in vivo* by the use of antibodies and *in vitro* by phosphorylation of p68 peptides with CK2 (*27*). Six S/T phosphorylation sites in human p68 have been identified by phosphoproteome analysis that are likely CK2 phosphorylation sites (*26*). p68 phosphorylation/dephosphorylation is therefore a potential mechanism for the regulation of Pol δ functions, and it has been reported that phosphorylation of human p68 may regulate its association to chromatin at the beginning of S phase (*27*).

We show that the p68 subunit of Pol δ is a novel PP1 binding protein and have identified the PP1 binding sequence. The interaction of p68 with PP1 allows the targeting of PP1 to the Pol δ holoenzyme. In addition, we have examined the phosphorylation of purified Pol δ by CK2 and show that p68, p125, and p12, but not p50, are phosphorylated by CK2. All three phosphorylated subunits of Pol δ are dephosphorylated by PP1. Our findings have significant implications as it identifies a new role for p68 and suggests that PP1 plays a role in the phosphorylation/dephosphorylation of p68, the other subunits of DNA polymerase δ, or even other components of the multiprotein replication and DNA repair complexes. We also show that CK2 phosphorylates the p125, p68, and p12 subunits of Pol δ and that phosphorylated Pol δ is dephosphorylated by PP1.

EXPERIMENTAL PROCEDURES

Recombinant Proteins. Rabbit muscle PP1α (*32*), human protein phosphatase-1 inhibitor-3 (*9*), and protein phosphatase-1 inhibitor-2 (*35*) were expressed in *Escherichia coli* and purified as previously described. His_6-tagged human p68 baculovirus was generated using the Bac-to-Bac system (Invitrogen Inc.) and used to infect Sf9 cells. The His-tagged p68 was purified on Ni-NTA agarose beads. Recombinant human Pol δ holoenzyme containing the p125, p50, p68, and p12 subunits was expressed in Sf9 cells and purified as previously described (*15, 33*). PP1β and PP1γ1 were obtained from Santa Cruz Biotech. Isoform-specific antibodies to PP1α (C-19), PP1β (N-19), and PP1γ (C-19) were obtained from Santa Cruz Biotech. Unless otherwise stated, a PP1 antibody that was nonisoform specific was used (BD Transduction Laboratories).

Construction of p68 Mutations and Deletions. The KIAA0039 cDNA in the pBluescript vector was used as a template for PCR cloning and was used to generate N-terminal His_6-tagged p68 and a series of C-terminal deletion mutants. The PCR constructs were subcloned and inserted into the pTactac vector (*32*). N-Terminal deletions containing the His tag were constructed using PCR and subcloned in frame into the pQE32 expression vector (Qiagen, CA). The site-directed mutants of p68 were generated by using the QuickChange site-directed mutagenesis kit (Stratagene, CA) with pTacTac-His_6p68(1−466) as the template. The mutations were verified by DNA sequencing. The primer pairs were as follows: p68(1−466) 5′-ATGCTACATATGCATCACCATCACCATCACCATCACGGAGGAGCGGACCAGCTTTATC-3′,3′-CCGAAGAAGGTCTCCTTTATTCGATCGTTCGAACCTAGGACGTA-5′; p68(129−466) 5′-AGTACAGGATCCCCCATATGTGCAGCAAATTTAGTGCTATACC-3′, 3′-CCGAAGAAGGTCTCCTTTATTCGATCGTTCGAACCTAGGACGTA-5′;p68(227−466)5′-AGTACAGGATCCCCCATATGAAGGCACCAGGGAAAGGG-3′, 3′-CCGAAGAAGGTCTCCTTTATTCGATCGTTCGAACCTAGGACGTA-5′; p68(1−226) 5′-ATGCTACATATGCATCACCATCACCATCACCATCACGGAGGAGCGGACCAGCTTTATC-3′, 3′-CGTAGACGTCGTCCGTTGATTCGAACTTAAGATCGA-5′; p68(1−334) 5′-ATGCTACATATGCATCACCATCACCATCACCATCACGGAGGAGCGGACCAGCTTTATC-3′, 3′-CTTAGACTATCGTCACTTCTAATTCTTAAGTTCGAATTCGTA-5′; p68(M1, 302−306 to DEAAA) 5′-GAAAAAAAAAGGGGGGACGAAGCAGCTGCGTCTGATGATGAGACA-3′, 5′-TGTCTCATCATCAGACGCAGCTGCTTCGTCCCCCCTTTTTTTTTC-3′; p68(M2, 322−326 DEAAA) 5′-CATGAGGAAAAAGAGGGACGAAGCAGCTGCTCCTGAATCTGATAGC-3′, 5′-GCTATCAGATTCAGGAGCAGCTGCTTCGTCCCTCTTTTTCCTCATG-3′; p68(M3, internal deletion of residues 298−328) 5′-GTTAAGGTGCTGCAGAAGGAAAGCAGTGAAGATGAAGTC-3′, 5′-GACTTCATCTTCACTGCTTTCCTTCTGCAGCACCTTAAC-3′.

Coimmunoprecipitation. MCF-10A mammary epithelial cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and were grown in DMEMF12 supplemented with 20 ng/mL EGF, 100 ng/mL cholera toxin, 0.01 ng/mL insulin, 500 ng/mL hydrocortisone, and 5% horse

serum. MCF-10A cells were harvested from two flasks (75 cm^2) and washed twice with ice-cold Tris saline (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1.5 mM phenylmethanesulfonyl fluoride). The cells were lysed by addition of 1 mL of Triton X-100 containing buffer (15 mM Tris-HCl, pH 7.5, 120 mM NaCl, 25 mM KCl, 2 mM EGTA, 2 mM EDTA, 0.1 mM dithiothreitol, 0.5% Triton X-100, 10 μg/mL leupeptin, 0.5 mM phenylmethanesulfonyl fluoride). The suspension was incubated at 4 °C for 30 min. The cellular debris was removed by centrifugation. The supernatant (1.7 mg of protein, 1 mL) was precleared to reduce nonspecific binding by addition of mouse IgG (0.5 μg) and 50 μL of protein A/G-agarose beads (Pierce Chemical Co., Rockford, IL). The suspension was incubated for 1 h, and the protein A/G-agarose removed by centrifugation at 2500 rpm for 5 min. Anti-PCNA (2.5 μg, PC-10; Santa Cruz Biotechnology), anti-p125 mAb (*34*), or anti-PP1 (2.5 μg, P35220; BD Transduction Laboratories) was added to the supernatant and the solution rotated for 1 h at 4 °C. Protein A/G agarose beads (50 μL) were then added, and the mixture was incubated at 4 °C overnight prior to centrifugation. The beads were washed six times with ice-cold PBS buffer, pH 7.4. The proteins were extracted with gel loading buffer (50 μL) and run on a 12% SDS−polyacrylamide gel and then transferred to nitrocellulose membranes. Western blotting for p125, p68, p50, and p12 was performed as previously described (*15*). The membranes were then stripped and overlay blotted with dig-PP1 as described below.

Overlay Blots with PP1-Digoxigenin. Purified recombinant PP1α was reacted with digoxigenin 3-*O*-methylcarbonyl-ε-aminocaproic acid *N*-hydroxysuccinimide (Roche Applied Science) as described by Zhao et al. (*7*) and repurified by DEAE-Sepharose chromatography (*3*). The membranes to be blotted were treated with dig-blocking buffer (Roche Applied Science) for 2 h and incubated with dig-PP1 (0.3 μg/mL) for 1 h at room temperature and washed three times with dig-wash buffer (Roche Applied Science) for 20 min. The membranes were then incubated with horseradish peroxidase conjugated sheep anti-dig Fab fragments (1:5000; Roche Applied Science) for 1 h at room temperature. The membranes were washed four times with dig-wash buffer for 20 min, incubated with enhanced chemiluminescence lighting solution (ECL; Pierce Biotechnology), and exposed to film.

PP1-Sepharose Pull Down of p68 from Sf9 Lysates. PP1α was coupled to Sepharose-CH beads (Amersham-Pharmacia) as previously described (*35*). A lysate (0.25 mL) of Sf9 insect cells expressing His-tagged p68 was diluted to 0.65 mL with water. PP1-Sepharose beads (100 μL) were added, and the suspension was rotated overnight at 10 °C. The beads were washed six times with ice-cold PBS, pH 7.4. Bound proteins were extracted from the beads by boiling with 50 μL of gel loading buffer and separated by electrophoresis on a 12% SDS−polyacrylamide gel. The proteins were transferred to nitrocellulose membranes and Western blotted with p68 antibody or analyzed by dig-PP1 overlay blot.

Pull-Down Assays Using His-Tagged p68. His-tagged p68 wt or mutants M1, M2, and M3 (1 μg) were mixed with recombinant PP1α (1 μg) in 0.8 mL of PBS buffer and incubated at 4 °C for 4 h. The His-p68 wt or mutants were pulled down by addition of 20 μg of Ni-NTA agarose beads. The mixture was rotated at 4 °C for 1 h. The beads were washed eight times with cold PBS containing 20 mM imidazole. The bound proteins were extracted by boiling in 40 μL of SDS gel loading buffer and separated on a 10% SDS−polyacrylamide gel. The proteins were transferred to nitrocellulose membranes and Western blotted with PP1 antibody.

Purification of Pol δ from HeLa Cells by Immunoaffinity Chromatography. Immunoaffinity chromatography was performed essentially as previously described (*36*). HeLa cells (1.6×10^9 cells) were obtained from the National Cell Culture Center (NCCC, Minneapolis, MN). The cells were lysed in 20 mM Tris-HCl, pH 7.8, 0.5 mM EGTA, 1 mM EDTA, 1 mM $MgCl_2$, 10% glycerol, and 200 mM NaCl. After removal of cell debris the lysate was diluted 4-fold with TGEE buffer (40 mM Tris-HCl, pH 7.8, 10% glycerol, 1 mM EDTA, 0.5 mM EGTA) and loaded onto a 7 mL column of anti-p125 antibody immobilized on agarose beads (*36*). The column was washed with 10 bed volumes of TGEE buffer/100 mM NaCl and then eluted with TGEE buffer containing 400 mM NaCl and 30% ethylene glycol. These preparations generally yielded from 20 to 30 μg of protein from 500 to 600 mg of lysate protein. Assays for DNA polymerase activity were performed by determination of [^{3}H]dTMP incorporated into poly(dA)/oligo(dT) template/primer in the presence and absence of PCNA as previously described (*13*). One unit of activity was defined as 1 nmol of dTMP incorporated per hour at 37 °C. The preparation had a specific activity of 11000 units/mg, approximately 50% that of homogeneous recombinant Pol δ (*33*). Western blots for PP1 were performed as previously described (*37*) and for the Pol δ p125, p50, p68, and p12 subunits as previously described (*36*).

Phosphorylation Assays. Phosphorylation reactions were performed in a total volume of 20 μL in 20 mM Tris-HCl, pH 7.5, 50 mM KCl, and 10 mM $MgCl_2$, with the indicated amounts of CK2 (New England Biolabs), 100 μM ATP, 15 μCi of [γ-^{32}P]ATP, and the indicated amounts of Pol δ or Pol δ subunits at 30 °C for 60 min. The reaction products were resolved by SDS−PAGE on 12% polyacrylamide gels and stained with Coomassie Blue. After destaining, the gels were dried and visualized by autoradiography or analyzed by phosphorimaging. p12 was expressed in *E. coli* as the GST-p12 fusion protein (*15*). GST-p12 was cleaved with factor Xa (Sigma-Aldrich) to yield the free p12; GST was removed using glutathione-Sepharose beads. The p12(S24D) mutant was constructed from the GST-p12 vector by site-directed mutagenesis.

Dephosphorylation Assays. Pol δ was phosphorylated with 250 units of CK2 (New England Biolabs, Ipswich, MA) at 30 °C for 60 min. TBB (4,5,6,7-tetrabromo-2-azabenzimidazole; Sigma-Aldrich) was then added to a final concentration of 200 μM to inhibit CK2 activity. PP1α (2.5 units) and $MnCl_2$ (final concentration 1 mM) were then added and the reactions incubated at 30 °C for an additional 60 min. Microcystin-LR (Sigma-Aldrich) was added where indicated to inhibit PP1 activity. Reaction products were analyzed by SDS−PAGE as described above.

Sample Preparation of Pol δ for Mass Spectrometry. Pol δ was purified by immunoaffinity chromatography as described above from 2×10^9 cells. The pooled fractions of Pol δ (30 μg of protein) were concentrated by centrifugation through a 30000 MW cutoff Centricon tube (Millipore Co.).

The concentrated protein (30 μg) was further purified by electrophoresis on a 5 × 100 mm cylindrical gel under nondenaturing conditions as described by Lee et al. (*38*). The gel was cut into 60 slices, extracted, and assayed for polymerase activity or silver stained. Fractions 16, 17, and 18 were combined and the proteins separated on SDS−PAGE and stained with Coomassie Blue. The gel region corresponding to MW 32−37 kDa was excised and analyzed by the Harvard Microchemistry Facility using microcapillary reverse-phase HPLC nanoelectrospray tandem mass spectrometry (μLC/MS/MS).

RESULTS

PP1 Is Associated with the Immunoaffinity-Purified Pol δ Complex Isolated from HeLa Cell Extracts, and This Association Is Mediated by a 68 kDa Polypeptide. We have previously used immobilized anti-p125 for the immunoaffinity chromatography of calf thymus Pol δ (*13*) and recombinant human Pol δ from Sf9 cell lysates (*15, 33*). This procedure was found to provide a one-step procedure for the isolation of Pol δ from cultured 293 cells (*36*) and was used here for the isolation of Pol δ from HeLa cells. HeLa lysates were directly applied to the column and eluted with buffers containing ethylene glycol (Experimental Procedures). The assay of the eluted fractions for Pol δ activity is shown in Figure 1A, in the presence and absence of PCNA. (The stimulation of polymerase activity by PCNA in this assay is a characteristic of Pol δ and is routinely used to monitor the quality of Pol δ enzyme preparations.) The elution conditions are mild and allow the isolation of Pol δ that is approximately 50% pure based on a comparison of the specific activity with that of purified recombinant Pol δ (Experimental Procedures). A protein stain of three of the peak fractions is shown in Figure 1B and shows that the four subunits of Pol δ are present at levels consistent with the estimated purity. The column fractions were immunoblotted with antibodies against the Pol δ subunits as well as against PP1. PP1 coeluted with the Pol δ subunits (Figure 1C).

We examined the HeLa Pol δ preparation to determine which PP1 isoforms were present in association with Pol δ. The pooled peak fractions (fractions 10−16, denoted as "P" and a bar in Figure 1A) were subjected to Western blotting using the nonisoform-specific PP1 antibody that was used in Figure 1C and with isoform-specific antibodies to PP1α, PP1β, and PP1γ (Figure 1D). PP1α and PP1γ1 were readily detected, while only a faint signal was observed for PP1β (Figure 1D). This suggests that PP1α and PP1γ1 are the primary isoforms associated with Pol δ. This is consistent with the findings that PP1α and PP1γ1 are predominantly associated with the nucleus (*39*).

Chemical evidence for the presence of PP1 in the HeLa Pol δ preparation was obtained by mass spectrometry analysis. A preparation of immunoaffinity-purified HeLa Pol δ was further purified by nondenaturing gel electrophoresis on a cylindrical gel (Experimental Procedures). The gel was cut into 60 slices, which were extracted with buffer and assayed for Pol δ activity (Figure 2A). An aliquot of each fraction was subjected to SDS−PAGE and silver stained (Figure 2B). Fractions 16, 17, and 18 were pooled and run on SDS−PAGE and stained with Coomassie Blue (Figure 2C). The region of the gel from ca. 32−37 kDa was excised and analyzed by microcapillary reverse-phase HPLC nanoelectrospray tandem mass spectrometry (μLC/MS/MS) at the Harvard Microchemistry Facility. Six peptides containing PP1 sequences were identified (Figure 2D). Three of the peptides were common to all three isoforms, one was common to PP1α and PP1γ1, one was for specific for PP1γ1, and one was specific for PP1β. The results provide chemical evidence for the presence of PP1 in this highly purified Pol δ preparation.

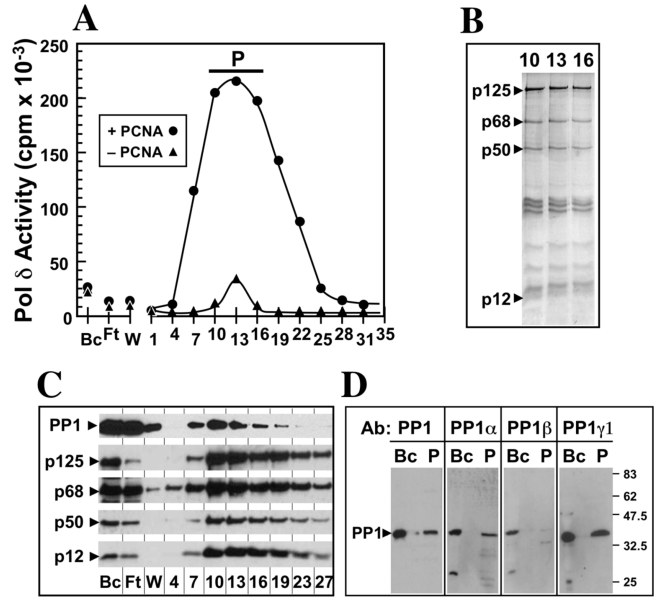

FIGURE 1: PP1 is associated with the immunoaffinity-purified Pol δ complex isolated from HeLa cell extracts. Panel A: Elution profile of the purification of Pol δ by immunoaffinity chromatography. A HeLa cell lysate was loaded onto an immunoaffinity column containing anti-p125 attached to Sepharose beads (Experimental Procedures). The eluted column fractions were assayed for Pol δ activity on poly(dA)/oligo(dT) template/primer by incorporation of [^{3}H]dTMP in the presence (circles) and absence (triangles) of PCNA. The letter "P" and the bar mark the fractions that were pooled for the experiment in panel D. "Bc" refers to the lysate before chromatography, "Ft" to the flow-through collected during the loading of the lysates, and "W" to the wash liquid. Panel B: Protein stain of the peak fractions of Pol δ activity. Fractions 10, 13, and 16 were run on 12% SDS−PAGE gels and stained with Coomassie Blue. Panel C: Association of PP1 with Pol δ during immunoaffinity chromatography. The column fractions were Western blotted with antibodies against the p125, p50, p68, and p12 subunits of Pol δ and for PP1 using a nonisoform specific antibody. Panel D: Identification of the PP1 isoforms that were present in the Pol δ preparation. The peak fractions (fractions 10−16, panel A) were pooled. Aliquots of the pooled enzyme were Western blotted with a nonisoform-specific antibody to PP1 and isoform-specific antibodies to PP1α, PP1β, and PP1γ and are shown as lanes marked "P". Lanes marked "Bc" are aliquots of the HeLa lysate before immunoaffinity chromatography. (Data shown for panels A−C are representative of more than three such experiments.)

PP1 Is Associated with Pol δ in Vivo, and This Association Is Mediated by a 68 kDa Polypeptide. MCF10A cell lysates were immunoprecipitated with anti-p125 followed by Western blotting with PP1 antibody in order to confirm the association of PP1 with Pol δ *in vivo*. The results show that PP1 could be coimmunoprecipitated with Pol δ (Figure 3A, lane 2). Next, the Western blots were stripped and then analyzed by overlay blot with digoxigenin-PP1 (dig-PP1) in order to identify the polypeptide to which PP1 was bound

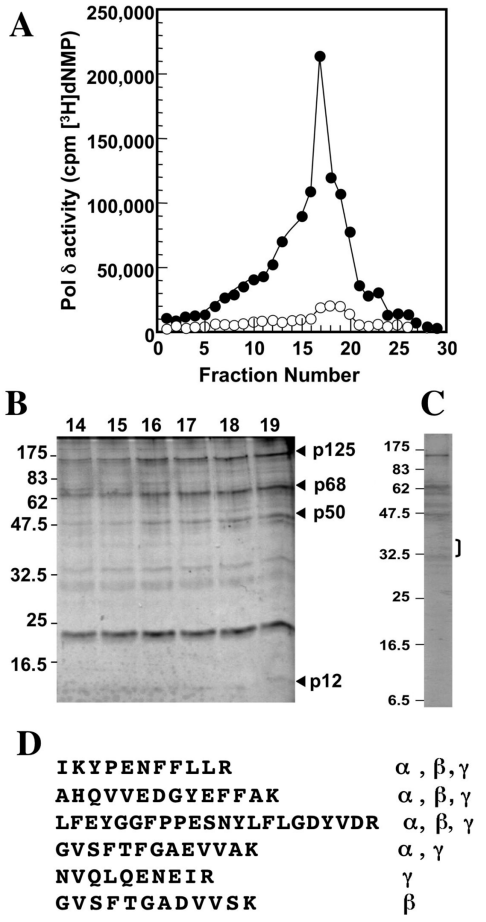

FIGURE 2: Identification of PP1 in Pol δ preparations by mass spectrometry. A Pol δ preparation that had been purified from HeLa cells by immunoaffinity chromatography was further purified by nondenaturing gel electrophoresis on a cylindrical gel (Experimental Procedures). The gel was sliced into 60 sections and the Pol δ extracted with buffer. Panel A: Pol δ activity was assayed in the extracts from the gel slices; numbering of slices is from the top of the gel. Only the first 30 fractions are shown as the rest of the fractions contained negligible amounts of activity. Assays in the presence of PCNA are shown as solid circles and in the absence of PCNA as open circles. Panel B: Aliquots of the peak fractions from 14 to 19 were subjected to SDS−PAGE and silver stained for protein. Panel C: Fractions 16, 17, and 18 were combined, subjected to SDS−PAGE, and stained with Coomassie Blue. The region of the gel marked by the bracket was excised and analyzed by microcapillary reverse-phase HPLC nanoelectrospray tandem mass spectrometry. Panel D: Sequences of the six PP1 peptides that were found, followed by symbols, indicate in which PP1 isoforms they occur.

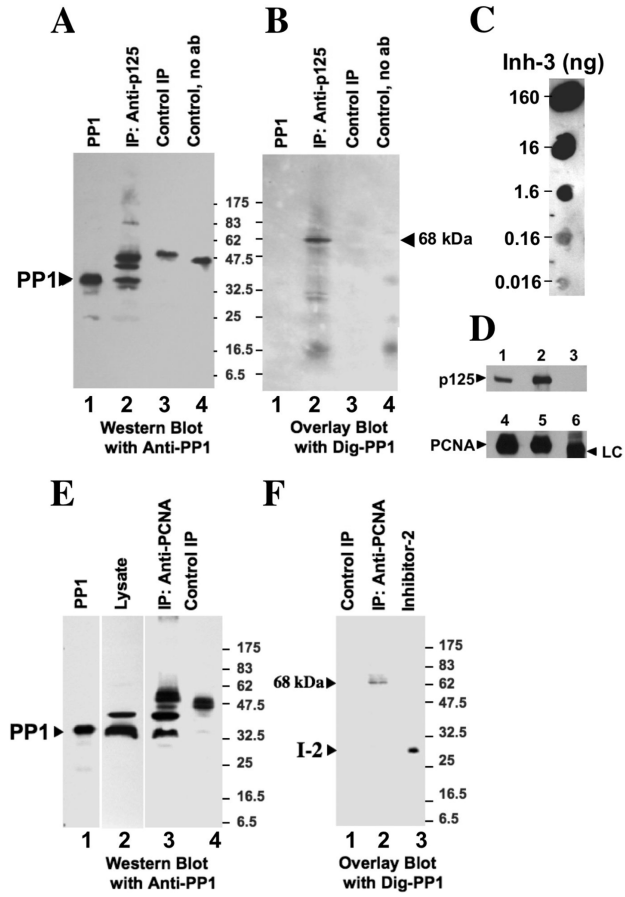

FIGURE 3: Identification of the PP1 binding polypeptide that mediates the coimmunoprecipitation of PP1 with Pol δ. Panel A: A MCF10A cell lysate (1.7 mg of protein) were immunoprecipitated with antibody against p125 and with Western-blotted antibody against PP1 (Experimental Procedures). Lane 1, purified recombinant rabbit muscle PP1α (1 ng); lane 2, the immunoprecipitate with anti-p125; lane 3, control immunoprecipitate with nonimmune IgG; lane 4, a control with no IgG added. Panel B: The blot in panel A was stripped and subjected to overlay blot by incubation with dig-PP1 and detection with anti-dig (Experimental Procedures). Lanes are as in panel A. Panel C: Dot blots of purified inhibitor-3 (Inh-3) with dig-PP1. The amounts are shown on the left in ng. Panel D: Control showing that the anti-p125 immunoprecipitates p125 (lanes 1−3) and the anti-PCNA immunoprecipitates PCNA (lanes 4−6). Lane 1, MCF10A lysate input; lane 2, immunoprecipitates of MCF10A lysate with anti-p125; lane 3, immunoprecipitate with nonimmune IgG, immunoblotted with anti-p125 (Western blotting was performed with anti-p125); lane 4, MCF10A lysate input; lane 5, immunoprecipitate of MCF10A lysate with anti-PCNA; lane 6, immunoprecipitate with nonimmune IgG; Western blotting was performed with anti-PCNA. LC refers the immunoglobulin light chain. Western blotting was performed with anti-PCNA. Panel E: MCF10A lysates were immunoprecipitated with anti-PCNA and Western blotted with anti-PP1. Lane 1, purified recombinant rabbit muscle PP1α; lane 2, MCF10A lysate input (10× dilution); lane 3, the immunoprecipitate with anti-PCNA; lane 4, immunoprecipitate with nonimmune IgG. Panel F: A MCF10A cell lysate was immunoprecipitated with antibody against PCNA and run on SDS−PAGE, and the proteins were transferred to a nitrocellulose membrane. This was directly overlay blotted with dig-PP1 (Experimental Procedures). Lane 1, immunoprecipitate with nonimmune IgG; lane 2, immunoprecipitate with anti-PCNA; lane 3, recombinant inhibitor-2 (80 ng). (Data shown are representative of more than three such experiments.)

(Figure 3B). The overlay blot revealed that PP1 was bound to a polypeptide of ca. 68 kDa (Figure 3B, lane 2). The overlay blot with dig-PP1 is highly sensitive as we were able to detect inhibitor-3, a known PP1 binding protein (*9*), at subnanogram levels in dot blots (Figure 3C). A similar experiment was performed by immunoprecipitation of MCF10A cell lysates with a PCNA antibody, as PCNA interacts with Pol δ (*31*). PP1 was coimmunoprecipitated with PCNA (Figure 3E, lane 3). In order to identify the polypeptide that bound PP1, a second coimmunoprecipitation was performed and subjected to overlay blotting with dig-PP1. This again identified a 68 kDa polypeptide as the PP1 binding polypeptide (Figure 3F, lane 2). These results establish that the association of PP1 with Pol δ is mediated by a 68 kDa polypeptide and that the interaction takes place *in vivo*.

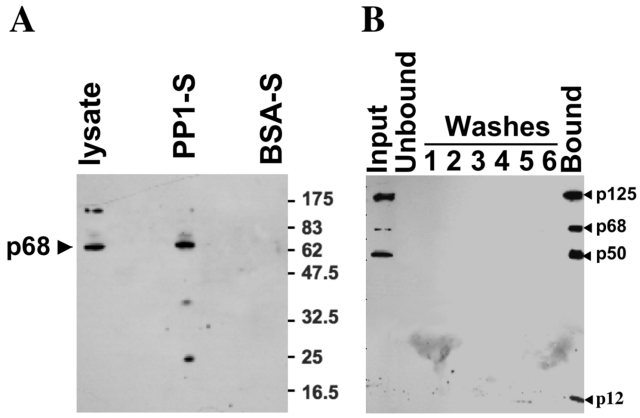

FIGURE 4: Identification of the p68 subunit of Pol δ as a PP1 binding protein. Panel A: Sf9 cells were infected with a baculovirus for the expression of human p68; a lysate from these cells was pulled down with PP1-Sepharose and Western blotted with antibody against p68 (Experimental Procedures). The lanes from left to right are the lysate, the PP1-Sepharose pull down ("PP1-S"), and a control pull down with BSA-Sepharose beads ("BSA-S"). Panel B: A lysate from Sf9 cells expressing all four subunits of Pol δ was pulled down with PP1-Sepharose beads. The beads were washed six times with 0.15 M NaCl, and the protein was extracted from the beads. The samples were subjected to SDS−PAGE and Western blotted with antibodies against the p125, p50, p68, and p12 subunits of Pol δ. From right to left the lanes show the Sf9 lysate ("input"), the supernatant of the pull down ("unbound"), the six washes, and protein extracted from the PP1-Sepharose ("bound"). (Data shown are representative of two such experiments.)

Identification of the PP1 Binding Protein as the p68 Subunit of Pol δ A likely candidate for PP1 binding polypeptide was the p68 subunit of Pol δ, based on its size. In order to demonstrate a direct interaction between PP1 and the p68 subunit, we used PP1-Sepharose (*35*) for pull-down assays. PP1-Sepharose was able to pull down the p68 subunit from lysates of Sf9 cells that expressed human p68 (Figure 4A, center lane). BSA-Sepharose was used as a control and did not pull down p68 (Figure 4A, right lane). Next, we determined that PP1 binds to p68 when the latter is part of the Pol δ holoenzyme. A lysate from Sf9 cells infected with recombinant baculoviruses for all four subunits of Pol δ was mixed with PP1-Sepharose, and the bound proteins were analyzed by Western blotting with antibodies against p125, p50, p68, and p12 (Figure 4B). The unbound fraction, representing the supernatant of the pull down, was depleted of all four Pol δ subunits (Figure 4B, second lane). No Pol δ subunits were found in the washes, and all four subunits were present in the pull down (Figure 4B, last lane). The results demonstrate that PP1 binds to the Pol δ holoenzyme and not just to the free p68 subunit.

Identification of the PP1 Binding Motif of p68. The ability of p68 to interact with PP1 suggested that it might harbor a variant of the canonical RV*X*F motif that has been shown to be present in most PP1 binding proteins (*6, 7*). Inspection of the amino acid sequence of human p68 indicated that there were four putative PP1 binding sequences, which are shown as I (residues 19−25), II (residues 57−63), III (residues 298−310), and IV (residues 318−328) in Figure 5A. These were selected based on their resemblance to the panel of sequences that were found by peptide library screening and of motifs present in PP1 binding proteins (*7*).

A set of His-tagged deletion mutants of p68 were constructed and purified (Figure 5B). These were run on

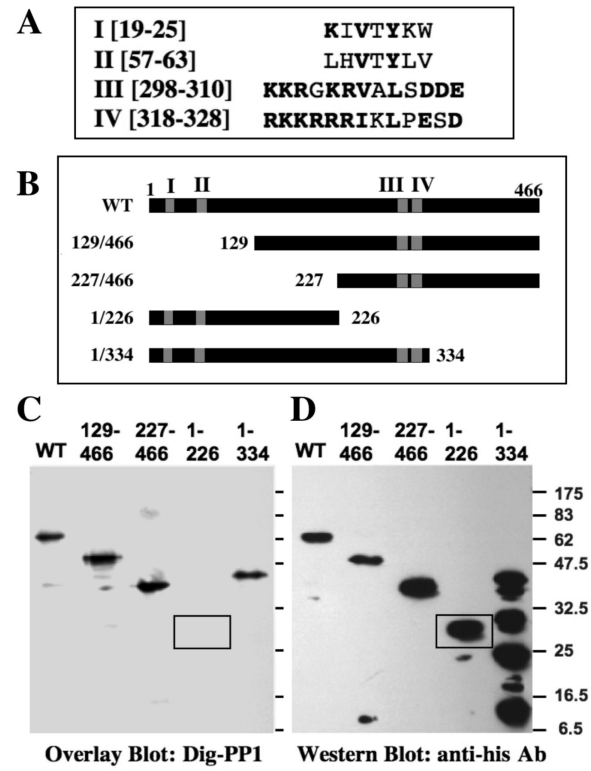

FIGURE 5: Identification of the PP1 binding region of p68 by analysis of deletion mutants. Panel A: The panel shows a list of four putative PP1 binding motifs based on the RV*X*F motif. These are labeled as I− IV. Panel B: Diagram of the His-tagged p68 deletion mutants used. Shaded regions show the locations of sequences I−IV. Panel C: The purified p68 deletion mutants were analyzed by overlay blotting with dig-PP1. The box indicates the expected position of migration of the $p68^{1-226}$ deletion mutant. Panel D: Western blotting of the SDS−PAGE of the p68 mutants with anti-polyhistidine antibody. The box indicates the position of migration of the $p68^{1-226}$ deletion mutant for comparison with the data of panel C.

SDS−PAGE and analyzed by overlay blotting with dig-PP1 (Figure 5C). The results show that sequences I and II could be excluded as PP1 binding sequences, since the $p68^{1-226}$ mutant did not bind dig-PP1, while the $p68^{129-466}$ and $p68^{227-466}$ mutants, both of which harbored only motifs III and IV, did bind to PP1. A Western blot of the deletion mutants is shown in Figure 5D to confirm the identity of the proteins bound by dig-PP1 in the overlay blots.

Site-directed mutagenesis of sequences III and IV was performed to determine which of these functioned as a PP1 binding motif. Three additional His-tagged p68 mutants were constructed. The first was M1, in which ^{302}KRVAL^{306} in sequence III was mutated to DEAAA. The second, M2, was one in which the sequence ^{323}RIKLP^{327} in sequence IV was mutated to DEAAA. The third, M3, was a mutant in which an internal deletion of residues 299−327 was made so that both sequences III and IV were removed (Figure 6A). The p68 mutants were isolated and purified (Figure 6B). These were analyzed for PP1 binding by pull-down assays. His-tagged p68 was mixed with PP1α, and the His-tagged p68-PP1 complexes were recovered by binding to Ni^{2+} beads, followed by Western blotting with anti-PP1 (Figure 6C). The M1 mutant (mutation of sequence III) and the M3 mutant (deletion of both sequences III and IV) did not pull down PP1. The M2 mutant (mutation of sequence IV) retained the ability to pull down PP1. These results show that sequence IV does not bind to PP1 and that

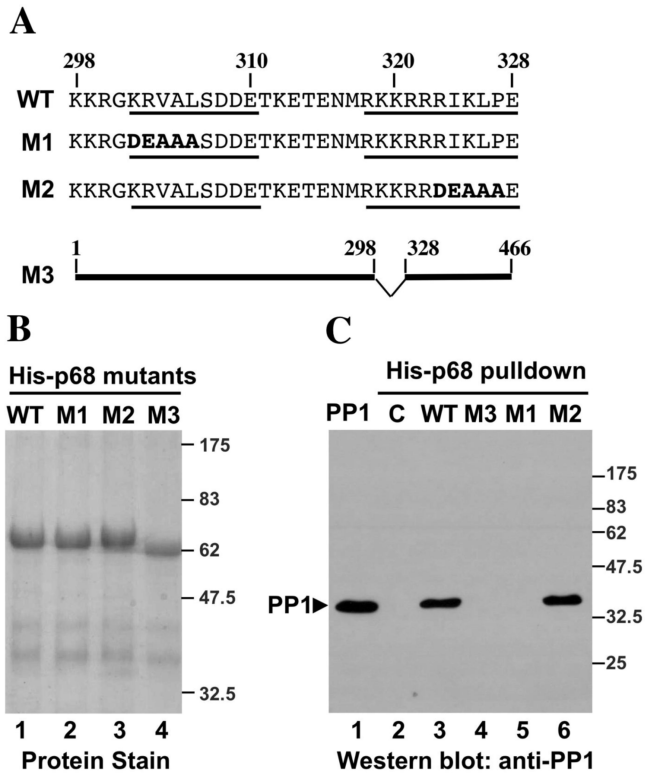

FIGURE 6: Identification of the sequence KRVAL as the sole PP1 binding sequence in p68. Panel A: Three p68 mutants were constructed: M1 in which the PP1 binding motif of sequence III was mutated (bolded residues) from KRVAL to DEAAA, M2 in which the putative RRIKL motif of sequence IV was mutated to DEAAA (bolded residues), and M3, in which residues 299−327 were internally deleted to remove both sequence III and sequence IV. Panel B: Coomassie Blue protein stain of purified His-tagged p68 and the three mutants. Each lane contained 2 μg of protein. Panel C: The purified His-tagged p68 proteins were used to pull down purified PP1α, followed by Western blotting with anti-PP1 (Experimental Procedures). Lane 1, purified PP1α as the input; lane 2 ("C"), control pull down in which no p68 was added; lane 3 ("WT"), pull down with full-length His-p68; lane 4 ("M3"), pull down with His-p68 M3 mutant; lane 5 ("M1"), pull down with His-p68 M1 mutant; lane 6 ("M2"), pull down with His-p68 M2 mutant. Each pull-down mixture (lanes 3−6) contained 1 μg each of His-tagged p68/mutant and PP1α.

p68 binds to PP1 via the motif III, which contains the KRVAL sequence (residues 302−306).

Phosphorylation of p68 and Pol δ by CK2 and Their Dephosphorylation by PP1. As noted in the introduction, little detailed information is available on the phosphorylation of Pol δ by various protein kinases. Since p68 has been found to contain at least six *in vivo* phosphorylation sites by phosphoproteomic analysis (*26*) that are in sequence contexts which conform to the CK2 consensus site (S/TxxD/E) (*40*), we examined the phosphorylation of p68 and the Pol δ holoenzyme by CK2. Purified recombinant p68 was readily phosphorylated by CK2. This is shown in Figure 7A, where p68 was phosphorylated with increasing concentrations of CK2 from 0 to 320 units per assay (Figure 7A). Analysis of the incorporation of radioactivity by phosphorimaging showed that this reached a plateau at ca. 80 units of CK2 per assay. Pol δ holoenzyme was phosphorylated with CK2 using 250 units of CK2 per assay to ensure maximal incorporation of radioactivity. It was consistently observed that the p125, p68, and p12 subunits, but not the p50 subunit, were phospho-

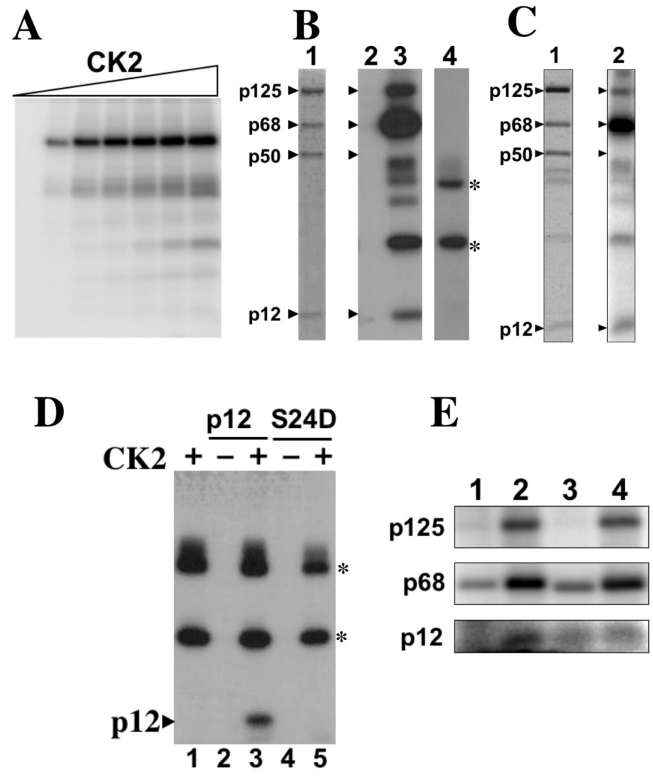

FIGURE 7: Analysis of the phosphorylation of Pol δ by CK2 and its dephosporylation by PP1. Panel A: Phosphorylation of p68. His-tagged p68 (0.5 μg) was phosphorylated with 0, 5, 20, 40, 80, 160, and 320 units of CK2 in the presence of [γ-^{32}P]ATP for 1 h at 30 °C (Experimental Procedures). The radioactive bands were visualized by phosphorimaging. Panel B: Phosphorylation of Pol δ holoenzyme by CK2. Purified recombinant Pol δ (1.4 μg) was phosphorylated with CK2 (250 units) and analyzed by SDS−PAGE followed by autoradiography on film. Lane 1, Coomassie Blue protein stain of the Pol δ enzyme used in the experiment; lane 2, control in which CK2 was omitted; lane 3, phosphorylation of Pol δ; lane 4, control in which Pol δ was omitted. (Bands marked with an asterisk are those of the autophosphorylation of the CK2α and CK2β subunits.) Panel C: The experiment in panel B was repeated, but the products were examined by phosphorimaging. Lane 1, Coomassie Blue protein stain of the Pol δ enzyme used in the experiment; lane 2, phosphorimage of the reaction products. The ratio of the amounts of radioactivity incorporated into the p12, p68, and p125 subunits was 1:7.0 ± 0.12:1.2 ± 0.3 (mean ± SD) from three independent experiments. Panel D: p12 has a single CK2 phosphorylation site. Purified recombinant p12 (0.5 μg) and the p12(S24D) (0.5 μg) mutant were phosphorylated with 250 units of CK2 (Experimental Procedures). The products were resolved on SDS−PAGE and visualized by autoradiography. Lane 1, control with CK2 alone; lane 2, control reaction mixture in which CK2 was omitted; lane 3, complete reaction mixture for phosphorylation of p12; lane 4, control reaction in which CK2 was omitted; lane 5, complete reaction mixture for the phosphorylation of p12(S24D). Panel E: Dephosphorylation of phosphorylated Pol δ by PP1. Pol δ (1.4 μg) was phosphorylated with 100 units of CK2 for 1 h (Experimental Procedures). Lane 1, control reaction in which 200 μM TBB was added to inhibit CK2 activity; lane 2, complete reaction mixture; lane 3, PP1 (2.5 units) and 200 μM TBB were added after the phosphorylation reaction as in lane 2, and the reaction mixture was incubated for a further 60 min; lane 4, same reaction as for lane 3, except that 10 μM microcystin-LR was added to inhibit PP1 activity. The panels for p125, p68, and p12 phosphorylation were taken from phosphorimaging analysis. (Exposures for p12 and p125 were higher than those used for p68.) The data were also quantitated by phosphorimaging analysis, because of the diffuse images for p12. A correction for the incomplete inhibition of CK2 by TBB (lanes 1) was made; i.e., the radioactivity in lane 1 was subtracted from that in lane 3 to obtain an estimate of the amount of radioactivity remaining after PP1 treatment. On this basis, the percentages of dephosphorylation of p125, p68, and p12 were 100%, 76%, and 75%, respectively.

rylated and that p68 was the most heavily phosphorylated. Representative data for a number of experiments are shown in Figure 7, where the reaction products were examined by both autoradiography (Figure 7B) and phosphorimaging (Figure 7C). The protein stains of the Pol δ complex that was used in the experiments are shown in lanes 1 in Figure 7B,C.

The p12 subunit is a small protein of 107 amino acid residues (*14*). Analysis of its sequence for putative phosphorylation sites by the NetPhos program (*41*) revealed that there was a single CK2 site at S24 with the sequence ^{24}SKGE^{27}. This site was mutated to aspartate. Examination of the phosphorylation of p12 and the p12 S24D mutant showed that the mutant was resistant to CK2 phosphorylation, confirming that p12 has a single CK2 phosphorylation site at S24 (Figure 7D).

We compared the amounts of radioactivity incorporated into the Pol δ subunits relative to that of the p12 subunit. The ratios of incorporation of radioactivity were p12:p68 = 1:7.0 ± 0.12, and p12:p125 = 1:1.2 ± 0.3 (Figure 7C). Since p12 has a single phosphorylation site, these results provide an estimate of the phosphorylation stoichiometry for p68 and for p125, based on the reasonable assumption that Pol δ was fully phosphorylated under the conditions used in our experiments. The stoichiometry of 7 that was found for p68 is in reasonable agreement with the phosphoproteomic data (*26*) which show there are at least six phosphorylation sites on p68. In this context, it is noted that a recent study has provided evidence based on *in vitro* phosphorylation of p68 peptides that human p68 is also phosphorylated at S389 and/or T391 (*27*). Our findings confirm that p68 is phosphorylated on multiple sites by CK2 and together with the phosphoproteomic data provide supporting evidence for a role of CK2 in the phosphorylation of p68 *in vivo*. In the case of the p125 subunit, a stoichiometry of 1.2 ± 0.3 was found. These data indicate that p125 has at least one and possibly two phosphorylation sites for CK2.

The ability of PP1 activity to dephosphorylate Pol δ that had been phosphorylated by CK2 was examined. In these experiments, TBB was used to inhibit CK2 after the phosphorylation of Pol δ, following which either PP1 or PP1 plus microcystin-LR, a potent inhibitor of PP1, was added (Figure 7E). PP1 readily dephosphorylated p125, p68, and p12 (Figure 7E, cf. lanes 3 and 2). Addition of microcystin-LR inhibited the dephosphorylation reactions, confirming that the reduction in radioactivity was due to the action of PP1.

DISCUSSION

We have identified the p68 subunit of Pol δ as a novel PP1 targeting protein. The interaction was initially detected by the finding that PP1 is associated with immunoaffinity-purified HeLa Pol δ. This was confirmed by coimmunoprecipitation of PP1 with the p125 subunit of Pol δ when antibodies against either the p125 subunit or PCNA were used. The presence of PP1 in Pol δ purified by electrophoresis on nondenaturing electrophoresis was also confirmed by mass spectrometry analysis. These experiments provide supporting evidence that this interaction takes place *in vivo*. The p68 subunit of Pol δ was identified as the polypeptide associated with PP1 by overlay blotting with dig-PP1, and the interactions between PP1 and p68, and PP1 with the Pol δ holoenzyme, were shown to take place with the purified proteins by the use of pull-down assays. The interaction of PP1 with p68 was therefore demonstrated to mediate an interaction of PP1 with the Pol δ holoenzyme. p68 did not inhibit PP1α activity, nor did addition of PP1α inhibit Pol δ activity (data not shown). Our findings support a role of p68 as a targeting subunit that recruits PP1 to the Pol δ holoenzyme.

The interaction site for PP1 was determined by examination of four sequences in p68 that contained putative RV*X*F PP1 binding motifs by deletion and site-directed mutagenesis. These experiments demonstrate that the PP1 interacting sequence is harbored in the 13-residue peptide ^{298}KKRGK**RVAL**SDDE^{310}, which contains the RVAL sequence, a variant of the RV*X*F motif. Site-directed mutagenesis of the KRVAL sequence abolished the interaction of p68 with PP1. The RVAL sequence conforms to the canonical PP1 motif, except that the conserved aromatic residue Phe is replaced by Leu. The replacement of the conserved aromatic residue Phe to Leu is also observed in two peptides (G1 and G2) identified in the peptide library screen by Zhao and Lee (*7*). The ^{298}KKRGKR**VAL**SDDE^{310} sequence has five basic residues preceding the VAL sequence, followed by two acid residues. This arrangement of basic and acid residues was found to be favored in the analysis of PP1 binding peptides from a random peptide display library (*7*).

Peptide interactions of the RVxF motif have been found to have K_d's in the micromolar range, whereas some regulatory proteins interact PP1 with K_d's in the nanomolar range (*6*). The greater strength of interaction of some PP1 binding proteins is due to the existence of additional binding interactions (*6, 42, 43*). We have observed that the interaction of PP1 with Pol δ persists through immunoaffinity chromatography and subsequent FPLC chromatography on MonoQ columns but that it is separated from Pol δ on FPLC gel filtration chromatography (Zhou and Lee, unpublished observations). In addition, we have coexpressed PP1α with all four subunits of Pol δ in Sf9 cells and isolated the Pol δ by immunoaffinity chromatography. Western blotting showed that PP1 was associated with Pol δ and that this persisted through MonoQ chromatography. However, the amount of PP1α protein that was present was barely detectable, suggesting that a pentameric complex of PP1α and Pol δ was not stable enough for its isolation (Zhou and Lee, unpublished observations).

The discovery that PP1 interacts with Pol δ via the p68 subunit raises the question of the identity of its phosphoproteins substrates, which in our current paradigm for PP1 functions will be those in the microvicinity of p68. p68 is a very hydrophilic protein with 46% of its residues composed of K, R, D, E, S, or T and has a calculated molecular mass of 51.4 kDa. It migrates anomalously on SDS−PAGE with an apparent molecular mass of ca. 68 kDa, and it is likely to have a highly extended and flexible structure, similar to the behavior of its yeast homologues, Pol32 and Cdc27 (*11, 18, 22*). An extended structure would be advantageous to a function of p68 as a targeting subunit for PP1, as it might permit for a range of movement that would allow the bound PP1 to dephosphorylate proteins in the vicinity of Pol δ.

The immediate targets of PP1 are likely to be the subunits of Pol δ itself, with p68 itself as a prime candidate. Phosphoproteome analysis has shown that there are six *in*

vivo phosphorylation sites in p68 (*26*), all of which were found to have high prediction scores as potential CK2 phosphorylation sites by the NetPhos software (*41*). In this study we report the first *in vitro* biochemical analysis of the phosphorylation of purified p68 and the Pol δ holoenzyme by CK2. Our findings confirm that p68 is a substrate for CK2 and that it is subject to multisite phosphorylation. In addition, we show that the p12 subunit is phosphorylated at a single site and that p125 is phosphorylated with a stoichiometry that indicates that there may be one or two phosphorylation sites. Further investigations will be needed to identify the *in vitro* phosphorylation sites of the p68 and p125 subunits.

Phosphorylation of residues within or close to the PP1 binding motif has been shown to disrupt the interaction of targeting/regulatory subunits with PP1 (*1, 6*), e.g., the PKA phosphorylation of the RVSF motif in Nipp1 (*44*). Thus, the possibility that phosphorylation might affect the protein interaction sites of p68 should be considered. p68, in addition to having a protein interaction site for PP1, also has protein interaction sites for Pol α (*20*) and for PCNA. Two of the six *in vivo* phosphorylation sites (S307 and T311) that were identified by phosphoproteomic analysis (*26*) lie immediately adjacent to the PP1 binding motif (^{298}KKRGKRVAL**S**DDE**T**^{311}). This location of S307 and T311 is significant, as their phosphorylation could disrupt the interaction between PP1 and p68. Thus, the interaction of PP1 and p68 may itself be regulated by protein phosphorylation. The Pol α interaction site has the DPIM sequence (underlined) ^{394}DGEGCIVTEKVYE**S**E**S**C**T**D**S**^{413} (*20*). The DPIM is followed by four *in vivo* phosphorylation sites, Ser407, Ser409, Thr411, and Ser413 (bolded) (*26*). The juxtaposition of the DPIM and these phosphorylation sites is potentially one that could regulate the interaction of Pol α and p68. A third protein interaction site on p68 that could be regulated by phosphorylation is the PCNA binding motif ^{456}QVSITGFF^{463} at the C-terminus (*13, 15, 45*). A recent study has reported that phosphorylation of p68 peptides containing this sequence by CK2 inhibits PCNA binding in ELISA assays, and this suggests that S458 and/or T460 phosphorylation might regulate p68/PCNA interaction (*27*).

There is no dearth of other potential substrates for PP1 that would be in its microvicinity when it is associated with Pol δ, as these include other protein components of the replication complex, a number of which are known to be phosphorylated (reviewed in ref *28*). These include the ssDNA binding protein RPA (replication factor A) (*46*), RFC (replication factor C) (*47*), Fen1 (Flap endonuclease-1) (*48*), and DNA ligase-1 (*49*), as well as Pol α (*50*).

In summary, we have identified a novel PP1 binding protein, the p68 subunit of Pol δ, and shown that it interacts with the Pol δ holoenzyme. These studies provide the first evidence for the targeting of PP1 to DNA polymerase δ. The ability of p68 to recruit PP1 to the Pol δ holoenzyme has significant implications as it supports a role for PP1 in the phosphorylation/dephosphorylation of p68, other subunits of DNA polymerase δ, or even other components of the multiprotein replication or DNA repair complexes in which the latter is a participant. In addition, we have shown that Pol δ is phosphorylated by CK2 *in vitro*. Our data for the phosphorylation of p68 are consistent with a role for CK2 in its *in vivo* phosphorylation.

REFERENCES

1. Ceulemans, H., and Bollen, M. (2004) Functional diversity of protein phosphatase-1, a cellular economizer and reset button. *Physiol. Rev. 84*, 1–39.
2. Sasaki, K., Shima, H., Kitagawa, Y., Irino, S., Sugimura, T., and Nagao, M. (1990) Identification of members of the protein phosphatase 1 gene family in the rat and enhanced expression of protein phosphatase 1 alpha gene in rat hepatocellular carcinomas. *Jpn. J. Cancer Res. 81*, 1272–1280.
3. Zhang, Z., Bai, G., Shima, M., Zhao, S., Nagao, M., and Lee, E. Y. (1993) Expression and characterization of rat protein phosphatases-1 alpha, -1 gamma 1, -1 gamma 2, and -1 delta. *Arch. Biochem. Biophys. 303*, 402–406.
4. Lee, E. Y., Zhang, L., Zhao, S., Wei, Q., Zhang, J., Qi, Z. Q., and Belmonte, E. R. (1999) Phosphorylase phosphatase: new horizons for an old enzyme. *Front. Biosci. 4*, D270–D285.
5. Alms, G. R., Sanz, P., Carlson, M., and Haystead, T. A. (1999) Reg1p targets protein phosphatase 1 to dephosphorylate hexokinase II in Saccharomyces cerevisiae: characterizing the effects of a phosphatase subunit on the yeast proteome. *EMBO J. 18*, 4157–4168.
6. Egloff, M. P., Johnson, D. F., Moorhead, G., Cohen, P. T., Cohen, P., and Barford, D. (1997) Structural basis for the recognition of regulatory subunits by the catalytic subunit of protein phosphatase 1. *EMBO J. 16*, 1876–1887.
7. Zhao, S., and Lee, E. Y. (1997) A protein phosphatase-1-binding motif identified by the panning of a random peptide display library. *J. Biol. Chem. 272*, 28368–28372.
8. Li, M., Satinover, D. L., and Brautigan, D. L. (2007) Phosphorylation and functions of inhibitor-2 family of proteins. *Biochemistry 46*, 2380–2389.
9. Zhang, J., Zhang, L., Zhao, S., and Lee, E. Y. (1998) Identification and characterization of the human HCG V gene product as a novel inhibitor of protein phosphatase-1. *Biochemistry 37*, 16728–16734.
10. Johnson, A., and O'Donnell, M. (2005) Cellular DNA replicases: components and dynamics at the replication fork. *Annu. Rev. Biochem. 74*, 283–315.
11. Garg, P., and Burgers, P. M. (2005) DNA polymerases that propagate the eukaryotic DNA replication fork. *Crit. Rev. Biochem. Mol. Biol. 40*, 115–128.
12. Lee, M. Y., Jiang, Y. Q., Zhang, S. J., and Toomey, N. L. (1991) Characterization of human DNA polymerase delta and its immunochemical relationships with DNA polymerase alpha and epsilon. *J. Biol. Chem. 266*, 2423–2429.
13. Mo, J., Liu, L., Leon, A., Mazloum, N., and Lee, M. Y. (2000) Evidence that DNA polymerase delta isolated by immunoaffinity chromatography exhibits high-molecular weight characteristics and is associated with the KIAA0039 protein and RPA. *Biochemistry 39*, 7245–7254.
14. Liu, L., Mo, J., Rodriguez-Belmonte, E. M., and Lee, M. Y. (2000) Identification of a fourth subunit of mammalian DNA polymerase delta. *J. Biol. Chem. 275*, 18739–18744.
15. Li, H., Xie, B., Zhou, Y., Rahmeh, A., Trusa, S., Zhang, S., Gao, Y., Lee, E. Y., and Lee, M. Y. (2006) Functional roles of p12, the fourth subunit of human DNA polymerase delta. *J. Biol. Chem. 281*, 14748–14755.
16. Burgers, P. M., and Gerik, K. J. (1998) Structure and processivity of two forms of Saccharomyces cerevisiae DNA polymerase delta. *J. Biol. Chem. 273*, 19756–19762.
17. Zuo, S., Bermudez, V., Zhang, G., Kelman, Z., and Hurwitz, J. (2000) Structure and activity associated with multiple forms of Schizosaccharomyces pombe DNA polymerase delta. *J. Biol. Chem. 275*, 5153–5162.
18. Bermudez, V. P., MacNeill, S. A., Tappin, I., and Hurwitz, J. (2002) The influence of the Cdc27 subunit on the properties of the Schizosaccharomyces pombe DNA polymerase delta. *J. Biol. Chem. 277*, 36853–36862.
19. Johansson, E., Garg, P., and Burgers, P. M. (2004) The Pol32 subunit of DNA polymerase delta contains separable domains for processive replication and proliferating cell nuclear antigen (PCNA) binding. *J. Biol. Chem. 279*, 1907–1915.
20. Gray, F. C., Pohler, J. R., Warbrick, E., and MacNeill, S. A. (2004) Mapping and mutation of the conserved DNA polymerase interaction motif (DPIM) located in the C-terminal domain of fission yeast DNA polymerase delta subunit Cdc27. *BMC Mol. Biol. 5*, 21.
21. Pohler, J. R., Otterlei, M., and Warbrick, E. (2005) An in vivo analysis of the localisation and interactions of human p66 DNA polymerase delta subunit. *BMC Mol. Biol. 6*, 17.

22. Johansson, E., Majka, J., and Burgers, P. M. (2001) Structure of DNA polymerase delta from Saccharomyces cerevisiae. *J. Biol. Chem. 276*, 43824–43828.
23. Lydeard, J. R., Jain, S., Yamaguchi, M., and Haber, J. E. (2007) Break-induced replication and telomerase-independent telomere maintenance require Pol32. *Nature 448*, 820–823.
24. van Welsem, T., Frederiks, F., Verzijlbergen, K. F., Faber, A. W., Nelson, Z. W., Egan, D. A., Gottschling, D. E., and van Leeuwen, F. (2008) Synthetic lethal screens identify gene silencing processes in yeast and implicate the acetylated amino terminus of Sir3 in recognition of the nucleosome core. *Mol. Cell. Biol. 28*, 3861–3872.
25. Liu, G., and Warbrick, E. (2006) The p66 and p12 subunits of DNA polymerase delta are modified by ubiquitin and ubiquitin-like proteins. *Biochem. Biophys. Res. Commun. 349*, 360–366.
26. Olsen, J. V., Blagoev, B., Gnad, F., Macek, B., Kumar, C., Mortensen, P., and Mann, M. (2006) Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. *Cell 127*, 635–648.
27. Lemmens, L., Urbach, S., Prudent, R., Cochet, C., Baldacci, G., and Hughes, P. (2008) Phosphorylation of the C subunit (p66) of human DNA polymerase delta. *Biochem. Biophys. Res. Commun. 367*, 264–270.
28. Henneke, G., Koundrioukoff, S., and Hubscher, U. (2003) Multiple roles for kinases in DNA replication. *EMBO Rep. 4*, 252–256.
29. Zeng, X. R., Hao, H., Jiang, Y., and Lee, M. Y. (1994) Regulation of human DNA polymerase delta during the cell cycle. *J. Biol. Chem. 269*, 24027–24033.
30. Wu, S. M., Zhang, P., Zeng, X. R., Zhang, S. J., Mo, J., Li, B. Q., and Lee, M. Y. (1998) Characterization of the p125 subunit of human DNA polymerase delta and its deletion mutants. Interaction with cyclin-dependent kinase-cyclins. *J. Biol. Chem. 273*, 9561–9569.
31. Loor, G., Zhang, S. J., Zhang, P., Toomey, N. L., and Lee, M. Y. (1997) Identification of DNA replication and cell cycle proteins that interact with PCNA. *Nucleic Acids Res. 25*, 5041–5046.
32. Zhang, A. J., Bai, G., Deans-Zirattu, S., Browner, M. F., and Lee, E. Y. (1992) Expression of the catalytic subunit of phosphorylase phosphatase (protein phosphatase-1) in Escherichia coli. *J. Biol. Chem. 267*, 1484–1490.
33. Xie, B., Mazloum, N., Liu, L., Rahmeh, A., Li, H., and Lee, M. Y. (2002) Reconstitution and characterization of the human DNA polymerase delta four-subunit holoenzyme. *Biochemistry 41*, 13133–13142.
34. Jiang, Y., Zhang, S. J., Wu, S. M., and Lee, M. Y. (1995) Immunoaffinity purification of DNA polymerase delta. *Arch. Biochem. Biophys. 320*, 297–304.
35. Zhao, S., Xia, W., and Lee, E. Y. (1996) Affinity chromatography of regulatory subunits of protein phosphatase-1. *Arch. Biochem. Biophys. 325*, 82–90.
36. Zhang, S., Zhou, Y., Trusa, S., Meng, X., Lee, E. Y., and Lee, M. Y. (2007) A novel DNA damage response: rapid degradation of the p12 subunit of dna polymerase delta. *J. Biol. Chem. 282*, 15330–15340.
37. Huang, H. S., Pozarowski, P., Gao, Y., Darzynkiewicz, Z., and Lee, E. Y. (2005) Protein phosphatase-1 inhibitor-3 is co-localized to the nucleoli and centrosomes with PP1gamma1 and PP1alpha, respectively. *Arch. Biochem. Biophys. 443*, 33–44.
38. Lee, M. Y., Tan, C. K., Downey, K. M., and So, A. G. (1984) Further studies on calf thymus DNA polymerase delta purified to homogeneity by a new procedure. *Biochemistry 23*, 1906–1913.
39. Trinkle-Mulcahy, L., Sleeman, J. E., and Lamond, A. I. (2001) Dynamic targeting of protein phosphatase 1 within the nuclei of living mammalian cells. *J. Cell Sci. 114*, 4219–4228.
40. Meggio, F., and Pinna, L. A. (2003) One-thousand-and-one substrates of protein kinase CK2? *FASEB J. 17*, 349–368.
41. Blom, N., Sicheritz-Ponten, T., Gupta, R., Gammeltoft, S., and Brunak, S. (2004) Prediction of post-translational glycosylation and phosphorylation of proteins from the amino acid sequence. *Proteomics 4*, 1633–1649.
42. Barford, D., Das, A. K., and Egloff, M. P. (1998) The structure and mechanism of protein phosphatases: insights into catalysis and regulation. *Annu. Rev. Biophys. Biomol. Struct. 27*, 133–164.
43. Terrak, M., Kerff, F., Langsetmo, K., Tao, T., and Dominguez, R. (2004) Structural basis of protein phosphatase 1 regulation. *Nature 429*, 780–784.
44. Van Eynde, A., Wera, S., Beullens, M., Torrekens, S., Van Leuven, F., Stalmans, W., and Bollen, M. (1995) Molecular cloning of NIPP-1, a nuclear inhibitor of protein phosphatase-1, reveals homology with polypeptides involved in RNA processing. *J. Biol. Chem. 270*, 28068–28074.
45. Ducoux, M., Urbach, S., Baldacci, G., Hubscher, U., Koundrioukoff, S., Christensen, J., and Hughes, P. (2001) Mediation of proliferating cell nuclear antigen (PCNA)-dependent DNA replication through a conserved p21(Cip1)-like PCNA-binding motif present in the third subunit of human DNA polymerase delta. *J. Biol. Chem. 276*, 49258–49266.
46. Binz, S. K., and Wold, M. S. (2008) Regulatory functions of the N-terminal domain of the 70-kDa subunit of replication protein A (RPA). *J. Biol. Chem. 283*, 21559–21570.
47. Munshi, A., Cannella, D., Brickner, H., Salles-Passador, I., Podust, V., Fotedar, R., and Fotedar, A. (2003) Cell cycle-dependent phosphorylation of the large subunit of replication factor C (RF-C) leads to its dissociation from the RF-C complex. *J. Biol. Chem. 278*, 48467–48473.
48. Henneke, G., Koundrioukoff, S., and Hubscher, U. (2003) Phosphorylation of human Fen1 by cyclin-dependent kinase modulates its role in replication fork regulation. *Oncogene 22*, 4301–4313.
49. Ferrari, G., Rossi, R., Arosio, D., Vindigni, A., Biamonti, G., and Montecucco, A. (2003) Cell cycle-dependent phosphorylation of human DNA ligase I at the cyclin-dependent kinase sites. *J. Biol. Chem. 278*, 37761–37767.
50. Voitenleitner, C., Rehfuess, C., Hilmes, M., O'Rear, L., Liao, P. C., Gage, D. A., Ott, R., Nasheuer, H. P., and Fanning, E. (1999) Cell cycle-dependent regulation of human DNA polymerase alpha-primase activity by phosphorylation. *Mol. Cell. Biol. 19*, 646–656.

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