Poly(ADP-ribose) polymerase-1 silences retroviruses independently of viral DNA integration or heterochromatin formation

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PARP-1 silences retrotransposons in Drosophila, through heterochromatin maintenance, and integrated retroviruses in chicken. Here, we determined the role of viral DNA integration and cellular heterochromatin in PARP-1-mediated retroviral silencing using HIV-1-derived lentiviral vectors and Rous-associated virus type 1 (RAV-1) as models. Analysis of the infection of PARP-1 knockout and control cells with HIV-1 harbouring WT integrase, in the presence or absence of an integrase inhibitor, or catalytic-dead mutant integrase indicated that silencing does not require viral DNA integration. The mechanism involves the catalytic activity of histone deacetylases but not that of PARP-1. In contrast to Drosophila, lack of PARP-1 in avian cells did not affect chromatin compaction globally or at the RAV-1 provirus, or the cellular levels of histone H3 N-terminal acetylated or Lys27 trimethylated, as indicated by micrococcal nuclease accessibility and immunoblot assays. Therefore, PARP-1 represses retroviruses prior to viral DNA integration by mechanisms involving histone deacetylases but not heterochromatin formation.

The cellular response to invading genomes is evolutionarily conserved, suggesting an early origin of these mechanisms (Ali et al., 2013; Gasiunas et al., 2014; Pinsker et al., 2001; Weitzman et al., 2010). A variety of DNA and RNA viruses activate DNA repair pathways in the absence of host DNA damage (Hoelzer et al., 2008; Lilley et al., 2005; 2011; Ohsaki et al., 2004; Orzalli et al., 2013; Pinsker et al., 2001; Ross et al., 2009; Schreiner et al., 2013; Weitzman et al., 2010), recruiting DNA repair proteins to their genomes (Lilley et al., 2005; 2011; Ohsaki et al., 2004; Orzalli et al., 2013; Ross et al., 2009; Weitzman et al., 2010), and evolving dependency of this cellular response for efficient viral replication (Cooper et al., 2013; Koyama et al., 2013; Lilley et al., 2005; 2011; Ohsaki et al., 2004; Orzalli et al., 2013; Ross et al., 2009; Sakurai et al., 2009; Schreiner et al., 2013; Weitzman et al., 2010). For example, the DNA damage sensor poly(ADP-ribose) polymerase-1 (PARP-1) (Ji & Tulin, 2010; Krishnakumar & Kraus, 2010) is recruited to the Kaposi’s sarcoma-associated herpesvirus and Epstein–Barr virus genomes and impairs viral replication by modifying viral proteins involved in genome replication and partitioning (Ohsaki et al., 2004), whereas hepatitis B virus depends on PARP-1 for efficient transcription (Ko et al., 2013). In addition, PARP-1 represses the expression of retrotransposons in Drosophila (Kotova et al., 2010; 2011; Tulin et al., 2002) and retroviruses in avian cells (Bueno et al., 2013).

Conflicting data regarding a role for PARP-1 in HIV DNA integration exist (Ariumi et al., 2005; Baekelandt et al., 2000; Gaken et al., 1996; Ha et al., 2001; Kameoka et al., 2004; 2005; Siva & Bushman, 2002), which is not surprising considering that, in mammals, this enzyme is the founding member of a family of 18 proteins (Ame et al., 2004) with significant functional redundancy (Ame et al., 2004; Boehler et al., 2012; Krishnakumar & Kraus, 2010; Ménessier de Murcia et al., 2003; Wang et al., 1995). Therefore, simpler organisms lacking this redundancy are advantageous to the study of PARP-1 (Bueno et al., 2013; Kotova et al., 2010; Pinnola et al., 2007; Tulin et al., 2002). One of these cellular models is the chicken B lymphoblastoid cell line DT40 that, within the PARP family, only expresses PARP-1 (Hochegger et al., 2006). These cells are a reliable model to study the role of PARP-1 in retroviruses, since it supports the early phase of the life cycle of gamma retrovirus- and lentivirus-derived viral vectors (Barr et al., 2005; Beitzel & Bushman, 2003; Bueno et al., 2013; Mitta et al., 2004, 2005; Randow & Sale, 2006).

We took advantage of the DT40 cellular model to understand better the mechanism of action of PARP-1 in retroviral silencing (Bueno et al., 2013), by determining whether PARP-1-mediated silencing requires viral DNA integration. Since PARP-1 represses integrated retro-elements, it is possible that integration-induced DNA damage recruits PARP-
1 to the invading genome causing transcriptional repression, as described for other types of DNA damage (Chou et al., 2010). Using procedures previously described (Bueno et al., 2013), DT40 cells WT, PARP-1 knockout (KO) or KO cells engineered to express human PARP-1 (h-1) (Hochegger et al., 2006) (Fig. 1a) were infected with p24-normalized amounts of vesicular stomatitis virus glycoprotein G (VSV-G)-pseudotyped, single-round infection HIV-1-derived viral vectors expressing luciferase from an internal CMV promoter. ATP-normalized luciferase was determined in cells infected with integrating (HIV luc IN WT) or non-integrating (HIV luc IN D64V or HIV luc IN WT in the presence of Raltegravir) 4 and 2 days post-infection, respectively. ATP-normalized luciferase was expressed relative to DT40 WT levels (b) or as arbitrary units (AU) (c). SD values indicate the variability of one experiment; data are representative of three (b) or one (c) independent experiments.

Fig. 1. Effect of PARP-1 on HIV-1 infection. (a) Immunoblot analysis of PARP-1 expression in DT40-derived cells detected with an anti-human PARP-1 monoclonal antibody (C2-10) that modestly cross-reacts with avian PARP-1. (b and c) DT40-derived cells were infected with VSV-G-pseudotyped HIV-1-derived viral vectors expressing luciferase from an internal CMV promoter. ATP-normalized luciferase was determined in cells infected with integrating (HIV luc IN WT) or non-integrating (HIV luc IN D64V or HIV luc IN WT in the presence of Raltegravir) 4 and 2 days post-infection, respectively. ATP-normalized luciferase was expressed relative to DT40 WT levels (b) or as arbitrary units (AU) (c). SD values indicate the variability of one experiment; data are representative of three (b) or one (c) independent experiments.

PARP-1 silences retroviral gene expression

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PARP-1 has been reported to silence integrated retroviruses and retrotransposons through epigenetic mechanisms implicated in the formation and maintenance of the host heterochromatin (Bueno et al., 2013; Kotova et al., 2010, 2011, 2011; Tulin et al., 2002). Unintegrated and integrated retrovirus genomes are associated with chromatin (Kantor et al., 2009; Schneider et al., 2012; Sloan & Wainberg, 2011); therefore, PARP-1 could silence these through similar mechanisms. However, potential variations in the chromatin organized at these two forms of viral genome could determine differences in the mechanisms implicated. To evaluate these alternatives, we determined the role of histone deacetylation and DNA methylation in PARP-1-mediated silencing of unintegrated HIV-1.

DT40 WT, KO and h-1 cells were infected with HIV-1 p24-normalized amounts of HIV luc IN D64V or IN WT viruses, and 2 or 4 days later, respectively, the cells were treated or not with the histone deacetylase inhibitor sodium butyrate (SB, 5 mM) for 24 h and then luciferase and ATP levels were measured. Statistical analysis of three independent experiments demonstrated significant differences in the infectivity of untreated DT40 WT and KO cells (p<0.005) and KO and h-1 cells (p<0.001) with either HIV luc IN D64V or WT virus. However, SB treatment increased luciferase activity at a higher magnitude in cells expressing PARP-1 infected with either integrating or non-integrating viruses, rendering these differences statistically nonsignificant (p<0.005) (Fig. 2a), and indicating that histone deacetylases mediate PARP-1 silencing of integrated and unintegrated HIV-1-derived vectors.

To evaluate the role of DNA CpG methylation in PARP-1-induced retroviral silencing, DT40-derived cells were infected with HIV luc IN D64V in the presence or not of the DNA methylation inhibitor 5-azacytidine (30 μM), and ATP and luciferase were measured 36 h later. 5-Azacytidine augmented retroviral luciferase expression, as expected (Schneider et al., 2012), but in a similar magnitude in cells expressing or not PARP-1 (Fig. 2c). Consequently, statistical analysis of two independent experiments showed significant differences between the infectivity of 5-azacytidine-treated or 5-azacytidine-untreated DT40 WT and KO (p<0.005), and KO and h-1 cells (p<0.001). These results indicated that, in contrast to integrated retroviruses (Bueno et al., 2013), the effect of DNA CpG methylation on the expression of unintegrated retroviruses is PARP-1 independent, suggesting important differences in the PARP-1-silencing mechanism of integrated and unintegrated retroviruses.

Furthermore, we determined the role of PARP-1 enzymatic activity in retroviral silencing. Non-infected DT40 cells were treated with DMSO or the nicotinamide-mimetic PARP inhibitor 1,5-isouquinolinediol (200 μM, inhibitor IV), and poly(ADP-ribose) (PAR) levels were measured 24 h later by ELISA (Trevigien). DMSO-treated WT and h-1 cells expressed 53.71 and 32.87 ng ml⁻¹ of PAR, respectively, but this was undetectable in KO cells (<10 pg ml⁻¹, ELISA’s detection limit), and inhibitor IV reduced PAR levels by 17- and 22-fold in DT40 WT and h-1 cells, respectively (Fig. 2d).

DT40 WT, KO and h-1 cells were treated with DMSO or inhibitor IV at the time of infection with HIV luc IN WT or D64V, and the input virus and drugs were removed 24 h later. Two (HIV luc IN D64V) or four (HIV luc IN WT) days later infection, luciferase and ATP were determined. Results (Fig. 2e) indicated that PARP-1 inhibition did not increase luciferase expression in infected DT40 WT or h-1 cells. Statistical analysis of two independent experiments revealed significant differences between DT40 WT and KO (p<0.005) or KO and h-1 (p<0.005) infected with HIV luc IN WT or D64V in the presence or not of inhibitor IV, suggesting that PARP-1 silences both integrating and non-integrating retroviruses in a catalytic-independent manner. Considering the essential role of the enzymatic activity of PARP-1 in DNA repair (Bürkle & Virág, 2013; Rass et al., 2012), these data also suggest that PARP-1 silences retroviruses independently of DNA damage which is supported by the fact that PAR levels were 1.1-fold higher in non-infected than in HIV luc IN WT-infected h-1 cells, 24 h post-infection.

Similar to the mechanism implicated in PARP-1-mediated silencing of retrotransposons in Drosophila, PARP-1 silences retroviruses in avian cells in a catalytic-independent manner potentially through epigenetic modifications [Fig. 2e and (Bueno et al., 2013)]. In Drosophila, lack of PARP-1 globally decreases heterochromatin levels, as evaluated by micrococcal nuclease (MNase) digestion, causing transcriptional de-repression of retro-elements (Kotova et al., 2010, 2011; Tulin et al., 2002). MNase cleaves the genomic DNA in the internucleosomal space, and its accessibility to this target is largely influenced by the degree of compaction of the chromatin (Chung et al., 2010). Therefore, we used this method to determine whether the role of PARP-1 in heterochromatin maintenance is conserved through evolution from insect to avian and humans.

Chromatin from DT40-derived cells was digested as described previously (Pinnola et al., 2007) using several MNase amounts (12.5, 25 and 50 U), incubation times (5, 10 and 15 min), and temperatures (19°C and 37°C), and then the digested DNA was purified and analysed by agarose electrophoresis. Results using these conditions were similar to those obtained with 50 U of MNase for 15 min at 37°C (Fig. 3a), and indicated no differences in the susceptibility of DT40 chromatin to MNase digestion as a function of PARP-1 levels. These findings indicated that, in contrast to Drosophila (Kotova et al., 2010, 2011; Tulin et al., 2002), the avian or human PARP-1 proteins do not affect the global abundance of heterochromatin in avian cells.

To further define the potential role of PARP-1 in regulating global levels of heterochromatin in avian cells, we determined the abundance of histone H3 post-translational modifications implicated in transcriptional regulation. Modification of histone H3 activity through position-specific methylation or
acetylation plays a fundamental role in the regulation of gene expression (Jenuwein & Allis, 2001; Kimura, 2013). In addition, the histone deacetylase inhibitor SB that reverts PARP-1-mediated retroviral silencing (Fig. 2a, b) is known to increase the cellular levels of N-terminal acetylated histones H3 and H4 (Drogaris et al., 2012). Therefore, histone H3 N-terminal acetylated (H3Ac), a marker of actively transcribed chromatin (Morales & Richard-Foy, 2000), and Lys27 trimethylated (H3K27me3) that is enriched in heterochromatin regions (Kim & Kim, 2012) were measured by immunoblot analysis with specific rabbit polyclonal antibodies (06-599 and 07-449, respectively, Millipore). Total histone H3 was also detected (clone A3.05-928, Millipore) as a loading control. Results (Fig. 3b) showed similar levels of H3Ac and H3K27me3 in the cells analysed, indicating that human or avian PARP-1 did not affect global levels of epigenetic marks implicated in transcriptional regulation in chicken cells. The lack of evolutionary conservation of the role of PARP-1 in regulating cellular levels of heterochromatin is expected considering the low conservation of chicken and Drosophila PARP-1 proteins (43 % identity and 60 % similarity) [ClustalW2 (Larkin et al., 2007) and BLASTP 2.2.19 (Altschul et al., 1997)].

To define whether PARP-1 affects chromatin organization in proviruses, we explored the effect of MNase digestion on the integrity of the LTRs of the Rous-associated virus type 1 (RAV-1) provirus. This virus was used to generate DT40 cells (Baba et al., 1985), and PARP-1 seems to repress its expression since KO cells produce higher levels of RAV-1 than WT or h-1 cells (Bueno et al., 2013). Genomic DNA was isolated from DT40-derived cells and subjected or not to MNase digestion using suboptimal (6.25 U for 15 min at 29°C) or optimal (50 U for 30 min at 37°C) conditions (Fig. 3c), and then used for real-time PCR analysis (Garcia-Rivera et al., 2010) with two sets of primers, DG24/DG25 or DG30/DG31. Primers DG24 (5′ TAGTCTTATGCAATACTCTAATGC 3′) and DG25 (5′ GGAAGGCAACAGACGGGTCTAA 3′) bind to nucleotides 5–28 and 179–200, respectively, in the U3 region, and primers DG30 (5′ TTATTAGGAAGGCAACAGACG 3′) and DG31 (5′ GCACATGCATGAAGCAGAAGGCTT 3′) bind to nucleotides 149–169 and 324–347 in the U3 and U5 sequences.

**Fig. 2.** Effect of sodium butyrate (SB) (a and b), 5-azacytidine (5-Aza) (c) and PARP-1 inhibition (e and f) on HIV-1 infection. Analysis of infection was carried out at 5 (a), 3 (b and c), 4 (e) or 2 (f) days post-infection. Fold infectivity in panels a–c and e and f was calculated by expressing ATP-normalized luciferase levels of each cell line relative to those found in untreated or DMSO-treated DT40 WT cells. (d) Effect of PARP-1 inhibition on cellular PAR levels. Values are expressed as percentage of the PAR levels detected in untreated DT40 WT cells. SD indicates the variability corresponding to one experiment; data are representative of three (a and b) or two (c–f) independent experiments.
respectively, amplifying an overlapping region that spans 96% of both RAV-1 LTRs. Data (Fig. 3d) indicate the relative fold change for each primer set, experimental conditions, and cell lines calculated by using the ΔC_T method, and normalized for the fold change found in the respective cell line’s undigested DNAs. Suboptimal conditions of MNase treatment increased detection of the LTRs as compared to non-digested samples, possibly due to a noted increase in the solubility of the template DNA. In contrast, at optimal conditions, LTR levels decreased for all the cell lines evaluated, being more marked in DT40 WT cells. However, levels of LTR in MNase-digested DNA did not correlate with the cellular levels of PARP-1 or RAV-1, indicating that the accessibility of the proviral LTRs to MNase is not influenced by PARP-1 levels. These data suggest that PARP-1 does not repress RAV-1 expression by organizing compacted chromatin at the provirus.

In summary, our results further support the importance of DNA repair proteins in the cellular response to invading genomes (Bueno et al., 2013; Cooper et al., 2013; Lilley et al., 2005, 2011; Ohasaki et al., 2004; Orzalli et al., 2013; Pinsker et al., 2001; Ross et al., 2009; Sakurai et al., 2009; Tulin et al., 2002; Weitzman et al., 2010), and demonstrate that PARP-1 restricts in a catalytic-independent manner the expression of retroviruses before viral DNA integration occurs. The enzymatic activity of histone deacetylases, but not of DNA methylases, mediates PARP-1 silencing of non-integrated retroviruses, indicating that PARP-1 silences retroviruses before and after integration by different mechanisms (Bueno et al., 2013).

Our data also indicate a major distinction between the mechanism implicated in PARP-1-mediated silencing of retrotransposons in Drosophila (Kotova et al., 2010, 2011; Tulin et al., 2002) and retroviruses in avian cells (Bueno et al., 2013). Although, in both cases, PARP-1-mediated silencing is independent of its catalytic activity, it involves the organization of heterochromatin at the retro-element genome in Drosophila but not in avian cells.

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**Fig. 3.** Effect of PARP-1 on heterochromatin maintenance in avian cells. (a) MNase chromatin accessibility assay. Agarose gel electrophoresis analysis of DNA obtained after MNase digestion of chromatin isolated from DT40-derived cells. (b) Immunoblot analysis of histone H3 total and post-translational modifications in DT40-derived cells. (c) MNase undigested (left panel) or digested (right panel) DNA isolated from DT40 WT cells. (d) Real-time PCR quantification of Rous-associated virus type 1 LTRs. Data are representative of six (a), two (b) or one (c) independent experiments. Means and SDs in panel d were calculated from two MNase digestion and real-time PCR independent experiments.
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