

CCCTC-binding Factor Activates PARP-1 Affecting DNA Methylation Machinery^{*S}

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Our previous data have shown that in L929 mouse fibroblasts the control of methylation pattern depends in part on poly(ADP-ribose)ylation and that ADP-ribose polymers (PARs), both present on poly(ADP-ribose)ylated PARP-1 and/or protein-free, have an inhibitory effect on Dnmt1 activity. Here we show that transient ectopic overexpression of CCCTC-binding factor (CTCF) induces PAR accumulation, PARP-1, and CTCF poly(ADP-ribose)ylation in the same mouse fibroblasts. The persistence in time of a high PAR level affects the DNA methylation machinery; the DNA methyltransferase activity is inhibited with consequences for the methylation state of genome, which becomes diffusely hypomethylated affecting centromeric minor satellite and B1 DNA repeats. *In vitro* data show that CTCF is able to activate PARP-1 automodification even in the absence of nicked DNA. Our new finding that CTCF is able *per se* to activate PARP-1 automodification *in vitro* is of great interest as so far a burst of poly(ADP-ribose)ylated PARP-1 has generally been found following introduction of DNA strand breaks. CTCF is unable to inhibit DNMT1 activity, whereas poly(ADP-ribose)ylated PARP-1 plays this inhibitory role. These data suggest that CTCF is involved in the cross-talk between poly(ADP-ribose)ylation and DNA methylation and underscore the importance of a rapid reversal of PARP activity, as DNA methylation pattern is responsible for an important epigenetic code.

Over the past decade our laboratory accumulated evidence that links poly(ADP-ribose)ylation with DNA methylation. A series of different experimental strategies suggests that blockage of poly(ADP-ribose)ylation induces *in vivo* DNA hypermethylation. Previous data showed that inhibition of PARP⁶ activity introduces an

anomalous hypermethylated pattern in genomic DNA (1, 2) and in some CpG island regions (3), suggesting that in the absence of ADP-ribose polymers some DNA regions are no longer protected from methylation. Further experiments showed that PARP activity can also affect the methylation pattern of transfected foreign DNA (4). The combined results of these different experimental approaches allowed us to propose the first method to induce DNA hypermethylation *in vivo* by treatment of cells in culture with PARP activity inhibitors (5) and to study by atomic force microscopy the effect of the addition of new methyl groups to DNA on chromatin structure *in vivo* (6).

To provide an explanation for how ADP-ribose polymers control and/or protect DNA methylation patterns, several experimental approaches were used. A mechanism has been suggested in which PARP-1 in its poly(ADP-ribose)ylated isoform makes DNMT1 catalytically inactive and, thus, inefficient in DNA methylation (7). In this model modified PARP-1 is considered as a molecular adaptor of high negative charge onto which chromatin proteins can be attracted and hosted (8). Several proteins show a greater affinity for ADP-ribose polymers than for DNA (9), so that these polymers compete with DNA for binding of these proteins (10). This noncovalent link, which is very strong (11), is not specifically guided by an attraction between charges; proteins showing high affinity for ADP-ribose polymers have an amino acid domain that is responsible for the interaction with these polymers (12). Deeper analysis demonstrated that the affinity of the noncovalent PAR interactions with specific binding proteins depends on the PAR chain length (13).

Dnmt1 possesses two possible consensus amino acid domains for binding with ADP-ribose polymers and shows a very strong affinity for PARs (7). *In vitro* experiments have shown that the interaction between DNMT1 and ADP-ribose polymers is stable even in the presence of a 30-fold excess of double strand DNA. Furthermore, the ADP-ribose polymers on automodified PARP-1 almost completely inhibit the catalytic activity of DNMT1, whereas unmodified PARP-1 is not able to inhibit the enzyme. These data, taken together with the facts that PARP-1 and DNMT1 co-immunoprecipitate *in vivo* and that in this complex PARP-1 is in its modified form (7), suggest that modified PARP-1, trapping DNMT1 through the ADP-ribose polymers, is responsible for the catalytic inactivation of the enzyme in chromatin. This mechanism could be responsible for

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⁶ The abbreviations used are: PARP, poly(ADP-ribose) polymerase; CTCF, CCCTC-binding factor; PAR, poly(ADP-ribose); Dnmt1, DNA methyltransferase 1; 5-AZA, 5-azacytidine; IP, immunoprecipitation; Co-IP, co-immunoprecipitation; Ab, antibody; PARG, poly(ADP-ribose) glycohydrolase.

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the protection of the nonmethylated state of CpG islands. In such a scenario, the lack of proper (ADP-ribosylation) may lead to new aberrant methylation; in other words, inhibition of PARP activity could allow new methyl groups to be inserted onto DNA. An additional mechanism involved in the functional interplay between PARP and DNA methylation is implied by the observation that inhibition of PARP activity increases both mRNA and protein levels of Dnmt1, the major maintenance methyltransferase, at the G₁/S phase border, anomalously increasing the formation of the active complex PCNA-Dnmt1 (14).

To establish whether poly(ADP-ribosylation) by itself is involved in the regulation of the promoter region of *Dnmt1* gene or of another gene whose product is, in turn, involved in the regulation of *Dnmt1* expression, we looked at transcription factors that undergo covalent poly(ADP-ribosylation) (15). CTCF, the highly conserved and ubiquitously expressed nuclear factor (16), attracted our attention as this protein, which is one of the major players in imprinting and insulator processes (17), brings together the following two epigenetic events in which we are interested: poly(ADP-ribosylation) and DNA methylation. As a chromatin insulator, CTCF links specific consensus sequences located in imprinting control regions. Importantly, it is able to link these regions only if they are unmethylated; furthermore, CTCF binding protects them from *de novo* methylation (18–20). It has recently been shown that CTCF, in the control of imprinting, binds the consensus sequences in its covalently poly(ADP-ribosylated) form (21, 22) (the ADP-ribose polymers, present on the N-terminal region of CTCF, increase its molecular mass from 130 to 180 kDa). The functional importance of poly(ADP-ribosylation) in the control of imprinting has been shown by experiments in which treatment of cells with 3-aminobenzamide, a competitive inhibitor of PARP activity, affects insulator function of most of the CTCF target sites. Further studies (23) indicate that following ectopic overexpression of full-length CTCF, the 180-kDa CTCF poly(ADP-ribosylated) form localizes to the nucleolus; the use of 3-aminobenzamide suggests that translocation of CTCF to the nucleolus is dependent on poly(ADP-ribosylation), and it is the epigenetic event involved in the control of transcription from rDNA in the nucleolus. The N-terminal domain of CTCF, which is the one that undergoes poly(ADP-ribosylation), seems to be necessary in determining the severe inhibition of cell proliferation and clonogenicity observed following ectopic overexpression of CTCF (24).

Our present data provide, for the first time, evidence that CTCF is involved in the cross-talk between poly(ADP-ribosylation) and DNA methylation. Here we show by transient ectopic overexpression of CTCF and *in vitro* experiments the following. (a) PAR accumulation is increased without higher *Parp-1* and *Parp-2* mRNA and protein expression. (b) PARP-1 and CTCF become poly(ADP-ribosylated). (c) CTCF is capable *per se* of activating PARP-1 automodification. (d) CTCF interacts with PARP-1 both *in vivo* and *in vitro*. (e) DNA methyltransferase activity is inhibited without down-regulation of *Dnmt1* mRNA and/or decrease of its nuclear level. (f) Genomic DNA is diffusely hypomethylated, and severe widespread hypomethylation characterizes both centromeric and B1 repetitive DNA

sequences. (g) Overexpression of CTCF in PARP-1^{-/-} cells shows direct involvement of PARP-1 in CTCF-induced PAR synthesis. As CTCF does not affect *per se* the activity of DNMT1, these data altogether confirm the important role played by PARs on DNMT1 activity (7).

EXPERIMENTAL PROCEDURES

Cell Culture, Treatments, and Transfection—L929 mouse fibroblasts and A1 mouse embryonic fibroblasts (PARP-1^{-/-}) were maintained as subconfluent culture in high glucose (4.5 g/liter) Dulbecco's modified Eagle's medium, with 10% fetal calf serum (Cambrex Corp.), 2 mM L-glutamine (Cambrex Corp.), and 50 units/ml Pen-Strep (Cambrex Corp.). Exponentially growing cultures were transfected by Lipofectamine Plus reagent (Invitrogen) adopting the manufacturer's protocol with the following plasmid expression vectors: pCI-CTCF-His tag (kindly provided by Elena M. Klenova, Department of Biological Sciences, University of Essex, UK) for the overexpression of human CTCF, pCI- empty vector as control, and pBabe-PURO for selection of transfectants. To obtain hypomethylated DNA, cells were cultivated for 72 h in standard medium containing 5 μM 5-AZA (Sigma).

Western Blot Analysis—Total cell lysates were obtained by direct lysis of cells in RIPA buffer (50 mM Tris-HCl (pH 8), 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% Nonidet P-40, 1 mM EDTA). Nuclei were collected from trypsinized and phosphate-buffered saline-washed cells by centrifugation following incubation (30 min) in isolation buffer containing 10 mM Tris-HCl (pH 7.9), 4 mM MgCl₂, 1 mM EDTA, 0.5 mM dithiothreitol, 0.25 mM sucrose, 1% Triton X-100. Nuclear fraction was lysed in RIPA buffer, and protein concentration was determined using the Bradford protein assay reagent (Bio-Rad) with bovine serum albumin (Promega) as standard. Both buffers were supplemented with protease inhibitors (complete EDTA-free, Roche Applied Science). Equal protein amounts were subjected to 6% SDS-PAGE and blotted onto Hybond-ECL nitrocellulose membranes (Amersham Biosciences). The antibodies employed were as follows: mouse monoclonal Ab anti-PARP-1 (C2–10, Alexis Biochemicals), mouse monoclonal Ab anti-PARP-2 (4G8, Alexis), mouse monoclonal Ab anti-PAR (10 HA, Trevigen), mouse monoclonal Ab anti-Dnmt1 (Imgenex), rabbit polyclonal Ab anti-CTCF (Upstate), rabbit polyclonal Ab anti-Sp1 (H-225, Santa Cruz Biotechnology), rabbit polyclonal Ab anti-lamin B1 (AbCam), and goat anti-mouse and anti-rabbit horseradish peroxidase-conjugated antibodies (Santa Cruz Biotechnology).

CTCF Expression in the Baculovirus System—pVLH-CTCF vector, kindly provided by Dr. Klenova (Department of Biological Sciences, University of Essex), was co-transfected with the BD BaculoGoldTM *Baculovirus* DNA (BD Biosciences) into SF9 cells. After the co-transfection, the recombinant baculoviruses were selected and amplified following the manufacturer's instructions. The baculovirus recombinant hCTCF His-tagged protein was purified from infected SF9 cells using the ProBondTM purification system (Invitrogen). *bv*CTCF was quantified by silver staining (SilverQuestTM, Invitrogen) using bovine serum albumin as standard.

In Vitro PARP-1 Activity Assay—*bv*CTCF (0.2, 0.4, or 0.8 pmol) was incubated for 2 h at 30 °C with 4 pmol of human recombinant PARP-1 (Alexis) in 200 μ l of poly(ADP-ribosyl)-ation buffer containing 50 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 10 mM C₂H₆OS, and 200 μ M NAD, in the presence or absence of 200 ng of DNase I-activated DNA or 20 μ M PJ-34 (Sigma). PARP-1 automodification reactions with or without PJ-34 (20 μ M) were used as control. Reactions were terminated by 20% trichloroacetic acid precipitation, and ADP-ribose synthesis was assayed by 6% SDS-PAGE followed by Western blotting with anti ADP-ribose antibody. An analogous radiometric assay was performed incubating *bv*CTCF (0.03, 0.05, 0.1, 0.2, 0.4 pmol) for 2 h at 30 °C with 1 pmol of human recombinant PARP-1 in 50 μ l of poly(ADP-ribosyl)ation buffer and in the presence of [³H]NAD (0.3 μ Ci). Following 20% trichloroacetic acid precipitation, samples were analyzed in a Beckman LS-6800 liquid scintillation spectrometer.

Co-immunoprecipitation (Co-IP)—Nuclei obtained from L929 cells were lysed in IP buffer (50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 300 mM NaCl, 1% Nonidet P-40, 1% Triton X-100) supplemented with protease-inhibitors (complete EDTA-free, Roche Applied Science). Lysates (1.5 mg) were pre-cleared with protein A- (for IP anti CTCF and anti PARP-1) or G (for IP anti Dnmt1)-agarose beads (Upstate) on a rotative shaker at 4 °C for 2 h and 30 min. Pre-cleared lysates were incubated with specific antibodies (rabbit polyclonal Ab anti-CTCF, Upstate; mouse monoclonal Ab anti-Dnmt1, Imgenex; and rabbit polyclonal Ab anti-PARP-1, Alexis) and with normal rabbit or mouse IgG (Santa Cruz Biotechnology) on a rotative shaker at 4 °C. The agarose beads, previously saturated with bovine serum albumin (1 μ g/ μ l) overnight, were added to the lysate/Ab solutions and incubated for 2 h on a rotative shaker at 4 °C. Subsequently, beads were washed in IP buffer and boiled in SDS-PAGE sample buffer, and the eluted proteins were analyzed by SDS-PAGE and Western blotting.

Pulldown Assay—50 μ l of ProBondTM resin (Invitrogen) was added to 1.2 ml of 1 \times native purification buffer (ProBondTM) in the presence or absence of 0.8 pmol of *bv*CTCF recombinant His-tagged protein. Mix was incubated overnight on a rotative shaker at 4 °C. Then the resin was collected and incubated with 4 pmol of human recombinant PARP-1 (Alexis) in 300 μ l of 1 \times native purification buffer for 3 h on a rotative shaker at 4 °C. After washing the resin with 1 \times native purification buffer, proteins were eluted from the beads by boiling them in SDS-PAGE sample buffer. Protein-protein interaction was analyzed by 6% SDS-PAGE and Western blot analysis.

DNA Methyltransferase Activity—Equal amounts of nuclear lysates from cells overexpressing CTCF and relative controls (3–5 μ g) were analyzed for the DNA methyltransferase activity by the EpiQuikTM DNA methyltransferase assay kit (Epigentek) following the manufacturer's conditions. This assay was also carried out to check the effect of *bv*CTCF (0.02, 0.04, and 0.08 pmol) or of same amounts of *in vitro* poly(ADP-ribosyl)ated PARP-1 (25) on 0.08 pmol of human recombinant DNMT1 (New England Biolabs). Human recombinant PARP-1 (Alexis) (0.08 pmol) was used as control.

RNA Extraction and Reverse Transcription—Total RNA (~1.8 μ g), purified by RNeasy mini kit (Qiagen), was subjected

to retrotranscription using Superscript first-strand synthesis system (Invitrogen).

Real Time PCR—Expression of mRNA for *Parp-1*, *Parp-2*, and *Dnmt1* genes was measured by real time PCR using TaqMan gene expression assays (Applied Biosystems) following the manufacturer's protocol for the absolute standard curve method on iCycler IQ detection system (Bio-Rad). The standard curve was generated using 1:1 serial dilutions (from 100 to 12.5 ng) of cDNA obtained from control at 24 h as reference. PCR efficiency was 90–100% for each set of primers and probe in any experiment. The amplification reaction was performed in duplicate for each sample in 96-well plates. The amount of *Parp-1*, *Parp-2*, and *Dnmt1* mRNAs was calculated adopting the standard curve method, and normalization was carried out using hypoxanthine-guanine phosphoribosyltransferase (*Hprt1*) as internal control gene. TaqMan gene expression assay IDs for each set of primers and probe were as follows: Mm00599763m1 (*Dnmt1*); Mm00500154m1 (*Parp-1*); Mm00456462m1 (*Parp-2*); and Mm00446968m1 (*Hprt1*).

DNA Extraction and Methyl-accepting Ability Assay—DNA was purified from cells using the DNeasy tissue kit (Qiagen), according to the manufacturer's instructions. Methyl-accepting ability assay was carried out in a final volume of 50 μ l of 10 mM Tris-HCl (pH 7.9), 10 mM MgCl₂, 50 mM NaCl, 1 mM dithiothreitol in the presence of 1 μ g of purified DNA and 1 unit of bacterial SssI methylase (New England Biolabs), using as methyl donor 16 μ M S-adenosylmethionine plus 10 μ Ci/ml of [*methyl*-³H]S-adenosylmethionine (GE Healthcare; specific activity 70–80 Ci/mmol). The reaction was incubated for 1 h at 37 °C and was stopped at 60 °C for 30 min after addition of 1% SDS and 250 μ g/ml of proteinase K. The incorporation of labeled methyl groups was evaluated on purified DNA in a Beckman LS-6800 liquid scintillation spectrometer.

Methylation-sensitive Southern Blot Analysis—The DNA methylation level of minor satellite and B1 repeats was evaluated by Southern blot analysis. DNAs (2 μ g) were digested with 40 units of MspI or HpaII restriction enzymes for 16 h at 37 °C. After 1.5% agarose gel electrophoresis, the digested DNA was blotted on Hybond-N nylon membrane (Amersham Biosciences), and the presence of new HpaII cutting sites was evidenced by hybridization to 3'-digoxigenin-labeled single strand synthetic oligonucleotides as probes. Labeling of probes and detection was performed using digoxigenin oligonucleotide 3'-end labeling kit and digoxigenin luminescent detection kit (Roche Applied Science). Sequence of probes were as follows: for minor satellite repeats, 5'-GGAAACATGATAAAAACCA-CAGTGTAGAACATATTAGATGAGTGAGTTACTACTGAAAACACATTCGTTGGAAACGGGATTTGTAGAACAGTGTATATCAATGAGTTACAATGAGAAACATC-3' (26); for B1 5'-AGTGAGTTCAGGACAGCCAG-3' (27). Oligos were made by custom primers synthesis service (Invitrogen). As positive control for DNA demethylation, digestion was performed in parallel on DNA from cells treated for 72 h with 5 μ M 5-AZA.

RESULTS

In the first experimental stage, our attention was focused on describing what happens in L929 fibroblast cells following tran-

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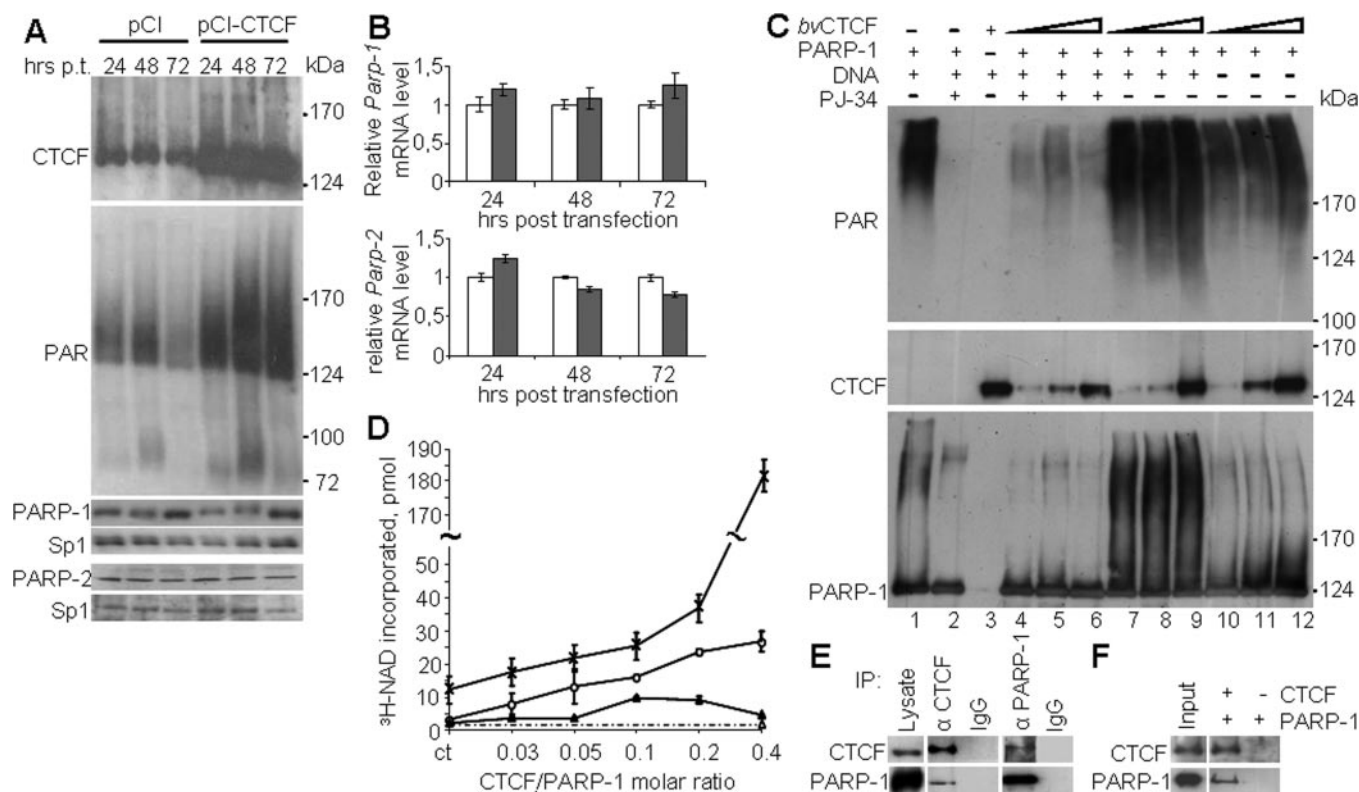


FIGURE 1. *In vivo* and *in vitro* experiments addressing the correlation between CTCF and poly(ADP-ribosylation). *A*, nuclear lysates from cultures at 24, 48, and 72 h post-transfection with pCI-CTCF and pCI were analyzed by SDS-PAGE and Western blot using antibodies against ADP-ribose polymers (PAR) and against the indicated proteins. Sp1 was used as endogenous control. *B*, real time RT-PCR for *Parp-1* and *Parp-2* performed on RNA samples from cells at 24, 48, and 72 h post-transfection of both pCI (white bar) and pCI-CTCF (gray bar) vectors. *Hprt1* mRNA was used as endogenous control. Data are reported as mean \pm S.E. of the ratio of *Parp-1* or *Parp-2* mRNA level to *Hprt1* mRNA level within each sample calculated from a minimum of three experiments carried out in duplicate. pCI samples were considered as 1.0. *C*, *in vitro* assay of PARP-1 activity in the presence of *bvCTCF*. Synthesis of PAR was detected by SDS-PAGE and Western blotting. Bottom panels report Western blot analysis for CTCF and PARP-1. *D*, radiometric PARP-1 assay carried out in the presence (X) or in the absence (O) of "activated DNA" and in the presence of PJ-34 (\blacktriangle). To exclude poly(ADP-ribosylation) activity of recombinant CTCF, 0.4 pmol of *bvCTCF* were assayed in poly(ADP-ribosylation) buffer (\triangle). Data are reported as mean \pm S.E. of two experiments performed in duplicate. *E*, reciprocal Co-IP of CTCF and PARP-1 performed on nuclear lysates. Normal IgGs were used as negative control. *F*, pull-down assay with *bvCTCF* (His-tagged) and recombinant PARP-1.

sient or semi-stable ectopic CTCF overexpression (24) (supplemental Fig. S1A). To quantify the proliferative capacity of CTCF-overexpressing cells, population doublings over time were calculated. We found that control cultures doubled in 1 day *versus* 3 days for the CTCF-overexpressing one. Cell growth diminished by about 50% at 72 h post-transfection (supplemental Fig. S1B); no substantial change in the distribution of the cell population among the different phases of the cell cycle was observed at this time despite the inhibition of cell growth (supplemental Fig. S1E). Clonogenic ability was also affected when analyzed at 48 h post-transfection of pCI-CTCF plus pBabe-PURO (1:20) and puromycin selection for 2 weeks. At this time the clonogenicity was decreased by about 57% (supplemental Fig. S1D). Unlike previously reported data, CTCF ectopic overexpression increased the percentage of dead (supplemental Fig. S1C) and apoptotic cells (supplemental Fig. S1F).

The extent of poly(ADP-ribosylation) was analyzed by Western blots of nuclear proteins obtained from adherent cells ectopically overexpressing CTCF and the respective control cells (Fig. 1A). The use of anti-PAR antibodies showed that CTCF overexpression induced PARP activity, leading to poly(ADP-ribosylation) of chromatin proteins, including PARP-1. Anti-CTCF antibodies allowed the identification of the poly(ADP-ribosyl)ated form of CTCF as a band running at ~180

kDa (Fig. 1A, top panel); it was impossible to detect this band using the anti-ADP-ribose polymer antibodies because the smearing of polymers, which occurs following PARP-1 auto-modification, covers the molecular weight region corresponding to the 180 kDa of the modified CTCF (21). PARP-1 poly(ADP-ribosylation) increased with CTCF overexpression time with a maximum reached at 48 h. Real time PCR and Western blot analyses showed that this increase was not correlated with greater PARP-1 and PARP-2 mRNA levels or with their higher nuclear levels (Fig. 1, A and B).

To directly test for the effect of CTCF on PARP-1 activity, we used immunometric and radiometric methods. Recombinant PARP-1 was incubated in the presence or absence of different amounts of recombinant *bvCTCF* under conditions that are typically adopted to induce PARP-1 automodification. The immunometric PARP-1 activity assay (Fig. 1C) was carried out in the presence of human recombinant PARP-1 and DNase I-activated DNA without (lane 1) or with (lane 2) PJ-34 (20 μ M), a competitive inhibitor of PARP activity. DNase I-activated DNA is DNA on which some nicks are present, this condition being necessary to induce the poly(ADP-ribosylation) of the nick-sensor PARP-1. The reaction was carried out in the presence (Fig. 1C, lanes 4–6) or in the absence (lanes 7–9) of PJ-34, plus addition of increasing amounts of recombinant CTCF

(CTCF/PARP-1 molar ratio from 0.05 to 0.2). Finally, PARP activity was assayed following addition of recombinant CTCF (at the same CTCF/PARP-1 molar ratio) but in absence of “activated” DNA (10–12). Fig. 1C, lane 3, reports the reaction carried out in the absence of PARP-1, indicating the absence of

PARP activity in *bv*CTCF preparation. As shown, increasing the molar ratio of CTCF/PARP-1 increased the level of poly-(ADP-ribosyl)ation of PARP-1. It was surprising that CTCF acted as an activator of PARP-1 modification *per se*, being able to induce poly(ADP-ribosyl)ation of PARP-1 even in the absence of nicked DNA, which is required to allow PARP-1 activity. The presence of CTCF reduces the effect of the competitive inhibitor PJ-34, meaning that there may be conformational change of PARP-1. In fact, poly(ADP-ribosyl)ation of PARP-1 was not completely inhibited by a PJ-34 concentration of 20 μ M. Fig. 1D refers to the radiometric assay carried out in presence of [³H]NAD. The molar ratio CTCT/PARP-1 of 0.4 increases PARP-1 activity in presence of activated DNA by about 15-fold. This assay confirms the finding that CTCF is capable *per se* of inducing poly(ADP-ribosyl)ation of PARP-1 even in absence of activated DNA. In fact, the presence of CTCF at 0.4 pmol allows ~8.5-fold increase in the modification of PARP-1. Co-immunoprecipitation and pulldown experiments have shown that CTCF and PARP-1 directly interact both *in vivo* and *in vitro*. (Fig. 1, E and F).

Our knowledge that DNA methylation and poly(ADP-ribosyl)ation are connected prompted us to further investigate the scenario where the ectopic overexpression of CTCF induced a burst of PARs and poly(ADP-ribosyl)ation of PARP-1, which remained high in time. We expected that this condition would induce permanent inhibition of Dnmt1 and consequently genomic DNA hypomethylation. We thus tested whether the increased amount of modified PARP-1 influenced the endogenous DNA methyltransferase activity. The activity assay, carried out on nuclear extracts obtained from cells ectopically overexpressing CTCF and the respective control cells, showed that DNA methyltransferase activity decreased by about 65–70% (Fig. 2C). Real time PCR (Fig. 2B) and Western blot analyses (Fig. 2A) excluded that inhibition of enzymatic activity was dependent on down-regulation of Dnmt1 levels. The possibility that CTCF *per se* was acting as an inhibitor of DNMT1 activity was disproved by *in vitro* assays; parallel experiments carried out with *in vitro* poly(ADP-ribosyl)ated PARP-1 confirmed that poly(ADP-ribosyl)ated PARP-1 is responsible for inhibitory effect on DNMT1 (7) (Fig. 2D). Co-immunoprecipitation experiments, carried out on nuclear extracts from L929 mouse fibroblasts, indicate that PARP-1 and Dnmt1 are associated *in vivo* (Fig. 2E), confirming our previous data (7).

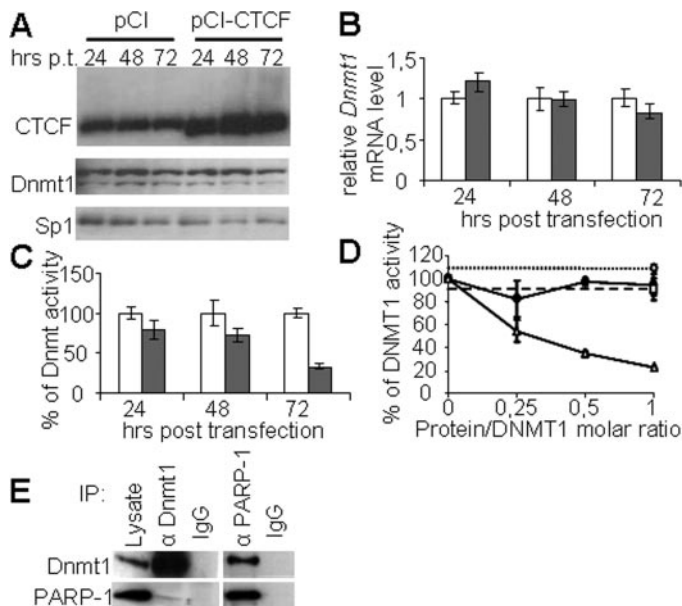


FIGURE 2. *In vivo* and *in vitro* experiments addressing the effect of CTCF overexpression on DNA methyltransferase activity. A, nuclear lysates from cultures at 24, 48, and 72 h post-transfection with pCI-CTCF and pCI were analyzed by SDS-PAGE and Western blot using antibodies against CTCF, Dnmt1, and against Sp1 as endogenous control. B, real time RT-PCR for *Dnmt1* performed on RNA samples from cells at 24, 48, and 72 h post-transfection with either pCI (white bar) or pCI-CTCF (gray bar) vectors using *Hprt1* mRNA as endogenous control. Data are reported as mean \pm S.E. of the arithmetic ratio of *Dnmt1* mRNA level to *Hprt1* mRNA level within each sample calculated from a minimum of three experiments performed in duplicate. pCI samples were considered as 1.0. C, endogenous DNA methyltransferase activity of nuclear extract from cultures at 24, 48, and 72 h post-transfection with pCI-CTCF (gray bar) and pCI (white bar) vectors. The pCI samples were considered as 100%. D, *in vitro* activity assay of recombinant DNMT1 in the presence of increasing amounts of *bv*CTCF (●), *in vitro* poly(ADP-ribosyl)-ated PARP-1 (Δ), PARP-1 at a PARP-1/DNMT1 molar ratio of 1 (□), and CTCF elution buffer (224). Data reported in B–D are mean \pm S.E. of three experiments performed in duplicate. E, reciprocal Co-IP of Dnmt1 and PARP-1 performed on nuclear lysates. Normal IgG were used as negative control.

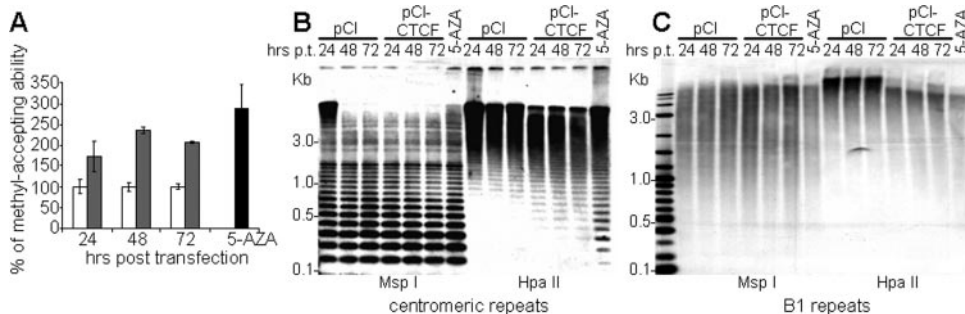


FIGURE 3. Effect of DNA methyltransferase activity inhibition on the methylation state of genomic DNA. A, methyl-accepting ability assay was carried out on genomic DNA purified from cells transfected for different times (24, 48, and 72 h) with pCI-CTCF (gray bar) or pCI (white bar) vectors. Results are displayed as percentage of activity versus relative controls taken as 100. DNA obtained from cells treated with 5-AZA was used as positive control for genome hypomethylation (black bar). Data are reported as mean \pm S.E. of three experiments performed in triplicate. B and C, Southern blot against minor satellite (B) and B1 (C) DNA repeats performed on genomic DNA purified from cultures at 24, 48, and 72 h post-transfection with pCI-CTCF and pCI and digested with HpaII or MspI restriction enzymes. DNA obtained from cells treated with 5-AZA was used as positive control for genome hypomethylation.

on nuclear extracts from L929 mouse fibroblasts, indicate that PARP-1 and Dnmt1 are associated *in vivo* (Fig. 2E), confirming our previous data (7).

Research was then focused on DNA methylation pattern and the possibility that inhibition of Dnmt1 activity leads to DNA hypomethylation. Fig. 3A reports data from methyl-accepting ability assays, carried out on DNA purified from cells overexpressing CTCF and control cells in the presence of labeled S-adenosylmethionine and human recombinant DNMT1. CTCF overexpression introduced hypomethy-

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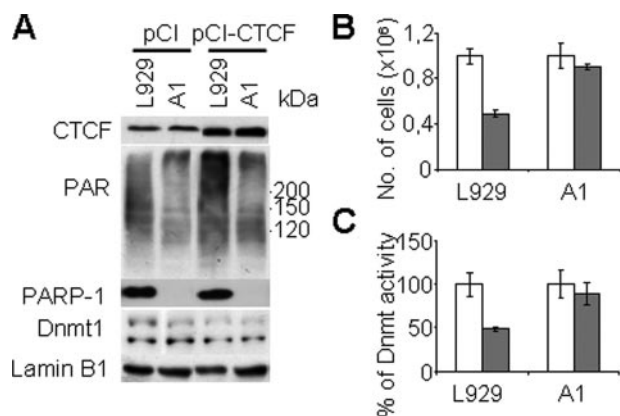


FIGURE 4. Effect of CTCF overexpression in PARP-1^{-/-} cells on PARs level, cell survival, Dnmt1 expression, and DNA methyltransferase activity. A, nuclear lysates from L929 and A1 (PARP-1^{-/-}) cells at 72 h post-transfection with pCI and pCI-CTCF vectors were analyzed by SDS-PAGE and Western blotting, using antibodies against PAR and against the indicated proteins. Lamin B1 was used as endogenous control. B, survival of L929 and A1 cells after 72 h post-transfection with pCI (white bar) and pCI-CTCF (gray bar) vectors. C, endogenous Dnmt1 activity of nuclear lysates from L929 and A1 cells at 72 h post-transfection with pCI (white bar) and pCI-CTCF (gray bar) vectors. Data reported in B and C are mean \pm S.E. of three independent experiments.

lation on genomic DNAs, and as a result, its methyl-accepting ability increased 3-fold at 24 h from transfection. The integrity and the amounts of purified genomic DNA were evaluated by agarose gel electrophoresis (data not shown). Southern blot analyses were performed on the same DNA samples to localize the new hypomethylated sequences. Fig. 3, B and C, reports experiments in which genomic DNA, previously digested with methylation-sensitive HpaII restriction enzyme and with its insensitive isoschizomer MspI, was analyzed, after electrophoretic separation of DNA fragments, by using 3'-digoxigenin-labeled single strand synthetic oligonucleotides as probes. As can be seen, both centromeric and B1 repetitive DNA sequences were hypomethylated. To verify how specific the PARP-1 poly(ADP-ribose)ylation is in affecting DNA methylation activity, we performed experiments of CTCF overexpression in PARP-1^{-/-} mouse cells. Ectopic overexpression of CTCF in PARP-1^{-/-} cells did not reduce the DNA methyltransferase activity, Dnmt1 level, and the cell growth significantly (Fig. 4, A–C and supplemental Experimental Procedures), although it induces a moderate increase of PARs because of an enhanced activity of PARP-2 and/or of other members of PARP family (Fig. 4A).

DISCUSSION

This research emphasizes the importance of proper dynamics between poly(ADP-ribose) polymerase and poly(ADP-ribose) glycohydrolase (PARG) activities so that cellular homeostasis is not affected by low or excessively high PAR levels. In physiological conditions, the active, poly(ADP-ribose)ylated PARP-1 molecules constitute a small proportion of the total population of PARP-1 molecules, whereas upon injury to the cell, the number of active PARP-1 molecules increases causing an up to 500 times higher level of PARs (28). In this environment PARP-1 plays an important role as it is responsible for about 90% of PAR production (29). However, the basal level of polymers is rapidly restored thanks to the intervention of

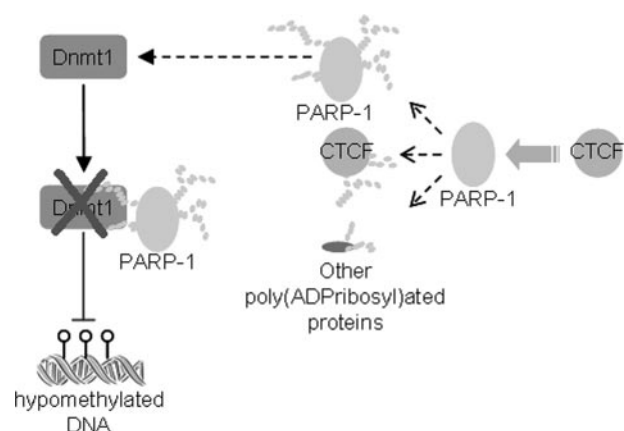


FIGURE 5. A possible model describing the sequence of events occurring in cells following CTCF ectopic overexpression. CTCF activates (gray arrow) PARP-1 inducing its automodification and poly(ADP-ribose)ylation of CTCF and of other nuclear proteins yet to be identified. The persistence of PARs in the nuclear environment induces “stable” inhibition of Dnmt1 activity. Modified PARP-1, binding noncovalently the Dnmt1, inhibits its enzymatic activity, and as a consequence, the genomic DNA becomes widely hypomethylated.

PARG (30–32), which shuttles from the cytoplasm to the nucleus (33, 34), digesting the excess of nuclear PARs in a few minutes (28, 35).

Over the years, it has been shown that the decrease and increase of PAR level can both threaten the normal cellular physiology. Treatment of cells with competitive inhibitors of PARP activity has demonstrated that delay in DNA damage repair and genomic instability occur when nuclear levels of PARs are low (36). Research on cells knocked out for PARP-1 (37) or PARP-2 (38), the two best-known members of the PARP family (39), has indicated that both enzymes are involved in the mechanism of DNA damage repair: knocking out of either enzyme is not lethal, whereas the cell cannot survive the double knock-outs (38). Likewise harmful is the accumulation of PARs, which is even perceived as a signal for cell death (40–42). Cell death, observed when PAR level is high, has long been attributed to NAD⁺ depletion and thus to energy insufficiency (43). Without excluding that more than one event leads to cellular death, it has been shown that PARs by themselves cause cell mortality (44). Cytoplasmic long and branched polymers (41), interacting with mitochondrial membrane stimulate the release of the pro-apoptotic factor AIF, an apoptotic signal downstream of PARP activation (42, 45, 46).

In the above-mentioned research attention was focused on what happens to the cell when high levels of PARs are present in the cytosol. We have focused our attention on events that occur in the nucleus. Based on our experimental data, we suggest a model (Fig. 5) that depicts the order in which molecular events take place. The starting point is when the increased nuclear level of CTCF induces a higher level of nuclear PARs. It is surprising that at all observation times, 24, 48, and 72 h following ectopic transfection of CTCF, the nuclear level of PARs remained high not depending on an increased PARP-1 expression, even though the PAR level returns to basal level in a few minutes following stress (28).

In vitro experiments show that CTCF is by itself capable of activating PARP-1. This activation happens even in absence of

activated DNA, and this is different from YY1 (47) and VP1 (48). Furthermore, the effect of the PJ-34 inhibitor is diminished in presence of CTCF. Co-immunoprecipitation and pull-down experiments confirmed interaction between CTCF and PARP-1 both *in vivo* and *in vitro*.

Our previous data show that both free PARs and PARs present on modified PARP-1, through a noncovalent interaction with DNMT1, inhibit its enzymatic activity. Here we investigated whether prolonged elevated PAR levels could stably inhibit the activity of Dnmt1. We show that DNA methyltransferase activity is decreased by about 70% in nuclear extract in cells overexpressing CTCF *versus* cells transfected with empty vector. A more thorough examination of Dnmt1 has shown that the inhibition is not because of a decreased level of *Dnmt1* expression. An *in vitro* approach allowed us to exclude direct inhibition of DNMT1 activity by CTCF, in line with our previous observation that PARs present on modified PARP-1 inhibit DNMT1 activity (7). The persistence of a high PAR level affects the DNA methylation machinery; Dnmt1 activity is inhibited, leading to wide hypomethylation of the genome, involving both centromeric and B1 repeats. Ectopic overexpression of CTCF in PARP-1^{-/-} cells demonstrates a specific functional relationship between CTCF and PARP-1.

This finding underscores the importance of a rapid reversal of PARP-1 automodification because it affects DNA methylation patterns considered to be part of the epigenetic code. DNA hypomethylation could decondense chromatin structure (49, 50), cause genomic instability (51), direct cells toward apoptosis (52), and cancer (53, 54). The unusual cellular environment, in which a large amount of ADP-ribose polymers are present for several days, mimics the situation in PARG knock-outs and provides a model for the study of damage because of excess of ADP-ribose polymers in cells. Our data suggest that deregulation of DNA methylation pattern is one of the molecular mechanisms causing lethality in PARG knock-out mice (40).

The finding that high PAR levels induce hypomethylation is intriguing, because in tumor cells the hypomethylation of repeat-rich heterochromatin contributes to genomic instability, through increased mitotic recombination events (51). The introduction of new methyl groups onto DNA or the diffuse hypomethylation in cancer cells (54, 56) could occur through deregulation of PARP or PARG activities. This demethylation is a passive mechanism (55) that does not depend on decreased expression of Dnmt1 but on the absence of an active Dnmt1.

In the cellular scenario dependent on ectopic overexpression of CTCF, the cytotoxicity is accounted for by a decreased level of intracellular NAD⁺ (43), whereas the increased number of apoptotic cells can be explained by recent data showing that the higher ADP-ribose polymer production (57) and DNA hypomethylation (52) induce apoptosis. The connection between DNA methylation and poly(ADP-ribosylation) inferred from our previous studies (1–6) is strengthened by our new findings showing that activation of PARP-1, dependent on CTCF, affects DNA methylation machinery. It is clear that, because of the complexity of pathways that occur in cells, despite evidence showing the connection between poly-ADP-ribosylated PARP-1 and Dnmt1, other additional and/or alternative mechanism(s) cannot be excluded. Based on our extensive research

on poly(ADP-ribosylation) and DNA methylation, we think that the control of DNA methylation pattern is among the multiple housekeeping roles of PARP-1; this role could be played by the inhibitory effect exerted by PARs on the Dnmt1 (7). The balance of PARs constitutively present in cells may be extremely important; their decrease causes anomalous DNA hypermethylation (1–6), whereas their increase leads to widespread DNA hypomethylation.

Further research will be carried out to define in depth the role played by CTCF in the cross-talk between poly(ADP-ribosylation) and DNA methylation and to establish whether and how the normal functions of PARG are affected in this new cellular scenario. We will assess whether CTCF represses PARG expression, inhibits its enzymatic activity, or impairs its physiological shuttling from the cytoplasm to the nucleus.

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