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Human Proliferating Cell Nuclear Antigen, Poly(ADP-ribose) Polymerase-1, and p21*waf1/cip1*

A DYNAMIC EXCHANGE OF PARTNERS*

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We addressed the analysis of the physical and functional association of proliferating cell nuclear antigen (PCNA), a protein involved in many DNA transactions, with poly(ADP-ribose) polymerase (PARP-1), an enzyme that plays a crucial role in DNA repair and interacts with many DNA replication/repair factors. We demonstrated that PARP-1 and PCNA co-immunoprecipitated both from the soluble and the DNA-bound fraction isolated from S-phase-synchronized HeLa cells. Immunoprecipitation experiments with purified proteins further confirmed a physical association between PARP-1 and PCNA. To investigate the effect of this association on PARP-1 activity, an assay based on the incorporation of radioactive NAD was performed. Conversely, the effect of PARP-1 on PCNA-dependent DNA synthesis was assessed by a DNA polymerase δ assay. A marked inhibition of both reactions was found. Unexpectedly, PARP-1 activity also decreased in the presence of p21*waf1/cip1*. By pull-down experiments, we provided the first evidence for an association between PARP-1 and p21, which involves the C-terminal part of p21 protein. This association was further demonstrated to occur also *in vivo* in MNNG (*N*-methyl-*N*′-nitro-*N*-nitrosoguanidine)-treated human fibroblasts. These observations suggest that PARP-1 and p21 could cooperate in regulating the functions of PCNA during DNA replication/repair.

Poly(ADP-ribose) polymerase-1 (PARP-1)[1] is a DNA-nick sensor protein that uses β-NAD^+ as a substrate for transferring ADP-ribose moieties to itself and to nuclear acceptor proteins (1). PARP-1 modulates the structure and function of many proteins involved in DNA metabolism (2, 3), co-purifies with some members of the DNA synthesome (4–6), and is a component of replication-competent complexes (7). It has been shown previously that PARP-1 co-immunoprecipitates with the proliferating cell nuclear antigen (PCNA) (4, 5), which is a pivotal protein in DNA replication, DNA repair, and cell cycle control. A number of proteins involved in DNA replication and repair interact with PCNA (reviewed in Refs. 8–11). Most of the PCNA-interacting proteins have a *QXX*(h)*XX*(a)(a) box that specifically binds the interdomain connector loop (12). PARP-1 shows a putative PCNA-binding consensus sequence (QDLIKMIF) at position 669 within the NAD-binding domain that is essential for the conversion of NAD into ADP-ribose and, consequently, for PARP-1 catalytic activity.

In this work we sought to attain a greater understanding of the interaction between PARP-1 and PCNA. We have demonstrated that PARP-1 and PCNA co-immunoprecipitate both from the soluble and the DNA-bound fraction isolated from S-phase-synchronized HeLa cells. These results were supported by immunoprecipitation experiments with purified proteins. To investigate the effect of this association on the properties of each protein, we evaluated the conversion of NAD into ADP-ribose (PARP assay) as well as PCNA-dependent nucleotide incorporation (pol δ assay), and we found a marked inhibition of both reactions. Unexpectedly, an inhibitory effect on PARP-1 activity was also noted in the presence of p21*waf1/cip1*. By pull-down experiments either with a mAb against PCNA or with p21-GST, we provided the first evidence for an association between PARP-1 and the C-terminal part of p21, *i.e.* the same region involved in binding to PCNA. Remarkably, the *in vivo* association between PARP-1 and p21 was further demonstrated by co-immunoprecipitation experiments on human fibroblasts treated with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG).

EXPERIMENTAL PROCEDURES

Cell Culture and Treatments—HeLa-S3 cells and primary cultures of human embryonic lung fibroblasts were grown as a monolayer in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum (HyClone), 4 mM glutamine, 2 mM sodium pyruvate, 100 units/ml penicillin, and 0.1 mg/ml streptomycin (all from Invitrogen). Cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2 and trypsinized when confluent. HeLa cells were synchronized in S-phase by a double thymidine treatment (7) followed by a further growth in complete medium for 3 h. High salt (0.35 M NaCl) extracts, formaldehyde cross-linking *in vivo*, and DNA-protein complexes were obtained as reported previously (7). In some experiments, HeLa cells were incubated for 3 h with 100 μM MNNG (Sigma) or for 12 h with 1 mM 3-aminobenzamide (Sigma), washed twice with phosphate-buffered saline, and used for further analyses. Fibroblasts were treated for 1 h with 5 μM MNNG and incubated further in drug-free medium for 4 or 8 h. Protein content was evaluated by the Bradford reagent (Bio-Rad).

Immunoprecipitation Experiments—Aliquots of 1×10^7 HeLa cells were lysed for 10 min in ice with 1 ml of hypotonic lysis buffer (50 mM Tris HCl, pH 7.4, 2.5 mM $MgCl_2$, 0.5% Nonidet P-40, 1 mM PMSF, 0.2 mM Na_3VO_4, 0.5 μM okadaic acid, and 150 μl of a protease inhibitor mixture (Sigma) (13). Samples were then centrifuged for 1 min at 4,500 rpm at 4 °C, and the supernatant containing the soluble fraction was kept apart. Pelleted nuclei were washed in isotonic buffer (10 mM Tris

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[1] The abbreviations used are: PARP-1, poly(ADP-ribose) polymerase-1; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; CTE, C-terminal peptide; GST, glutathione *S*-transferase; IP, immunoprecipitate; LEI, leukocyte elastase inhibitor; mAb, monoclonal antibody; MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; PCNA, proliferating cell nuclear antigen; rPCNA, recombinant PCNA; p21, p21*waf1/cip1*; PMSF, phenylmethylsulfonyl fluoride; pol δ, DNA polymerase δ.

HCl, pH 7.4, 150 mM NaCl) and then incubated for 20 min at 37 °C in 0.5 ml of digestion buffer containing 10 mM Tris HCl, pH 7.4, 10 mM NaCl, 5 mM $MgCl_2$, 0.2 mM PMSF, and 200–400 units of DNase I (Sigma) (14). Nuclei were pelleted at 14,000 rpm, and the supernatant, containing the DNA-released material, was taken. For immunoprecipitation, equal amounts of soluble and DNA-bound fractions were incubated for 1 h at room temperature and then overnight at 4 °C, with 5 μg of the mAb PC10 to PCNA (Dako) or F1–23 to PARP-1 (Alexis, Vinci Biochem). As a negative control, IP was performed with anti-mouse IgG (Jackson ImmunoResearch). After adding protein A-Sepharose (Amersham Biosciences; 600 μg in 0.1 M potassium phosphate buffer, pH 8.0), samples were incubated for 1 h at room temperature under agitation, centrifuged at 13,000 rpm, washed, and finally analyzed by SDS-PAGE and Western blot. Experiments of *in vivo* interaction among p21, PARP, and PCNA were performed on human fibroblasts treated for 1 h with 5 μM MNNG and incubated further in drug-free medium for 4 or 8 h. Detergent-soluble and chromatin-bound fractions were prepared from aliquots of 1×10^7 cells as above described. Samples (1 mg each) were incubated for 1 h at room temperature and then overnight at 4 °C with a mixture (2 μg each) of C-19 and N-20 rabbit polyclonal antibodies to p21 (Santa Cruz Biotechnology), previously bound to protein A-Sepharose. As a negative control, rabbit IgG (Dako) were used. After three washings in lysis buffer, the immunoprecipitated peptides were resolved by SDS-PAGE. Immunoblot analysis was then performed with the following antibodies: C-2-10 to PARP-1, C-19 to p21, and PC10 to PCNA (see below).

Pull-down Experiments—PARP-1 (60 ng), PCNA (120 ng) and p21 CTE (C-terminal peptide)-GST fusion protein (15) (25 ng) were incubated in IP buffer (10 mM Tris HCl, pH 7.4, 150 mM NaCl, 2 mM PMSF, protease inhibitor mixture) in different combinations for 2 h at 4 °C. Then, 4 μg of the mAb PC10 or anti-mouse IgG (used as isotype control, Jackson ImmunoResearch) were added, and tubes were rotated gently overnight at 4 °C. Samples were then incubated for 1 h at 4 °C after the addition of 2 mg of protein A-Sepharose. Pull-down experiments were also performed by using the recombinant p21CTE protein bound to glutathione-Sepharose (16). PARP-1 (60 ng) and/or PCNA (120 ng) were preincubated in IP buffer for 2 h at 4 °C. After the addition of 20 μl of p21-GST, samples were incubated for 30 min at 4 °C. After three washings, beads were analyzed by Western blot.

Western Blot Analysis—Samples were electrophoresed in a minigel and transferred onto a nitrocellulose filter (Bio-Rad) (17). After saturation for 1 h with PTN (phosphate-buffered saline containing 0.2% Tween 20 and 10% newborn calf serum), the membrane was incubated overnight with the following mAbs: C-2-10 to PARP-1 (Alexis), diluted 1:2500 in PTN; PC10 to PCNA (Dako), diluted 1:1000; 187 to p21 (Santa Cruz Biotechnology), diluted 1:100; AE-4 to histone H1 (Santa Cruz), diluted 1:1000. The membrane was washed and incubated for 1 h with horseradish peroxidase-conjugated anti-mouse IgG (Sigma). Visualization of immunoreactive polypeptides was obtained by the ECL system (Sigma).

pol δ Assay—In a final volume of 25 μl, the reaction mixture contained 250 mM bis-Tris, pH 6.6, 5 mM dithiothreitol, 20% glycerol, 1 mg/ml bovine serum albumin, 10 μM [^{3}H]dTTP (Amersham Biosciences, 1.5 Ci/mmol), 10 mM $MgCl_2$, 0.25 μg of poly(dA)/oligo(dT) (10:1 base ratio), 0.05 units of pol δ (corresponding to ~150 ng of protein), and 30–60 ng of rPCNA (15). Recombinant PARP-1 (Alexis; specific activity 6 units/μl; 30–240 ng) was preincubated with PCNA for 5 min at room temperature. After a reaction at 37 °C for 30 min, the precipitated material was collected on Whatman GF/C filters, and radioactivity was quantitated by liquid scintillation counting. Three independent experiments were carried out in duplicate.

PARP Assay—In a final volume of 50 μl, the reaction mixture contained 100 mM Tris HCl, pH 8.0, 1 mM dithiothreitol, 10 mM $MgCl_2$, 100 μg/ml of activated DNA, 100 μM NAD^+, 15 μCi of ^{32}P-NAD^+ (PerkinElmer Life Sciences, 800 Ci/mmol), rPARP-1, and 6 μg of either rPCNA or p21. In some experiments, to rule out possible aspecific effects, 6 μg of recombinant leukocyte elastase inhibitor (LEI, kindly provided by Dr. A. Torriglia) was used. The reaction was carried on for 10 min at 25 °C. Aliquots of 20 μl were processed for the evaluation of acid-insoluble radioactivity. Three independent experiments were carried out in duplicate.

RESULTS

Co-immunoprecipitation of PARP-1 and PCNA—PCNA is present in the cell as a free/detergent-soluble form, and as a DNA-bound/detergent-insoluble form associated to the replication forks during S-phase. To enrich the DNA-bound fraction, which normally accounts for a low percentage of the total PCNA, we synchronized HeLa cells in the S-phase and isolated two cellular fractions containing either soluble or DNA-bound/insoluble proteins (13, 14). DNA-associated proteins were further released by DNase I, and PCNA was immunoprecipitated from both fractions with the specific mAb PC10. Western blot analysis of immunoprecipitated samples (*IP*) revealed the presence of both PCNA and PARP-1 in the soluble fraction (*S*) and, to a lesser extent, in the insoluble fraction (*I*) (Fig. 1*A*). To address whether the association between PCNA and DNA could be mediated by poly(ADP-ribose), we treated the cells with the PARP-1 inhibitor, 3-aminobenzamide, and found an amount of immunoprecipitated DNA-bound PCNA similar to that of untreated cells (Fig. 1*B*). To investigate whether the association of PARP-1 and PCNA occurred when the two proteins were in a chromatin-bound status, we released bound proteins with a high salt treatment. PARP-1 and PCNA were co-immunoprecipitated by both the anti-PCNA and the anti-PARP-1 antibody (Fig. 1*C*) and were also detectable in a cross-linked DNA-protein complex (Fig. 1D). Interestingly, both proteins were cross-linked more efficiently in MNNG-treated cells (Fig. 1*D*), an effect that cannot be ascribed to the general behavior of nuclear proteins as demonstrated by the levels of histone H1 (Fig. 1*D*). These results could suggest an active role of PARP-1 and PCNA in DNA repair induced by alkylating agents. In fact, this type of DNA damage triggers the recruitment of PCNA from a soluble to an insoluble chromatin-bound complex (18) and is a potent activator of poly(ADP-ribosylation) (19).

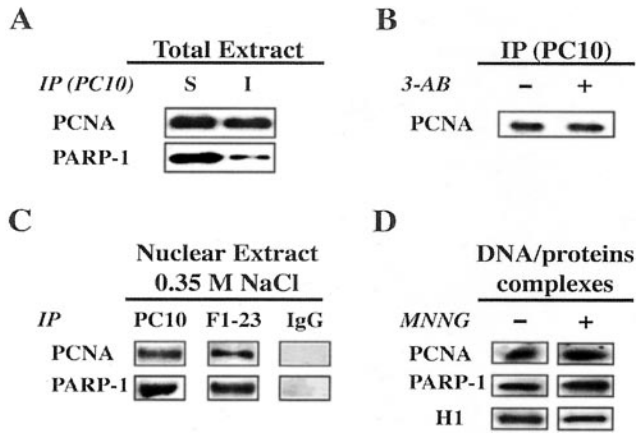

FIG. 1. **Co-immunoprecipitation of PARP-1 and PCNA from S-phase-synchronized HeLa cells.** *A*, PARP-1 and PCNA in the soluble (*S*) and insoluble (*I*) fractions after immunoprecipitation with the mAb PC10 to PCNA. *B*, PCNA after immunoprecipitation from the insoluble fraction of cells treated with 3-aminobenzamide (*3-AB*). *C*, PCNA and PARP-1 after immunoprecipitation from 0.35 M NaCl nuclear extracts by either PC10 (to PCNA) or F1–23 (to PARP-1). *D*, PARP-1 and PCNA in DNA-protein complexes from control and MNNG-treated cells. As a negative control, immunoprecipitation was performed with anti-mouse IgG. Immunoblot with anti-histone H1 was used as a loading control. A typical result is shown.

PARP-1 Inhibits pol δ Activity—To investigate whether the association between PARP-1 and PCNA affects the activity of PCNA as a co-factor of pol δ, we performed a PCNA-dependent DNA synthesis assay after pre-incubating PARP-1 and PCNA at different concentrations. Fig. 2*A* shows the results of a typical experiment. With an excess of PCNA (PARP-1:PCNA molar ratio, 1:6 or 1:3), PARP-1 did not affect pol δ activity. However, a higher PARP-1 amount, up to 240 ng, caused a net inhibition of pol δ activity, thus suggesting that an excess of PARP-1 allowed the sequestration of most PCNA molecules, with a consequent impairment of pol δ processivity. Indeed, the Dixon plot analysis of the results of three independent experiments carried out with increasing amounts of both PARP-1

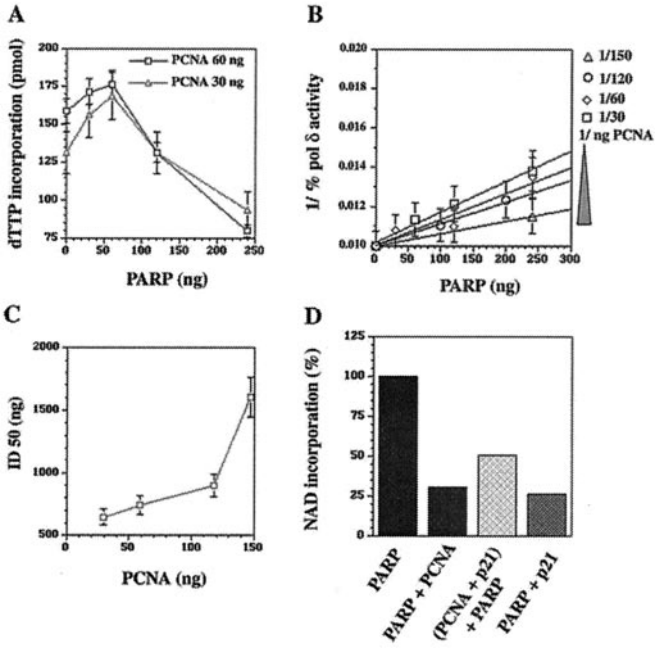

FIG. 2. **The association between PARP-1 and PCNA inhibits PCNA-dependent DNA synthesis and NAD incorporation.** *A*, pol δ assay was carried out using 30 or 60 ng of PCNA in the presence of increasing amounts of PARP-1 (30–240 ng). *B*, Dixon plot analysis using of pol δ activity using increasing amounts of PCNA (30–150 ng) and PARP-1 (30–240 ng). *C*, ID_{50} of PARP-1 on pol δ activity. *D*, PARP-1 assay. Proteins were incubated for 5 min at 4 °C in different combinations, and then ^{32}P-NAD incorporation was measured. The results of a typical experiment of three are shown. Data shown in *A–C* are expressed as the mean ± S.D. from three independent experiments.

(30–240 ng) and PCNA (30–150 ng) clearly showed that the inhibition of pol δ activity by PARP-1 is dependent strictly on PCNA concentration (Fig. 2*B*). This effect is more clearly illustrated in Fig. 2*C*, where the ID_{50} of PARP on pol δ activity was expressed as a function of PCNA concentration. The data show that for increasing PCNA concentration, an increasing amount of PARP-1 is required to inhibit PCNA-dependent DNA synthesis, possibly because of a physical association between the proteins.

PCNA and p21 Inhibit PARP-1 Activity—The interaction between PARP-1 and PCNA is likely to occur through a short peptide motif, which is typically present within the sequence of PCNA partners (12). Indeed, inspection of the PARP-1 sequence reveals a putative PCNA-binding consensus sequence at the position 669, *i.e.* within the NAD-binding domain, that is essential for PARP-1 activity. Thus, one would expect that binding of PCNA to PARP-1 in this region would affect the catalytic activity of PARP-1. To test this hypothesis, we measured the conversion of ^{32}P-NAD, *i.e.* the substrate for the poly(ADP-ribosylation) reaction, into ^{32}P-poly(ADP-ribose). As illustrated in Fig. 2*D*, radioactive NAD incorporation decreased by 4-fold when rPCNA was added to the reaction mixture. To confirm the physical association between PARP-1 and PCNA, we attempted to sequester PCNA by adding the C-terminal peptide of p21 (p21CTE), which is known to interact with a region of the PCNA molecule representing a shared binding site for many PCNA partners. Surprisingly, under these conditions, the PCNA-dependent decrease in NAD incorporation was not fully prevented. A possible direct effect of p21 on PARP-1 was then tested by adding p21CTE to the PARP assay. Under these conditions, we found that p21 inhibits PARP-1 activity to the same extent as PCNA, thus suggesting a possible interaction of p21CTE and PARP-1 (Fig. 2*D*). To rule out a nonspecific inhibitory effect of any recombinant protein on

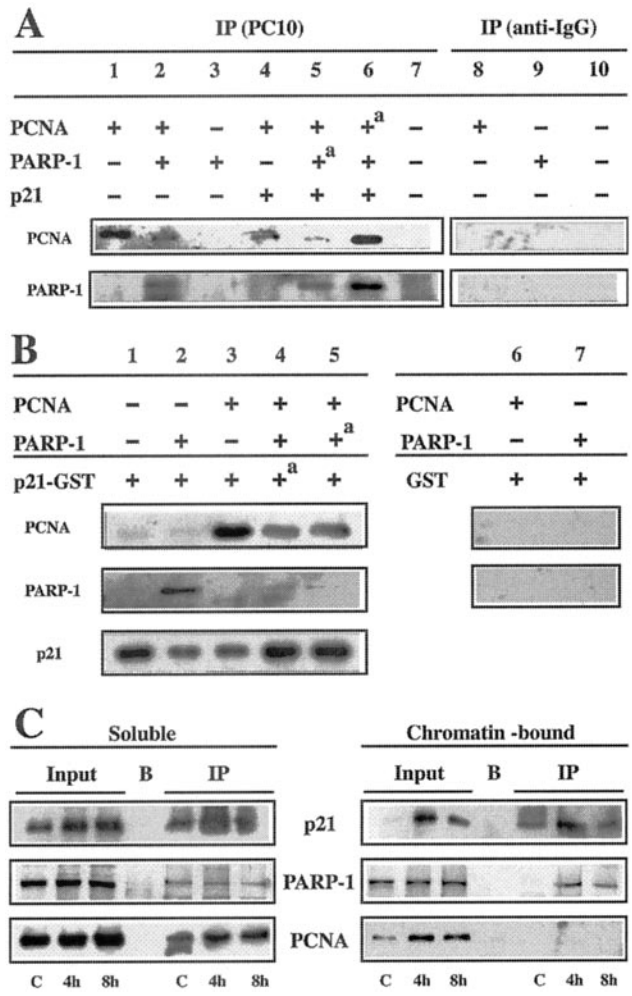

FIG. 3. **Pull-down of PCNA, PARP-1, and p21CTE and co-immunoprecipitation of p21 and PARP-1 from human fibroblasts.** Recombinant proteins were incubated as described under "Experimental Procedures." The *a* denotes the last protein added. Immunoprecipitation was carried out by using either the mAb PC10 (*A*) or p21CTE-GST (*B*). The IP was analyzed for PCNA, PARP-1, and p21. As a negative control, IP was performed with anti-mouse IgG (*A*) or GST (*B*). A typical result is shown. *C,* soluble and chromatin-bound fractions (*Input*) were isolated from control fibroblasts (*lane C*), and MNNG-treated fibroblasts (*4 h* and *8 h*) and used to immunoprecipitate p21. Each IP was analyzed for the presence of p21, PARP-1, and PCNA. The input corresponds to 40 μg of extract before immunoprecipitation. As a negative control, immunoprecipitation was performed with anti-rabbit IgG (*B*). A typical result is shown.

PARP-1 activity, we carried out the assay in the presence of the recombinant protein LEI and found that PARP-1 activity was not decreased (not shown).

p21 Associates with PARP-1—The finding of a direct effect of p21 on PARP-1 activity prompted us to perform pull-down experiments with recombinant proteins. A typical Western blot of the IP is shown in Fig. 3*A*, where *lane 1* represents the positive control of immunoprecipitated PCNA. Its amount was greatly reduced by the presence of PARP-1 (*lane 2*) or p21 (*lane 4*). A similar result was obtained by adding PARP-1 to PCNA bound to p21 (*lane 5*). However, when PARP-1 and p21 were first preincubated, the amount of immunoprecipitated PCNA was very high (*lane 6*). These data suggest that the association of PARP-1 with p21 makes PCNA free to react with the mAb PC10. On the contrary, the binding of PCNA to one partner (either PARP-1 or p21) possibly masks the epitope recognized by PC10. Indeed, p21 binds PCNA through residues 119–133 (20, 21), which overlap the region spanning residues 111–125,

representing the epitope that reacts with PC10 (22). The immunodetection of PARP-1 in the IP provided results consistent with those obtained for PCNA (Fig. 3*A*). To confirm the interaction between p21 and PARP-1, we performed pull-down experiments with p21CTE-GST. As illustrated in Fig. 3*B*, the incubation of either PARP-1 (*lane 2*) or PCNA (*lane 3*) with p21CTE-GST resulted in the pull-down of the proteins. When the three proteins were present, the levels of pulled down PARP-1 and PCNA were reduced, as shown for *lanes 4* and *5*. The decrease occurred to a similar extent when PARP-1 and PCNA (*lane 4*) or PCNA and p21 (*lane 5*) were first preincubated. These results further support the finding that p21 and PARP-1 associate and that they compete for the interaction with PCNA.

To investigate the existence of an *in vivo* interaction between p21 and PARP-1, we immunoprecipitated p21 from total extracts prepared from human fibroblasts and found that PARP-1 was co-immunoprecipitated with p21. Moreover, the amount of co-immunoprecipitated p21 and PARP-1 was higher in fibroblasts treated with MNNG than in untreated control cells (not shown). These results prompted us to repeat the experiments on isolated detergent-soluble and chromatin-bound fractions from control and MNNG-treated fibroblasts. As shown in Fig. 3*C*, the Western blot analysis of p21 revealed the presence of the protein in samples before (*Input*) and after immunoprecipitation (*IP*), thus attesting to the specificity of the applied procedure. The amount of p21 was higher in MNNG-treated cells, where it could be actively involved in DNA repair (23). As expected, PARP-1 was detected in the input of both fractions, and it co-immunoprecipitated with p21, thus confirming the *in vivo* interaction between these proteins. Based on the amount of PARP-1 that is present in the lysate from each fraction (input) compared with the respective IP, we estimate that p21-interacting PARP-1 represents no more than 5% of the soluble fraction and accounts for 20–25% of the chromatin-bound counterpart. That the interaction occurs mainly in MNNG-treated cells suggests that it could have a functional role in DNA repair. Remarkably, PCNA was not co-immunoprecipitated with p21 in the chromatin-bound fraction, although it was in the soluble fraction. Because chromatin-bound PCNA is the form actively involved in DNA repair, our results suggest that the association of chromatin-bound p21 with PARP-1 may avoid an untimely interaction of p21 with PCNA (14), thus enabling PCNA to be freely recruited to DNA repair sites.

DISCUSSION

PARP-1 interacts with and modifies a number of proteins, thereby regulating their activity. However, little is known about a possible direct association between PARP-1 and its partners. The association of PARP-1 with nuclear proteins could be mediated by the noncovalent binding of poly(ADP-ribose) through a poly(ADP-ribose)-binding domain (24), which has not been found in the PCNA sequence but is present at the C terminus of $p21^{waf1/cip1}$ (24), where it coincides with the residues involved in the binding to the interdomain connector loop of PCNA (20, 21).

We aimed at elucidating the functional role of the physical interaction of PARP-1 and PCNA, which was previously suggested by their co-immunoprecipitation from replication complexes (4, 5). In the present study, we demonstrated the following. (i) PARP-1 associates *in vitro* and *in vivo* with PCNA in HeLa cells; the increase in the DNA-bound form of both PARP-1 and PCNA observed in MNNG-treated cells suggests a role for this association in DNA metabolism. (ii) PARP-1 affects PCNA-dependent pol δ activity *in vitro*, in agreement with previous data suggesting a binding of PARP-1 to DNA free ends of pol δ substrate (3). Because the physical association of PARP-1 and PCNA is concentration-dependent, and an excess of PARP-1 allows the sequestration of PCNA (its interaction with pol δ is essential for processive DNA synthesis), we propose an alternative explanation, *i.e.* that the region of PCNA involved in the association with PARP-1 overlaps with the pol δ-interacting domain. In this view, PARP-1 could be a negative regulator of PCNA-dependent pol δ activity when replicative DNA synthesis has to be inhibited, *e.g.* under damage conditions. (iii) The inhibitory effect of PCNA on PARP-1 activity suggests that a physical interaction occurs through a putative PCNA-binding sequence located within the catalytic domain of PARP-1. In this respect, PCNA could act as a negative regulator of PARP-1.

Unexpectedly, we found that p21CTE, which corresponds to the PCNA-binding portion of p21, interacts directly with PARP-1, and that this interaction occurs also *in vivo*. This observation is new and intriguing. The C-terminal domain of p21 associates with the interdomain connector loop of PCNA and inhibits pol δ-catalyzed DNA synthesis *in vitro* (25). As suggested by the co-immunoprecipitation data, PARP-1 could compete with the C-terminal portion of p21 in binding the interdomain connector loop of PCNA. Competition between pol δ and p21 for PCNA binding has been proposed to regulate the differential inhibition of DNA replication *versus* DNA repair. Our observations that DNA binding of p21, PCNA, and PARP-1 is increased upon DNA damage, together with the finding that PARP-1 binds both PCNA and p21, might suggest a role for these interactions in coordinating the cell response to DNA damage. Thus, PARP-1 and p21 could cooperate in regulating PCNA functions during DNA replication/repair.

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