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Poly (ADP-ribose) Polymerase Is Involved in PMA-induced Activation of HIV-1 in U1 Cells by Modulating the LTR Function

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Phorbol 12-myristate 13-acetate (PMA)-induced HIV-1 production in U1 cells was markedly suppressed by inhibitors of poly (ADP-ribose) polymerase (PARP). Northern blot analysis revealed that the PARP-inhibitors suppressed the virus production at a level of transcription. In order to examine the effect of PARP on transcriptional regulation of HIV-1 genes, we transfected a reporter plasmid containing HIV-1-LTR-promoted luciferase gene to L-1210 cell clones, which expressed varying decreased level of PARP. In wild type L-1210 cells, the expression of LTR-promoted luciferase gene was stimulated approximately 4-fold in response to PMA, whereas the PMA-dependent response was almost abolished in mutant cells, which expressed only 8% of PARP of the wild type cells. The effect of decrease in PARP content on the function of HIV-1-LTR was confirmed also in human wild type cells, Jurkat and J111, which were co-transfected with the reporter plasmid and a plasmid expressing a PARP-antisense RNA: Down-regulation of PARP in the cells by the expression of the antisense RNA significantly suppressed the PMA-dependent, LTR-function of the reporter plasmid in both Jurkat and J111 cells. NF-κB, which is known to mediate the PMA-induced activation of HIV-1 in U1 cells, was found to be activated approximately 5-fold in PMA-treated U1 cells. PARP-inhibitor, unexpectedly, did not suppress but rather stimulated (approximately 2-fold) the NF-κB activation. Combining the results with the finding that the LTR-function was minimum in a PARP-defective mutant cells in spite of a very high level of the activated NF-κB in the cells, we suggest that PARP, in addition to activated NF-κB, is essential for the function of HIV-1 LTR.

The transcription of human immunodeficiency virus type-1 (HIV-1) is regulated by complex regulatory mechanisms involving various cellular factors (1) and a virus-encoded transactivator, Tat (2). U1 cells, which are latently infected with HIV-1 LAI strains (3), are considered to be a good model system in vitro to study the mechanism of activation of HIV-1 genome. So far, several agents including interleukins (4), TNF-α (5), dioxin (6), Tat (7), UV (8), and phorbol 12-myristate 13-acetate (PMA, ref. 9) have been shown to be the inducers of the HIV-1 production in U1 cells.

On the other hand, recent studies on the function of poly(ADP-ribose) polymerase (PARP) suggest that this enzyme is involved in the regulation of some genes through its interaction with various transcription factors (10–15). Recently, we also found that NF-κB is poly(ADP-ribosyl)ated by PARP (M. Kameoka *et al.*, manuscript submitted[2]). In the present study, we found that PARP-inhibitors markedly suppressed the PMA-dependent-induction of HIV-1 mRNA in U1 cells. Further analyses with the use of HIV-1-LTR-promoted reporter plasmid revealed that the PMA-dependent, LTR-function is significantly modulated by the cellular PARP level.

MATERIALS AND METHODS

Cell lines and culture. U1 cells, which were established by Folks *et al.* and were latently infected with HIV-1LAI strain (3), J111, a human acute monocytic leukemia cell line, and Jurkat, a human acute lymphoma cell line, were cultured in a "complete medium"

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Abbreviations: PMA, phorbol 12-myristate 13-acetate; PARP, poly (ADP-ribose) polymerase; pADPR, poly (ADP-ribose); HIV-1, human immunodeficiency virus type-1; TNF-α, tumor necrosis factor-α; 3AB, 3-aminobenzamide; 3ABA, m-aminobenzoic acid; Bam, benzamide; BA, benzoic acid; 1,8NI, 1,8-naphthalimide; IQ, isoquinoline; RT, reverse transcriptase; PSL, photostimulative luminescence; RLU, relative light units; PMSF, phenylmethylsulfonyl fluoride; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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(RPMI-1640 medium supplemented with 10% fetal bovine serum) in a CO_2-incubator at 37°C. Murine lymphocytic leukemic cells, L1210 and L1210-derived PARP-deficient cell clones, Cl-3, Cl-352 and Cl-3527, which were previously established in our laboratory (16), were cultured in "complete medium" at 33°C.

Reagent. PARP inhibitors (17), 3-aminobenzamide (3AB), benzamide (Bam), and 1,8-naphthalimide (1,8NI) were purchased from Sigma Chemical Co. (MO), Nacalai Tesque Inc. (Kyoto, Japan), and Aldrich Chemical Comp. (WI), respectively. Weakly inhibitory analogs (17), 3-aminobenzoic acid (3ABA), benzoic acid (BA), and isoquinoline (IQ) were obtained from Sigma Chemical Co. (MO), Nacalai Tesque Inc. (Kyoto, Japan), and Wako Pure Chemical Industries (Osaka, Japan), respectively. PMA was obtained from Sigma. DEAE-dextran, poly(rA).oligo(dT), poly(dI-dC) were purchased from Pharmacia-Amersham (Uppsala, Sweden).

Reverse transcriptase (RT) assay. RT activity was assayed using poly(rA).oligo(dT) as a template and [^{32}P] dTTP, as described previously (18). Relative RT activity was calculated based on photostimulative luminescence (PSL) value measured by a BAS-1000 imaging analyzer (Fujix, Tokyo, Japan).

Northern blot analysis. Northern blot analysis of HIV-1 mRNA was carried out as described previously (19), with a *Hind*III fragment of HIV-1 DNA (nucleotides 531–9609 of pNL432; ref. 20) as a probe. Relative amount of HIV-1 mRNA synthesized was calculated based on PSL value of the 9.2 kb mRNA. Calibration of the assay was performed by measuring GAPDH mRNA on the blot with the use of GAPDH cDNA (Clontech Lab. Inc., CA) as a probe.

Plasmid constructs. pRSV-PARPas, a plasmid capable of generating an antisense RNA (0.75 kb) with a sequence complementary to the 5′ end of PARP cDNA, was constructed as follows: A 3.1 kb *Xho* I-*Xho* I fragment of pBXP3.0 (16), which contains a full length PARP cDNA, was placed into pUC119 (pUC119-PARP). Then, a 0.75 kb *Xba* I-*Hind* III fragment prepared from pUC119-PARP was placed into a mammalian expression vector, pRc/RSV (RDB No. 850; Riken Gene Bank, Ibaraki, Japan), in the antisense direction. pLTR-luc, containing the LTR of HIV-1 (pNL432) in the upstream of luciferase gene, was a generous gift from Dr. H. Sakai (Institute for Virus Research, Kyoto University, Japan).

Methods for DNA transfection. DNA transfection into Jurkat and J111 cells was carried using Lipofectamine Plus reagent (GIBCO BRL, MD) according to the manufacturer's protocol. DNA transfection into L1210 clones was carried out by DEAE-dextran method. Briefly, cells were suspended in 1 ml of DNA/DEAE solution (prepared to contain 7.5 μg of plasmid DNA, 50 μg/ml DEAE-dextran, 50 mM Tris-HCl buffer, pH 7.3, and 0.9 ml of RPMI-1640 medium in a total volume of 1.0 ml). The sample was incubated at 33°C for 30 min, with mixing at an interval of 10 min and, then, mixed with 1 ml of 20% (V/V) DMSO in hypertonic solution (70 mM Tris-HCl pH 7.3, 1 M sucrose, 20% PEG #6000 and 0.2 M NaCl). The sample was incubated further at 33°C for 10 min and then, the cells were washed 3 times with serum-free RPMI-1640 medium and cultured in "complete medium."

Luciferase assay. Luciferase assay was carried out using Luciferase Assay System (Pica gene LT7.5; Toyo Ink Mgf. Co., Japan). Relative light units (RLU) of luciferase expressed was measured by TR717 microplate luminometer (Perkin-Elmer, WA).

Assay for PARP activity. The transformant with a plasmid expressing PARP-antisense-RNA was collected with the use of Capture-Tec pHook-1 System (Invitrogen Corp., CA). Briefly, pRSV-PARPas and pHook-1 were co-transfected into Jurkat cells, the cells were cultured for 48 hr, and then, the transformant expressing a single chain antibody (sFv) was captured by magnetic beads coated with phOx, according to the manufacturer's protocol. A control experiment was carried out with the use of a control plasmid pRc/RSV and pHook-1. The assay of the full activity of PARP in the cells was carried out under the supplementation of enough amounts of nicked DNA to the reaction mixture as described previously (16).

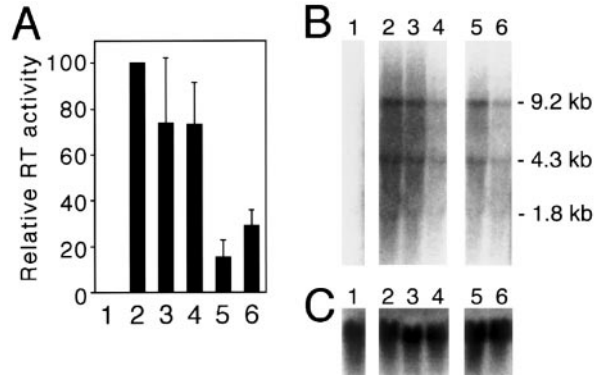

FIG. 1. PARP-inhibitors suppress PMA-induced HIV-1 activation in U1 cells. (A) U1 cells were treated with (lanes 2–6) or without (lane 1) 2 nM of PMA for 24 hr. The culture medium contained either 5 mM of BA (lane 3), 3ABA (lane 4), Bam (lane 5), 3AB (lane 6) or DMSO (vehicle; lanes 1 and 2). Relative RT activities in the cultured medium were assayed as described under Materials and Methods. Error bars represent standard deviations of the mean of three independent experiments. (B and C) Northern blot analysis was carried out with total cellular RNA (15 μg) of U1 cells after treatment with (lanes 2–6) or without (lane 1) 2 nM of PMA for 24 hr. The culture medium contained 5 mM of BA (lane 3), Bam (lane 4), IQ (lane 5), 1,8NI (lane 6) or DMSO (lanes 1 and 2). The blotted samples were hybridized with the probe for HIV-1 mRNA (B) and the probe for GAPDH mRNA (C) as described under Materials and Methods.

Electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared according to the previous report (21). The double-stranded oligonucleotides with the consensus sequences of NF-κB-, SP-1- and AP-1-binding sites were purchased from Promega (WI). These oligonucleotides were end-labeled with [γ-^{32}P]ATP with T4 polynucleotide kinase (Takara Shuzo, Co., Japan) and used as the probes. Nuclear extracts (5 μg protein) were incubated in a 25 μl-reaction mixture containing 20 mM Hepes-KOH, pH 7.8, 168 mM KCl, 1 mM EDTA, 1 mM DTT, 2 mM $MgCl_2$, 8% glycerol, 0.2 mM PMSF, 2 μg of poly (dI-dC) and 0.5 ng of ^{32}P-labeled DNA probe for 25 min at room temperature. After electrophoresis, polyacrylamide gels were dried and the radioactive bands were visualized with an imaging analyzer (BAS-1000). Competition assays were performed as described previously (21).

RESULTS

Treatment of U1 Cells with PARP-inhibitors Suppressed PMA-induced HIV-1 Production

U1 cells, which were latently infected with HIV-1, slightly produced the virus when they were maintained in a culture medium with no inducing agents. Treatment of the cells with 2 nM PMA for 24 hr induced the production of a large number of HIV-1 virus as reported by other investigators (9). As shown in Fig. 1A, the PMA-treatment induced more than 100-fold of the virus production as indicated by the increase of reverse transcriptase (RT) activity in the culture medium. The PMA-induced HIV-1 production was markedly suppressed by a co-treatment with either benzamide

(Bam) or 3-aminobenzamide (3AB), which are known to be relatively specific PARP-inhibitors (17). Approximately 85 and 71%-suppression was observed with 5 mM Bam and 3AB, respectively (Fig. 1A). Weakly inhibitory analogs (17), benzoic acid (BA) and 3-aminobenzoic acid (3ABA), were less effective, although a relatively weak suppression of approximately 25% was detected. Similar suppression by PARP-inhibitors of the induction of the virus was also found when U1 cells were induced by TNF-α and Tat, which are known to be other potent inducers of HIV-1 in U1 cells (5, 7), suggesting that PARP is involved in a common step of the virus induction in U1 cells (data not shown).

Next, we examined whether PARP-inhibitors affected the transcription of viral mRNA in PMA-treated U1 cells. As shown in Fig. 1B, essentially no production of viral mRNA was detected in U1 cells without PMA-treatment and PMA induced more than 50-fold of activation of the synthesis of specific viral mRNAs of 9.2, 4.3 and 1.8 kb (22). A PARP-inhibitor, Bam (5 mM, lane 4), suppressed the PMA-induced synthesis of the viral mRNA by 66%, whereas the weakly-inhibitory analog BA was much less effective (compare lanes 2, 3, and 4 in Fig. 1B). Additional experiments with another set of a PARP-inhibitor, 1,8-naphthalimide (1, 8-NI) and a weakly-inhibitory analog, isoquinoline (IQ) (Y. Tanaka, unpublished results) were carried out. The inhibitor (1,8-NI) markedly suppressed the synthesis of HIV-1 mRNAs, whereas IQ did not (compare lanes 5 and 6 in Fig. 1B). The expression of GAPDH mRNA, measured for calibration, was affected by neither of the above treatments significantly (Fig. 1C).

These results indicated that PARP-inhibitors specifically suppressed the PMA-induced HIV-1 production in U1 cells at a step or upstream of the transcription of the viral mRNA and suggested that PARP may be involved in a positive regulatory mechanism for the transcription of HIV-1 mRNA.

PMA-induced, HIV-1 LTR-function Is Markedly Suppressed in PARP-Deficient L-1210 Cell Clones

In order to study further the mechanism how PARP is involved in regulation of the transcription of HIV-1 mRNA, we transfected a plasmid carrying HIV-1-LTR-promoted luciferase gene (pLTR-luc) to wild type L-1210 and L-1210-derived, PARP-defective cell clones, which were established in our laboratory previously (16). When the ability of LTR to respond to PMA in these cells was examined, PMA could enhance approximately 4-fold the expression of the reporter-gene, indicating that the regulatory system, involving HIV-1-LTR promoter and PMA, functioned normally in wild type L-1210 cells also (Fig. 2). The PMA-dependent activation of reporter gene, however, decreased with decreasing PARP content in the mutant cells and was almost abolished in Cl-3527, which possessed only 8% of PARP of wild type cells (Fig. 2). Furthermore, the basal level of the activity of HIV-1-LTR in this clone (as estimated from the basal expression of reporter gene in this clone without PMA-treatment) was as low as 12% of that of wild type cells (data not shown) in spite of the fact that the DNA-binding activity of NF-κB in the mutant was as much as approximately 35-fold of that of wild type cells[2]. All of these findings suggested that, besides the activity of NF-κB, PARP also was essential for the function of HIV-1 LTR.

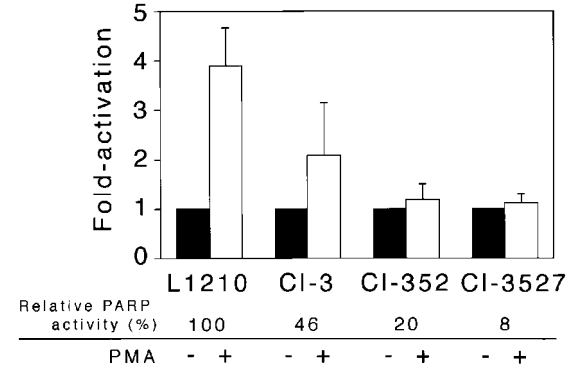

FIG. 2. PMA-responsive, HIV-1 LTR-regulated luciferase gene expression is suppressed in L1210 cell clones with reduced PARP activities. L1210 (parental), Cl-3, Cl-352 and Cl-3527 (each 4×10^6 cells) were transfected with pLTR-luc (7.5 μg) as described under Materials and Methods. After 15 hr treatment with (□) or without (■) 2 nM PMA, luciferase assay was performed and the relative value of the PMA-dependent activation was calculated as described in Materials and Methods. RLU was normalized by protein content. Relative PARP activity in each cell clone was indicated at the bottom of figure. Error bars represent standard deviation of the mean of three independent experiments.

Down-regulation of PARP by Expressing Antisense RNA in Jurkat and J111 Cells Results in Suppression of PMA-dependent Promoter Function of HIV-1 LTR

In order to confirm that cellular PARP content affect the PMA-dependent activation of HIV-1 LTR in normal human cells also, we examined whether a down-regulation of PARP by antisense RNA affects the function of the reporter plasmid in Jurkat and J111 cells or not. A transient expression of PARP-antisense RNA in Jurkat cells suppressed the PARP content to $32 \pm 10\%$ of control cells when PARP activity of transformants was assayed as described in Materials and Methods. As shown in Fig. 3, the down-regulation of PARP resulted in the suppression by approximately 60 to 70% of the PMA-stimulated activity of the reporter gene in Jurkat and J111 cells.

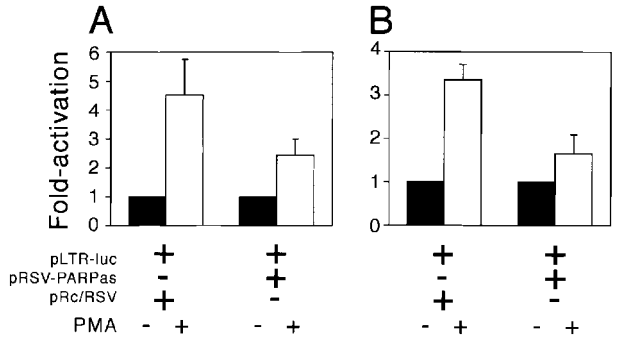

FIG. 3. Expression of antisense RNA of PARP gene suppresses PMA-enhanced, HIV-1 LTR-regulated luciferase gene expression. Jurkat (3×10^5 cells; A) and J111 (3×10^5 cells; B) cells were transfected with pLTR-luc (0.5 μg) and pRSV-PARPas (0.5 μg) or pRc/RSV (0.5 μg) as indicated in the lower panel. After the transfection, cells were cultured for 33 hr, then, each sample was divided in two separate cultures, and treated with (closed columns) or without (open columns) 2 nM PMA for 15 hr. The luciferase assay and calculation of a relative value of PMA-dependent activation were carried out as described in the legend of Fig. 2. Error bars represent standard deviations of means of 5 independent experiments.

Effect of PARP-inhibitor on PMA-induced Activation of the DNA-binding Activity of NF-κB in U1 Cells

Since it is shown that the treatment of U1 cells with PMA results in the concomitant activation of the DNA-binding activity of NF-κB in the cells and that the up-regulation is important for the gene expression of the HIV-1 (9), we examined the effect of the treatments of cells with PMA and PARP-inhibitors on the activation of NF-κB in U1 cells using EMSA. As shown in Fig. 4A, the PMA-treatment increased approximately 5-fold the DNA-binding activity of NF-κB in U1 cells. Unexpectedly, the level of NF-κB was further increased approximately 2-fold by the co-treatment with a PARP-inhibitor, Bam (Fig. 4A, lane 6), while a non inhibitory analog, BA was not significantly effective (Fig. 4A, lane 4).

The EMSA of AP-1 and SP-1, examined as a control, revealed that both these transcription factors did not respond so significantly to PMA (Fig. 4B and 4C), although both Bam and BA showed a slight stimulative effect on the DNA-binding activity of SP-1 (Fig. 4C, lanes 3–6) by some unknown mechanism (Fig. 4C).

DISCUSSION

There are a few reports suggesting the involvement of PARP or pADPR metabolism in HIV-1 replication. Yamagoe, Kohda, and Oishi (23) showed that UV-induced, but not tat-mediated, expression of a HIV-1-LTR-promoted gene in HeLa cells was markedly suppressed by PARP-inhibitors, suggesting that PARP positively regulates the promoter function in the cells; it is noteworthy, however, that the suppression was not detected at the level of transcription, but observed at the level of translation. On the other hand, Uchiumi *et al.* (24) reported that an inhibitor of pADPR-glycohydrolase, tannic acid, inhibited the HIV-1-LTR function through the interaction with a tannic acid-responsive DNA element locating near the NF-κB site in HIV-1-LTR. Their results may suggest that PARP and/or poly(ADP-ribosyl)ation of a protein negatively regulates HIV-1-LTR.

Although the involvement of PARP in expression of HIV-1 gene seems to be complicated depending on the systems, including cells, inducers, and inhibitors, used for the study as mentioned above, our results revealed that (1) PARP-inhibitors strongly suppressed the PMA-induced production of HIV-1 in U1 cells (Fig. 1), (2) the suppression occurred at a level of the synthesis of viral mRNA (Fig. 1), and (3) analyses with a reporter plasmid carrying HIV-1-LTR-promoted luciferase gene indicated that a normal level of PARP is essential for the PMA-dependent LTR-function (Figs. 2 and 3).

All of these results strongly suggested that the PMA-dependent activation (and, presumably, TNF-α- and tat-dependent activation also) of the transcription of HIV-1 gene is under the positive control of PARP and that a transcription factor, which may be regulated by PARP, is involved in the activation the HIV-1-LTR in response to PMA. In this respect, several reports (10–14) suggested the involvement of PARP in transcriptional regulations of genes through an interaction of the enzyme with transcription factors and, in addition,

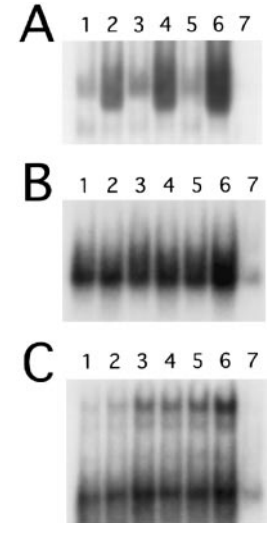

FIG. 4. The effect of PMA and PARP-inhibitors on the activation of NF-κB, AP-1 and SP-1 in U1 cells. Activation of NF-κB (A), AP-1 (B) and SP-1 (C) in U1 cells was assayed by EMSA with the nuclear extracts. The cells were treated with DMSO (lanes 1 and 2), BA (5 mM; lanes 3 and 4) or Bam (5 mM; lanes 5 and 6) for 18 hr followed by the treatment with (lanes 2, 4, 6 and 7) or without (lanes 1, 3 and 5) 2 nM of PMA for 1 hr. Competition with 100-fold excess amounts of respective cold probe (shown in lane 7) was examined with the nuclear extracts used in lane 2.

it has been shown that the PMA-induced production of HIV-1 in U1 cells is controlled through the activation of NF-κB (9). Concerning PARP/NF-κB relation, we recently found that down-regulation of PARP in L-1210 cells resulted in a marked up-regulation of the DNA-binding activity of NF-κB and that PARP poly(ADP-ribosyl)ated NF-κB proteins and suppressed the DNA-binding activity *in vitro*[2].

In the present study we observed that the DNA-binding activity of NF-κB was increased approximately 5-fold by the treatment of U1 cells with PMA. A PARP-inhibitor did not suppress but further stimulated approximately 2-fold the activation of NF-κB (Fig. 4). We also observed that the basal level of the expression of reporter gene in PARP-defective Cl-3527 cells was about 12% of that in wild type cells (data not shown) in spite of the fact that activated NF-κB in the mutant was approximately 35-fold of that in wild type cells. These data may be interpreted as that both the activation of NF-κB and the presence of a certain level of PARP in a cell are prerequisite for the function of HIV-LTR.

Although we do not have, so far, enough evidence to explain how PARP participates in the NF-κB-mediated activation of HIV-1 LTR, we recently found that PARP poly(ADP-ribosyl)ated p65 subunit of NF-κB *in vitro* and that the modification markedly suppressed the DNA-binding activity of p50/p65 heterodimer and p65/p65 homodimer of NF-κB[2]. Thus, a feasible scheme to explain the suppressive effect of PARP-inhibitors on PMA-induced HIV-1 production in U1 cells may be that poly(ADP-ribosyl)ation of NF-κB is required at some step of the NF-κB-dependent activation of HIV-1 LTR although another possibility that PARP may interact with other unidentified factor(s), which is involved in the PMA-dependent activation of HIV-1-LTR besides NF-κB, cannot be excluded. Further study with the use of a purified transcription system *in vitro* may be required to clarify the complicated effect of PARP, PARP-inhibitors, and pADPR metabolism on the function of HIV-1-LTR.

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