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ORIGINAL PAPER

Proliferating cell nuclear antigen destabilizes c-Abl tyrosine kinase and regulates cell apoptosis in response to DNA damage

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Abstract The tyrosine kinase, c-Abl, plays important roles in many aspects of cellular function. The activity of c-Abl is tightly controlled, but the underlying mechanism is unclear. Recent studies suggest that c-Abl function is regulated by distinct lipids in different cell types. In the present study, we show that the DNA replication factor, proliferating cell nuclear antigen (PCNA), interacts with c-Abl and destabilizes c-Abl by promoting its polyubiquitination and degradation. Moreover, deletion of a domain in c-Abl, the PIP box, disrupts its interaction with PCNA, abolishes the PCNA-induced degradation of nuclear c-Abl, and substantially increases the nuclear c-Abl apoptotic function. These findings indicate that PCNA negatively regulates the stability of c-Abl and thereby inhibits apoptosis in the response to DNA damage.

Keywords c-Abl · PCNA · Apoptosis

Introduction

The tyrosine kinase, c-Abl, plays important roles in many aspects of cellular function. The activity of c-Abl is under stringent control, but the regulatory mechanism is not understood [1]. The c-Abl protein is ubiquitously expressed in many cell types, and is distributed in multiple subcellular compartments, including the nucleus, plasma membrane and actin cytoskeleton [2]. Recent studies suggest that the activity of the membrane-associated c-Abl is negatively regulated by phosphatidylinositol [3] and by the intramolecular association of the amino-terminal myristoyl group with the catalytic domain [4, 5]. By contrast with the membrane-associated c-Abl, the control of nuclear fraction of c-Abl appear to involve distinct mechanisms. In this regard, the tyrosine kinase activity of c-Abl tyrosine kinase has been shown to be activated by the deletion of the SH3 domain, as in the Abelson murine leukemia virus, or by the translocation of breakpoint cluster region (Bcr) sequences to the *N*-terminus of c-Abl, as in Philadelphia chromosome-positive human leukemia cells [6, 7]. Additionally, the nuclear c-Abl was found to associate with several proteins, including Rb, Pag/Msp23, and AAP1 [8, 9], and these interaction partners were shown to exert negative influence on c-Abl activity.

Proliferating cell nuclear antigen (PCNA) functions as a DNA sliding clamp for DNA polymerase delta and is an essential component for DNA replication [10]. PCNA is a multi-function protein. Besides acting as an accessory protein for DNA polymerase, PCNA has been shown to be involved in joining Okazaki fragments, DNA repair, DNA methylation, chromatin assembly, and cell cycle progression [11]. Regulations of PCNA functions include cofactor exchange by cofactor phosphorylation or by PCNA ubiquitination, an irreversible intervention into PCNA associations proceeds via protein degradation involving the ubiquitin-proteasome pathway. It has been reported that PCNA acts as a Cofactor in Cdt1 degradation by CUL4/DDB1-mediated *N*-terminal ubiquitination [12], also in

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degradation of Xic1, a *Xenopus leavis* homolog of the human CDK inhibitor p27 [13]. Therefore, PCNA is a matchmaker for many proteins involved in DNA and chromatin metabolism, also serves to promote the targeted degradation of associated proteins.

In this report, we show that the nuclear c-Abl tyrosine kinase associates with PCNA through the SH2 domain. The interaction leads to the degradation of the c-Abl kinase. Additionally, we show that deletion of the PIP box within c-Abl abrogates the interaction between PCNA and c-Abl, abolishes the PCNA-induced degradation of nuclear c-Abl, and notably, increases the nuclear c-Abl apoptotic function upon DNA damage.

Materials and methods

Cell culture and transfection

293T and MCF-7 were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone), 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. Cells were treated with MG132 (Sigma) as noted in the text. Transient transfections were performed with Lipofectamine 2000 (Invitrogen). Vectors and Epitope Tagging of Proteins Flag-tagged c-Abl and their mutants, GFP-tagged c-Abl, His-tagged PCNA, GFP-tagged PCNA, and Myc-tagged PCNA were expressed by cloning the genes into the pcDNA3-based vector (Invitrogen). Glutathione S-transferase (GST) fusion proteins were generated by expression in pGEX4T-2-based vectors (Amersham Biosciences Biotech, Inc.) in Escherichia col BL21 (DE3). Cells were treated with 100 mM cisplatin (CDDP; Sigma) or 10 mM STI571 (Gleevec; Novartis, Basel, Switzerland).

Immunoprecipitation and immunoblot analysis

Cell lysates were prepared in lysis buffer (50 mM Tris–HCl [pH 7.5], 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 10 mM sodium fluoride, 10 mg/ml aprotinin, 10 mg/ml leupeptin, and 10 mg/ml pepstatin A) containing 1% Nonidet P-40. Soluble proteins were subjected to immunoprecipitation with anti-Flag (M2, Sigma), anti-Myc (Santa Cruz), anti-GFP, anti-c-Abl (K-12, Santa Cruz), anti-PCNA (Santa Cruz), or anti-mouse IgG antibody (Sigma). An aliquot of the total lysate (5%, v/v) was included as a control. Immunoblot analysis was performed with anti-Myc, HRP-conjugated anti-Flag (Sigma), anti-c-Abl (Santa Cruz), anti-α-Tubulin (Sigma), anti-phosphotyrosine (P-Tyr) antibody (cell signaling) or anti-PCNA (SantaCruz) antibody. The antigen–antibody complexes were visualized by chemiluminescence (PerkinElmer Life Sciences).

Yeast two-hybrid screening

The Matchmaker two-hybrid system kit (Clontech) was used for detecting specific proteins interacting with the PCNA bait protein as described by manufacturer. Briefly, the bait plasmid pAS2-PCNA and a human mammary cDNA prey library (Clontech) were sequentially transformed into the *Saccharomyces cerevisiae* strain CG1945. Transformants were plated on synthetic medium lacking tryptophan, leucine, adenine, and histidine. Approximately 0.6 million transformants were screened. The candidate clones c-Abl and c-Abl SH2 domain deletion plasmids were rescued from the yeast cells and reintroduced back to the same yeast strain to verify the interaction between the candidates and the PCNA bait. The specificity of the interaction was determined by comparing the interactions between the candidates and various bait constructs. The unrelated prey plasmid pACT2-lamin and the empty vector pACT2 were examined as negative controls.

Protein binding assays

In GST pull-down experiments, cell lysates were incubated for 2 h at 4°C with 5 μg purified GST or GST fusion proteins bound to glutathione beads. The adsorbates were washed with lysis buffer and then subjected to SDS–PAGE and immunoblot analysis. An aliquot of the total lysates (5%, v/v) was included as a loading control on the SDS–PAGE.

In direct binding assays, immunoprecipitates were separated by SDS–PAGE and then blotted onto nitrocellulose membranes. Membranes were subsequently incubated with purified GST-fusion proteins for 2 h at room temperature. The GST fusion proteins binding to nitrocellulose were probed with anti-GST antibody.

In vivo ubiquitination assays

For in vivo ubiquitination assay, 293T cells were cotransfected with plasmids expressing Flag-c-Abl, His-PCNA, and HA-tagged ubiquitin. Cells were treated with MG132 (20 μM) for different times (6 and 12 h) at 48 h after transfection, and cells were then immunoprecipitated with anti-Flag antibody and analyzed with immunoblotting.

Flow cytometry

For propidium iodide staining, cells were harvested by trypsinization, fixed with ice-cold 70% ethanol and resuspended in a solution containing 50 μg/ml propidium iodide, 0.1% Triton X-100, 50 μg/ml RNAse A, and 5 mM EDTA at room temperature (RT) for 1 h. Cells were then diluted 1:1 in 1% BSA PBS for cytometric analysis. DNA

content was assessed by staining 70% ethanol-fixed cells with propidium iodide and monitoring by FACScan (Becton Dickinson). The percentage of cells with sub-G1 DNA was determined by the MODFIT LT Program (Verity Software). Statistical, significance was determined using the unpaired Student's *t*-test and GraftPad software. The results were expressed as the mean of three independent experiments.

Annexin V staining was performed as indicated with the Annexin V-Biotin Apotosis Detection Kit (Oncogene). Briefly, cells were washed with cold PBS, resuspended in 1× Binding Buffer and then Annexin V Dye was added, after 15 min incubation, cells were washed and propidium iodide were added, cells were analysed by flow cytometry. The results were expressed as the mean of three independent experiments.

Results

PCNA associates with c-Abl

We previously noted in a yeast two-hybrid screening that PCNA associates with c-Abl (data not shown). To examine which region of c-Abl binds to PCNA, yeast two-hybrid experiments were performed again. As shown in Fig. 2d, the PCNA interact with WT c-Abl, but not with SH2 domain deleted c-Abl. To substantiate the c-Abl–PCNA interaction, lysates from the human cell line 293T were subjected to immunoprecipitation with anti-c-Abl antibody. By immunoblotting with an anti-PCNA antibody, PCNA was found in the immunoprecipitates, but not in the control immunoprecipitates (Fig. 1a). To further confirm the association between PCNA and c-Abl, 293T cells were cotransfected with plasmids expressing Flag-tagged c-Abl and GFP-PCNA fusion proteins. Western analysis with anti-GFP antibody revealed a substantial amount of PCNA in the anti-flag immunoprecipitates, which is indicative of association between Flag-c-Abl and GFP-PCNA proteins (Fig. 1b). Immunofluorescence co-localization studies using anti-c-Abl and anti-PCNA antibody also showed that c-Abl colocalized with PCNA, predominantly in the cell nucleus (data unshown).

To further characterize the interaction between PCNA and c-Abl, lysates from 293T cells were incubated with glutathione S-transferase (GST) or GST-PCNA fusion protein. The experiment showed that c-Abl binds to GST-PCNA, but not GST (Fig. 1c). To rule out an indirect association mediated by other components in the cell lysates, anti-Flag immunoprecipitates prepared from cells expressing Flag-c-Abl were subjected to SDS–PAGE and then blotted onto a nitrocellulose membrane. After incubation with soluble GST-PCNA fusion protein, the nitrocellulose membrane was treated with an HRP-anti-GST antibody. The results showed that c-Abl binds to PCNA directly (Fig. 1d, top). As a control, Flag-c-Abl does not bind to GST (Fig. 1d, middle). Therefore, we conclude that PCNA binds to c-Abl.

PCNA binds the SH2 PIP box domain of c-Abl

To define the interaction domain of c-Abl with PCNA, lysates from 293T cells expressing Flag-PCNA were incubated with GST-c-Abl SH3, GST-c-Abl SH2 fusion proteins or GST. Analysis of the bound proteins by immunoblotting with anti-Flag antibody demonstrated binding of PCNA to c-Abl SH2 (Fig. 2a), which is in consistence with yeast two hybrid results (Fig. 2d). These findings confirmed a direct binding of PCNA to the SH2 domains of c-Abl. The association between PCNA and c-Abl suggests that PCNA could be a substrate for the tyrosine kinase. Flag-PCNA was coexpressed with c-Abl and whole cell lysates were immunoprecipitated with anti-Flag antibody and immunoblotted with an anti-phosphotyrosine (P-Tyr) antibody. A known c-Abl substrate, PSMA7, was used as a positive control for tyrosine kinase activity in this experiment [14]. As shown in Fig. 2b, PCNA could not be phosphorylated by c-Abl, in contradiction to the report that tyrosine kinase of EGFR controls PCNA function through its phosphorylation [15].

To further map the PCNA-binding sites in c-Abl, we analyzed the *N*-terminal SH2 PCNA binding region. The sequence, QxxL/I (residues 179–186), resembles a previously characterized PCNA binding motifs: the PIP box [16]. To evaluate the role of this motif in the interaction between c-Abl and PCNA, we either deleted the motif or mutated the residues in the motif into alanines. As shown in Fig. 2c, Myc-tagged PCNA protein interacts with the wild-type c-Abl, but not with these two c-Abl mutants. Thus, the interaction with PCNA requires the SH2 PIP box domain of the c-Abl tyrosine kinase.

PCNA-mediated c-Abl degradation involves ubiquitin of the c-Abl protein

To examine the biological function of the interaction between PCNA and c-Abl, an increasing amount of the PCNA expression plasmid was contransfected with 0.2 μg of c-Abl expression plasmid, we found that as low as 200 ng of PCNA expression plasmid could significantly reduce the protein levels of c-Abl and this reduction was proportional to the amount of cotransfected PCNA expression construct (Fig. 3a). In order to avoid the potential artifact of the transfection procedure, a control plasmid encoding an unrelated protein p21 that does not interact with c-Abl was contransfected together with c-Abl. We found that p21 expression does not down-regulate

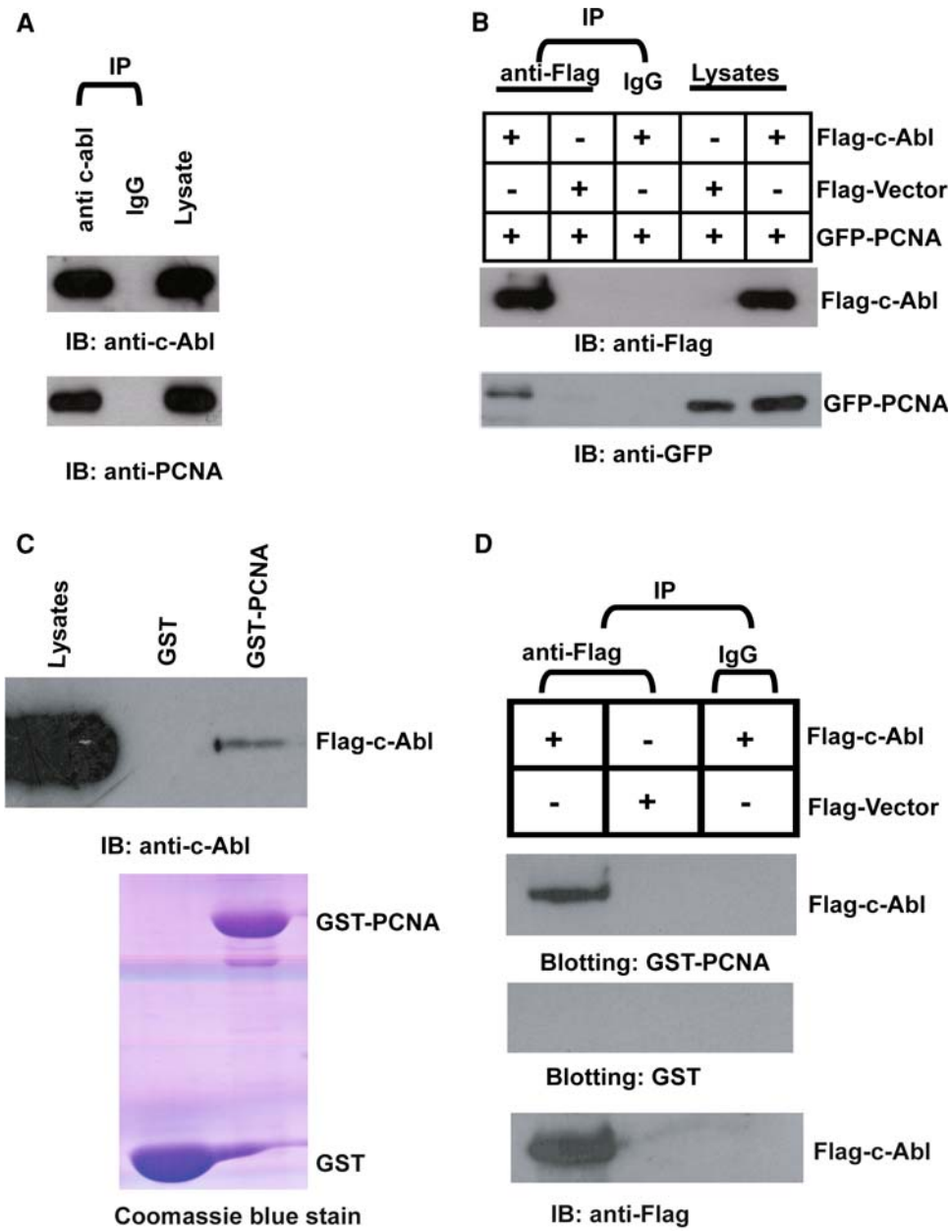

Fig. 1 PCNA Associates with c-Abl. **a** Lysates from 293T cells were subjected to immunoprecipitation with anti-c-Abl or IgG, fractionated by SDS–PAGE, and subsequently analyzed by immunoblotting with anti-PCNA antibodies. **b** 293T cells were cotransfected with Flag-c-Abl and GFP-PCNA expression plasmids or Flag-vector, and anti-Flag or IgG immunoprecipitates were analyzed by immunoblotting with anti-GFP or anti-Flag antibody. **c** Lysates from 293T cells transfected with Flag-c-Abl expressing plasmid were incubated with a GST or GST-PCNA fusion protein for 2 h. The absorbates were analyzed by immunoblotting with anti-c-Abl (*top*). Loading of the GST proteins was assessed by Coomassie blue staining (*bottom*). **d** Anti-Flag or IgG immunoprecipitates prepared from cells transfected with Flag-c-Abl or Flag-vector expressing plasmids were subjected to SDS–PAGE and blotted onto nitrocellulose membrane. The nitrocellulose membrane was incubated with soluble GST-PCNA or GST for 2 h and then analyzed with anti-GST or anti-Flag antibody

c-Abl levels as PCNA (data unshown), indicating that the c-Abl down-regulation is a specific effect elicited by PCNA. To ensure the physiological relevance of this PCNA-mediated c-Abl down-regulation, we tested the effect of PCNA on endogenous c-Abl abundance in 293T cells. As shown in Fig. 3b, expression of PCNA was associated with a marked decrease of endogenous c-Abl level. It was of interest to examine whether c-Abl PIP box deletion mutant could also be affected by PCNA over-expression. Flag-PCNA and c-Abl PIP box deletion mutant were contransfected to analyze for their response to c-Abl destabilization. In contrast to wild-type c-Abl, the abundance of mutant c-Abl was unchanged (Fig. 3c). These findings indicate that PCNA may regulate cell apoptosis to DNA damage through nuclear c-Abl downregulation.

To further delineate the mechanism of PCNA-mediated c-Abl degradation, 293T cells were cotransfected with plasmids expressing Flag-c-Abl, His-PCNA, and HA-ubiquitin together with the proteasome inhibitors MG115 (20 μM for 6 and 12 h), c-Abl was then immunoprecipitated by an anti-Flag antibody and blotted with an anti-HA antibody. As shown in Fig. 3d, PCNA led to increased steady level of c-Abl ubiquitination. These results indicate that PCNA may have a major role in c-Abl ubiquition and its proteasome-mediated degradation.

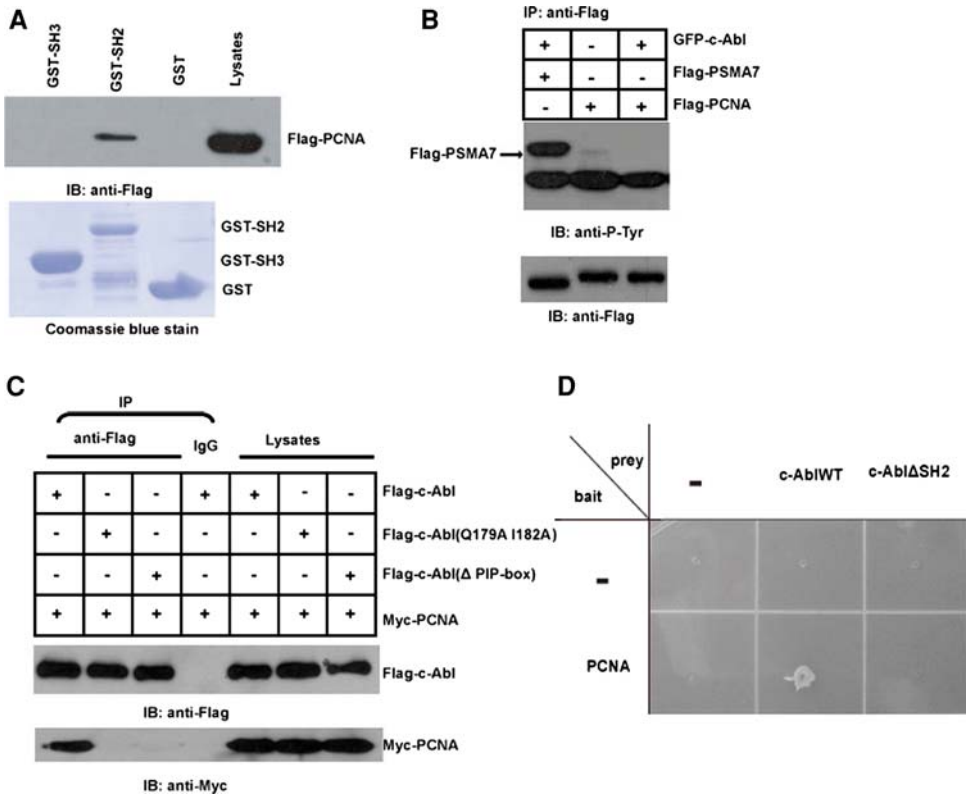

Fig. 2 PCNA binds to the SH2 PIP box domain of c-Abl. **a** 293T cells were transfected with Flag-PCNA expressing plasmid. The GST fusion protein absorbates from cell lysates were analyzed by immunoblotting with anti-Flag antibody (*top*). Loading of the GST proteins was assessed by Coomassie blue staining (*bottom*). **b** 293T cells were cotransfected with GFP-c-Abl and Flag-PCNA expression plasmids, or Flag-PSMA7, anti-Flag immunoprecipitates were analyzed by immunoblotting with anti-P-Tyr antibody. **c** 293T cells were cotransfected with Myc-PCNA expression plasmids and Flag-c-Abl, or Flag-c-Abl mutants, anti-Flag immunoprecipitates were analyzed by immunoblotting with anti-Myc or anti-Flag antibody. **d** CG1945 was cotransformed with the c-Abl WT and SH2 domain deletion c-Abl prey plasmids as indicated. Positive interaction showed colony formation on synthetic medium lacking tryptophan, leucine, adenine, and histidine

Binding of PCNA to c-Abl attenuates DNA damage-induced apoptosis

To assess the role of c-Abl–PCNA interaction in the regulation of apoptosis upon DNA damage, cells were treated with CDDP, a chemical compound that can induce DNA damage and cells with different c-Abl expression was analyzed. First, a MCF7 c-Abl knockdown (MCF7/c-Abl-kd) clone by stable transfection with c-Abl siRNA was generated. To determine whether the phenotype generated by RNA interference was due to the loss of c-Abl protein, we constructed a Flag epitope tagged c-Abl cDNA expression plasmid with a silent nucleotide substitution in the RNAi region of c-Abl cDNA that is resistant to RNA interference. MCF7, MCF7/c-Ablkd, and MCF7/c-Ablkd/WT c-Abl cells were treated with CDDP, c-Abl deficient cells showed significantly attenuated CDDP-induced apoptosis compared with MCF7 or MCF7/c-Ablkd cells rescued with WT c-Abl (Fig. 4a). To further analyze the effect of PCNA on c-Abl-induced apoptosis, apoptosis was analyzed in MCF7/c-Abl kd rescued with WT c-Abl or c-Abl mutants together with PCNA. As shown in Fig. 4b, expression of c-Abl with deletion or mutation at PIP box domain led to increased cell apoptosis compared with WT c-Abl expression. To further confirm these results, annexin V staining method was used, as was shown in Fig. 4c, c-Abl deficient cells rescued with c-Abl with deletion or mutation at PIP box domain led to increased cell apoptosis compared with WT c-Abl introduction in the presence of PCNA, while pretreatment with Gleevec of MCF7/c-Ab-lkd/WT c-Abl cells led to decreased cell apoptosis in response to DNA damage, taken together, these findings demonstrate that binding of PCNA to c-Abl and degradation of c-Abl block the apoptotic function of c-Abl in the response to CDDP induced-DNA damage.

Discussion

PCNA destabilizes c-Abl by direct binding

Nuclear c-Abl is activated by diverse genotoxic agents and induces apoptosis [17–19]. Conversely, overexpression of

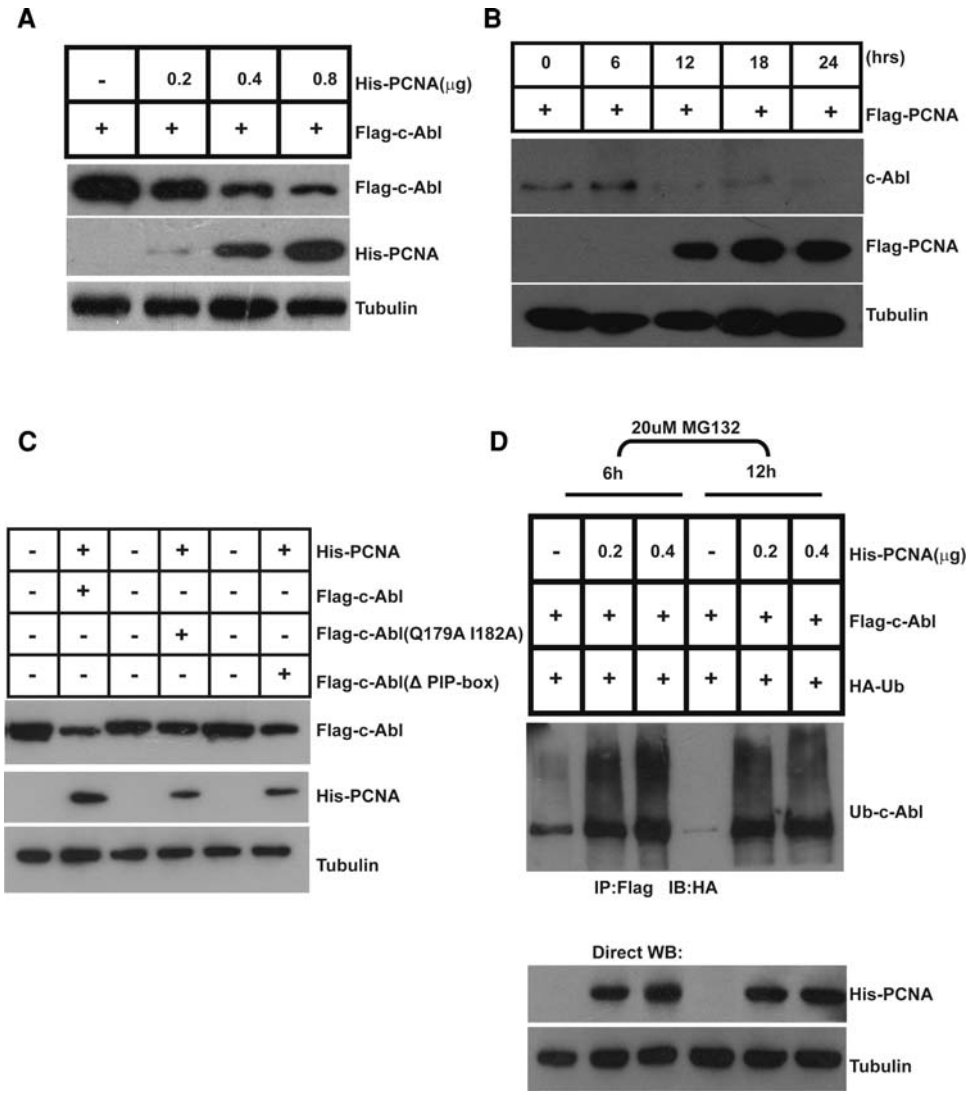

Fig. 3 PCNA promotes c-Abl ubiquitination and degradation. **a** HEK293T cells were transfected with plasmids expressing increasing amount of His-PCNA (0.2, 0.4, 0.8 μg) and 0.2 μg Flag-c-Abl. Whole cell lysates were analyzed by immunoblotting with anti-Flag or anti-His antibody, Tubulin was used as equal loading control. **b** HEK293T cells were transfected with different amounts of plasmids expressing Flag-PCNA whole cell lysates were analyzed by immunoblotting with anti-Flag or anti-c-Abl antibody, Tubulin was used as equal loading control. **c** HEK293T cells were transfected with plasmids expressing His-PCNA together with WT Flag-c-Abl or Flag-c-Abl mutants. Whole cell lysates were analyzed by immunoblotting with anti-Flag or anti-His antibody, Tubulin was used as equal loading control. **d** Increasing amounts (indicated on the *top*) of His-PCNA were cotransfected with plasmids encoding Flag-c-Abl and HA-tagged ubiquitin. Cells were grown in medium containing MG132 (20 μM) for 6 or 12 h. And anti-Flag immunoprecipitates were analyzed by immunoblotting with anti-HA antibody, whole cell lysates were subjected to immunoblotting with anti-His antibody, Tubulin was used as equal loading control

PCNA, as found in most human carcinomas, blocks the induction of apoptosis by genotoxic anticancer agents [20, 21]. The present studies demonstrate PCNA regulates proteasome-mediated c-Abl degradation by binding the Abl tyrosine kinase SH2 domain. Importantly, the present findings demonstrate that the binding of PCNA to c-Abl and degradation of c-Abl in the nucleus block the proapoptotic function of c-Abl in response to CDDP induced-DNA damage. Recent studies on the interaction of MUC1 and c-Abl suggest that MUC1 inhibits DNA damage-induced apoptosis by sequestering c-Abl in the cytoplasm [22]. The existence of a cellular Abl inhibitor has been revealed by several lines of evidence, c-Abl can be negatively regulated by its partners include Rb, Pag/Msp23, AAP1, and Abi etc [8, 9]. The present study demonstrates that PCNA promotes the targeted degradation of associated nuclear c-Abl in ubiquitin-proteasome pathway, adding another level of c-Abl regulation.

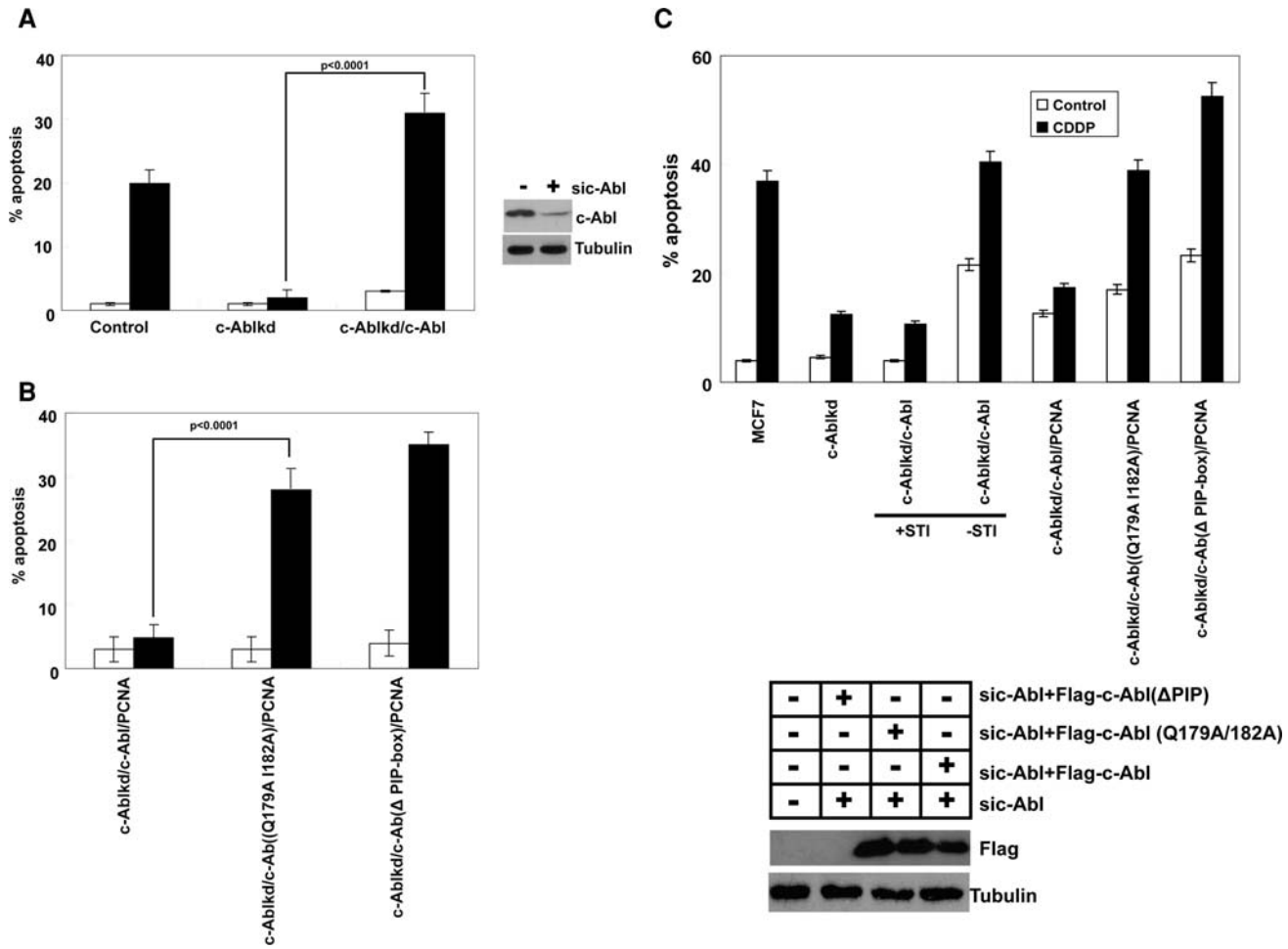

Fig. 4 Binding of PCNA to c-Abl attenuates CDDP induced apoptosis. **a** The results from sub-G1 DNA analysis of MCF7, MCF7/c-Abl kd cells, and MCF7/c-Abl kd/WT c-Abl cells treated with CDDP for 0 h (*open bars*) and 12 h (*solid bars*) were expressed as the percentage apoptosis. The results were expressed as the mean of three independent experiments. Whole cells lysates from MCF7/c-Abl kd and MCF7 cells were analyzed by immunoblotting with anti-c-Abl antibody, Tubulin was used as equal loading control. **b** The results from sub-G1 DNA analysis of MCF7/c-Abl kd/WT c-Abl, MCF7/c-Abl kd/c-Abl (PIP box deletion) mutant and MCF7/c-Abl kd/c-Abl (Q179A/182A) cells together with PCNA expression plasmids treated with CDDP for 0 h (*open bars*), 12 h (*solid bars*) or 24 h (*hatched bars*) were expressed as the percentage apoptosis. The results were expressed as the mean of three independent experiments. **c** The results from Annexin V staining analysis of MCF7, MCF7/c-Abl kd cells, MCF7/c-Abl kd/WT c-Abl, MCF7/c-Abl kd/c-Abl (PIP box deletion) mutant and MCF7/c-Abl kd/c-Abl (Q179A/182A) cells treated with CDDP for 0 h (*open bars*) and 12 h (*solid bars*) were expressed as the percentage apoptosis. The results were expressed as the mean of three independent experiments. Whole cells lysates from MCF7/c-Abl kd, MCF7/c-Abl kd/WT c-Abl, MCF7/c-Abl kd/c-Abl (PIP box deletion) mutant and MCF7/c-Abl kd/c-Abl (Q179A/182A) cells were analyzed by immunoblotting with anti-Flag antibody, Tubulin was used as equal loading control

PCNA inhibits c-Abl-induced apoptosis upon DNA damage

PCNA protects genomic integrity and facilitates the repair of damaged DNA induced by a variety of genotoxic agents. For example, low dose ultraviolet irradiation leads to p21 degradation and PCNA accumulation, which promotes DNA repair. Here we provide the molecular mechanism of PCNA function and the physiological significance of the interaction between PCNA and c-Abl: PCNA promotes DNA repair by blocking DNA damage-induced apoptosis through down-regulation of nuclear c-Abl. In this context, the apoptotic function of c-Abl is enhanced by c-Abl PIP box mutation that is responsible for PCNA–c-Abl interaction and PCNA-mediated c-Abl degradation. PCNA abundance changes in response to cell stress, and inhibition of c-Abl pathway may be integrated with PCNA1 as a mechanism for attenuating signals that induce an apoptotic response.

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