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Post-Integrational DNA Repair of HIV-1 Is Associated with Activation of the DNA-PK and ATM Cellular Protein Kinases and Phosphorylation of Their Targets

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Abstract—Integration of the DNA copy of HIV-1 genome into the cellular genome results in series of damages, repair of which is critical for successful replication of the virus. We have previously demonstrated that the ATM and DNA-PK kinases, normally responsible for repairing double-strand breaks in the cellular DNA, are required to initiate the HIV-1 DNA postintegrational repair, even though integration does not result in DNA double-strand breaks. In this study, we analyzed changes in phosphorylation status of ATM (pSer1981), DNA-PK (pSer2056), and their related kinase ATR (pSer428), as well as their targets: Chk1 (pSer345), Chk2 (pThr68), H2AX (pSer139), and p53 (pSer15) during the HIV-1 DNA postintegrational repair. We have shown that ATM and DNA-PK, but not ATR, undergo autophosphorylation during postintegrational DNA repair and phosphorylate their target proteins Chk2 and H2AX. These data indicate common signaling mechanisms between the double-strand DNA break repair and postintegrational repair of HIV-1 DNA.

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Keywords: HIV-1, DNA-PK, ATM, kinases, DNA repair, post-integrational DNA repair

INTRODUCTION

Life cycle of the human immunodeficiency virus type 1 (HIV-1) is a complex and multistage process aiming at integration of the viral genetic material into the cell genome and production of new virions. In the first stages after the virus penetrates into the cell, its RNA genome is converted into a double-stranded DNA (cDNA) under the action of the viral reverse transcriptase. This cDNA in complex with another important viral enzyme, integrase, is transported into the cell nucleus, where the integrase catalyzes integration of the cDNA into the cell genome. This process, termed integration, results in emergence of damages in the cellular DNA at the sites of insertion of the viral cDNA: five-nucleotide long single-stranded regions flanking the integrated cDNA, as well as unpaired dinucleotides CA at the 5′-ends of cDNA [1-3].

These damages must be repaired in order to complete integration, restore integrity of the genome, and ensure virus replication [4]. The process of damage repair and restoration of genome integrity has been termed postintegrational repair (PIR). It is known that cellular proteins play a crucial role in PIR [4-6], however, precise mechanism of this process has not been elucidated yet, as well as there is no consensus on the

Abbreviations: ATM, ataxia telangiectasia mutated protein; ATR, ataxia telangiectasia and Rad3-related protein; cDNA, DNA copy of HIV-1 genome; Chk1 and Chk2, checkpoint kinases 1 and 2; DNA-PK, DNA-dependent protein kinase; DNA-PKcs, catalytic subunit of DNA-PK; PIR, postintegrational DNA repair.

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issue, which particular cellular proteins fulfill repair of DNA damages and restoration of the genome integrity.

In the early 2000s certain indications of possible participation of protein kinases from the PIKK family (key regulators of the double-strand DNA breaks repair) in replication of HIV-1 appeared in the literature. Those included the DNA-dependent protein kinase (DNA-PK) consisting of the proteins Ku70, Ku80, and catalytic subunit of DNA-PK (DNA-PKcs), and ataxia telangiectasia mutated protein (ATM) [7-10]. However, it remained unclear in what stage of the viral life cycle these proteins participate. In particular, participation of these proteins in the PIR process seemed doubtful, because single-strand lesions appear in the DNA as a result of integration, but not double-strand DNA breaks. Nevertheless, we were able to demonstrate that the cellular proteins Ku70, Ku80, DNA-PKcs, and ATM participate in the PIR process; moreover, we established that recruitment of these proteins to the DNA damage sites occurs due to formation of the complex between the HIV-1 integrase and Ku70 subunit of DNA-PK [11, 12]. Inhibitors of phosphorylating activity of both DNA-PK and ATM suppressed virus replication by affecting the stage of postintegrational repair of HIV-1 DNA [11, 12]. This allowed us to conclude that both protein kinases, DNA-PK and ATM, are activated as a result of interaction with integrase, and thus initiate the processes of DNA repair and regulation of cell cycle. However, it is still unclear, which targets of these kinases are phosphorylated during PIR, and to what extent this process in general resembles the repair of DNA double-strand breaks. In this study we examined whether the standard targets of the cell response to DNA double-strand beaks orchestrated by the ATM and DNA-PK protein kinases are phosphorylated during HIV-1 PIR. Additionally, phosphorylation status of the third protein kinase from the PIKK family, ATR (ataxia-telangiectasia and Rad3-related protein) and its main target, Chk1, was examined. This kinase initiates repair of single-strand breaks in DNA, such as, for example, during replication fork stalling, but does not participate in the PIR process. Hence, we evaluated accumulation of phosphorylated forms of the kinases themselves (pSer2056-DNA-PKcs, pSer1981-ATM, and pSer428-ATR) and of their main downstream targets (pSer345-Chk1, pThr68-Chk2, pSer139-H2AX, and pSer15-p53) in response to cell transduction with the lentiviral vector based on HIV-1. It was established that both the protein kinase ATR and its target, checkpoint kinase 1 (Chk1), are practically not modified in the course of PIR, while ATM and DNA-PKcs undergo autophosphorylation. All tested targets of ATM and DNA-PK (Chk2, H2AX, and p53) are phosphorylated at the sites indicated above. It is important to note that phosphorylation of ATM, DNA-PKcs, checkpoint kinase 2 (Chk2), and H2AX histone occurs only in the case of successful integration and following formation of the complex of integrase with Ku70, whereas phosphorylation of p53 is not associated with integration, but occurs in response to accumulation of the linear form of HIV-1 cDNA. We also succeeded to demonstrate for the first time that the phosphorylated histone H2AX forms loci in the nuclei of transduced cells typical for the DNA double-strand break repair, moreover, their numbers and average intensity of fluorescence correlate with the PIR efficiency and could be used as markers of the efficiency of this process.

MATERIALS AND METHODS

Cell cultivation and production of VSV-G pseudotyped replication-defective vectors based on HIV-1 genome. HEK293T cells were cultivated in a DMEM medium containing 10% FBS, 100 units/ml of penicillin and 100 μg/ml of streptomycin in atmosphere of 5% CO_2 at 37°C. To produce VSV-G pseudotyped replication-defective lentiviral vectors based on HIV-1 genome with natural variant integrase (HIV_wt), HEK293T cells were co-transfected using calcium-phosphate method with plasmids pCMV-VSV-G (Addgene, USA; #8454), pCMVΔR8.2 (Addgene; #12263), and LeGo-G/BSD (Addgene; #27354) at a mass ratio 1 : 2 : 3. In order to assembly a vector with mutant forms of integrase HIV_mut, HIV_E152A, HIV_F185A the pCMVΔR8.2 plasmid was replaced with a similar plasmid containing sequence with an indicated mutation obtained in our laboratory. Culture medium was replaced with a fresh one 6 h after transfection. Lentiviral vectors were collected 48 and 72 h after transfection. Vectors were concentrated with ultracentrifugation using a fixed-angle rotor Type 45 Ti (Beckman, USA) for 2 h at 30,000*g* and 4°C.

Determination of titer of lentiviral vectors. To determine titer of a lentiviral vector HIV_wt, 100,000 HEK293T cells were placed into wells of a 24-well plate (Corning, USA). A series of 10-fold dilutions of the vector were prepared and 5-μl aliquots of the initial sample and dilutions were used for transduction of HEK293T cells 24 h after the start of cultivation. Percent of GFP-positive cells (containing green fluorescent protein, GFP) was evaluated in the cell populations 48 h after the transduction using a CytoFlex flow cytometer (Beckman); concentration of transducing units in the sample was calculated based on these data. Given that the HIV_mut vectors, HIV_E152A and HIV_F185A, contain mutant forms of integrase in their composition and one of the early stages of the HIV-1 cycle is disrupted in them, it was not possible to determined titer of pseudoviral particles in this case with flow cytometry. That is why the titer was determined using ELISA of HIV-1 p24-antigen (Vektor-Best, Russia).

Western-blot assay. HEK293T cells (1 million) were transduced with the lentiviral vector HIV_wt or with an equivalent amounts of lentiviral vectors HIV_mut, HIV_E152A or HIV_F185A using multiplicity of infection (MOI) of 10. After 2 h medium with unbound vector was removed, cells were washed twice with a 1× PBS (phosphate buffered saline, pH = 7.4), followed by placing cells were into a culture medium. Ten hours after transduction cells were lysed at 4°C with a RIPA-buffer (Servicebio, China) supplemented with Phosphatase inhibitor cocktails 2 and 3 (Sigma, USA), as well as with a Halt protease inhibitor cocktail (Pierce, USA). Prior to analysis cell debris was removed with ultracentrifugation (14,000*g*, 10 min at 4°C). For each experimental point protein amount was assayed in the samples using a DC protein assay kit (Bio-Rad, USA); equal protein amounts were applied onto the gel. Analyzed protein samples were separated in a gradient (4-15%) Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad) and transferred onto a PVDF-membrane (Bio-Rad) using a semidry transfer procedure with a Trans-Blot Turbo Transfer System (Bio-Rad). Investigated proteins were visualized using primary mouse antibodies against phosphorylated form of p53 (pSer15), and primary rabbit antibodies against DNA-PK (pSer2056), ATM (pSer1981), ATR (pSer428), Chk1 (pThr68), Chk2 (pSer345), γH2AX (pSer139) (Cell Signaling Technology, USA). HRP-conjugated secondary antibodies against rabbit and mouse antibodies were next used (Cell Signaling Technology). Visualization of target protein bands was carried out with the help of a horseradish peroxidase Clarity Western ECL substrate (Bio-Rad) using a ChemiDoc MP system (Bio-Rad).

To evaluate effects of DNA-PK and ATM activity on accumulation of the phosphorylated forms of p53 (pSer15) and H2AX (pSer139), inhibitors of DNA-PK (Nu7441, Sigma) and ATM (Ku-55933, Sigma) were used during cell transduction with lentiviral vectors at concentrations providing 50% inhibition of PIR [12]. Inhibitors were dissolved in dimethyl sulfoxide. Dimethyl sulfoxide in the medium in all experiments was maintained at the level of 0.5%.

Evaluation of formation of γH2AX loci. HEK293T cells were seeded onto cover slips treated with fibronectin, 24 h later cells were transduced with lentiviral vectors HIV_wt, HIV_mut, HIV_E152A, HIV_F185A at MOI = 15. Twelve hours after transduction cells were fixed with a 4% solution of paraformaldehyde (PFA) in 1× PBS for 15 min at room temperature. In parallel with the indicated samples, a sample of non-transduced cells was prepared to evaluate endogenous level of the loci (negative control), as well as cells treated with 50 μM etoposide for 1 h (positive control). Staining of the samples was carried out using protocol for a HCS DNA Damage Kit (Invitrogen, USA). At the last stage, samples were embedded into a Mowiol mounting medium (DAPI 1 μg/ml, DABCO 1 mg/ml, Sigma). Fluorescence of Alexa Fluor 555 and DAPI was assessed with an Eclipse-Ti2 microscope (Nikon, Japan) equipped with a 60×/1.4 objective and sCMOS-camera Neo (Andor, Ireland) (pixel effective size – 110 nm). Enumeration of loci number and locus intensity was carried out with the ImageJ software.

RESULTS

DNA-PKcs, ATM, and their targets Chk2, H2AX, and p53 are phosphorylated in response to cell transduction with vectors based on the HIV-1 genome. Previously, when studying the possible participation of members of the PIKK kinase family in the early stages of HIV-1 replication, particularly in the PIR regulation, we have shown that the PIR process depends of phosphorylating activity of ATM and DNA-PK, but not of ATR [12]. Considering that these two kinases usually are activated in response to DNA double-strand breaks [13], and that there are no such breaks in the product of integration of HIV-1 cDNA, it was only logical to assume that the following spectrum of cellular reactions could differ from the reactions typical for the DNA double-strand break repair. That is why it was necessary to test whether the well-known targets of ATM and DNA-PK are phosphorylated when cells are treated with the lentiviral vectors based on the HIV-1 genome.

We considered the DNA-PKcs and ATM enzymes themselves as such targets, since they are autophosphorylated upon activation, as well as their downstream targets Chk2, p53, and the repair histone H2AX. In addition, we decided to analyze phosphorylation status of ATR (autophosphorylated upon activation) and its target Chk1 as a negative control, because the ATR kinase does not participate in the HIV-1 replication and, hence, should not be activated during PIR. HEK293T cells were transduced with the VSV-G pseudotyped replication-defective lentiviral vectors based of the HIV-1 genome with wild-type integrase variant (HIV_wt), and after 12 h phosphorylation status of Ser2056 in DNA-PKcs, Ser1981 in ATM, Thr68 in Chk2, Ser139 in H2AX, and Ser15 in p53, as well as of Ser428 in ATR, and Ser345 in Chk1 was examined. Non-transduced cells were used as a control (Fig. 1). Analysis of phosphorylated forms was carried out 12 h after transduction, because the maximum efficiency of PIR is observed during 10-18 h after transduction [12].

In the samples treated with the HIV_wt vector phosphorylated forms of pSer2056-DNA-PKcs, pSer1981-ATM, pThr68-Chk2, pSer139-H2AX (γH2AX) were detected, as well as of pSer15-p53; moreover, their amounts were significantly higher than the background levels in the control samples (Fig. 1). Under these

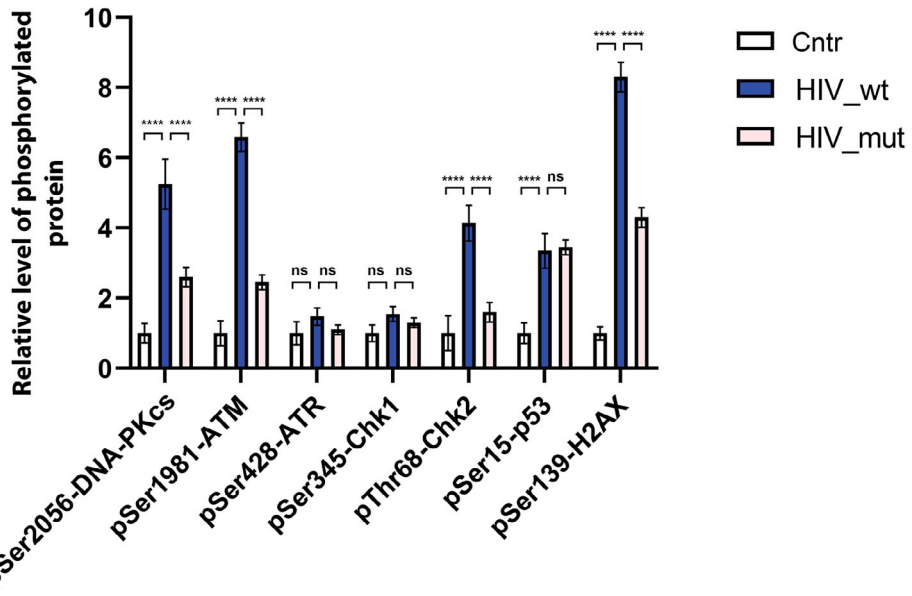

Fig. 1. Analysis of the levels of phosphorylation of DNA-PKcs, ATM, ATR, Chk1, Chk2, p53, and H2AX in the cells transduced with HIV_wt, HIV_mut, and in the non-transduced control (Cntr) 12 h after addition of lentiviral vectors. Relative phosphorylation levels of the proteins in the cells transduced with HIV_wt or HIV_mut in comparison with the non-transduced control are shown in the graph (mean ± SD, n = 3). Statistically significant differences in the changes of phosphorylation levels of the proteins were assessed with the help of two-way ANOVA with Tukey test for multiple comparisons; ns, no statistically significant differences observed; **** p-value < 0.0001; ns, no statistically significant differences observed.

conditions, as expected, neither ATR, not its target Chk1 were practically phosphorylated.

Changes of phosphorylation status of the indicated proteins could be caused both by the PIR process in the course of which DNA-PK and ATM are phosphorylated, and by other factors not associated with PIR. In order to understand whether the protein modifications are caused by PIR, we additionally analyzed phosphorylation status of these targets in the cells transduced with the HIV_mut vector encoding the integrase with E212A/L213A amino acid substitutions preventing its interaction with Ku70 [14]. Such mutations in integrase disrupt PIR stage of the HIV-1 life cycle [11]. In this case almost all targets, with exception of p53, demonstrated lower levels of phosphorylation (Fig. 1).

Accumulation of γH2AX and pSer15-p53 occurs via two independent pathways. To elucidate the reasons for differences in phosphorylation of H2AX and p53 during cell transduction with HIV-1-based vectors, we also constructed the HIV_E152A vector encoding catalytically inactive form of integrase (E152A substitution), which is unable to integrate the viral cDNA [15], and the HIV_F185A vector encoding integrase with F185A substitution. This amino acid substitution prevents integrase binding to the HIV-1 reverse transcriptase, which is required for correct reverse transcription [16]. As a result of cell transduction with the HIV_E152A vector, a double-stranded linear cDNA is accumulated in the cells, but it cannot be integrated into the host cell genome, while in the case of the HIV_F185A vector reverse transcription does not occur in the cells, and the cells only contain the viral RNA genome.

The HEK293T cells were transduced with the HIV_wt, HIV_mut, HIV_E152A, or HIV_F185A vectors, and after 12 h amounts of γH2AX and pSer15-p53 were determined in the cell lysates using Western blotting (Fig. 2). H2AX histone was effectively phosphorylated only in the case of HIV_wt, while phosphorylation of p53 occurred in all cases, when cDNA was synthesized in the cells, that is in the cases of cell transduction with HIV_wt, HIV_mut and HIV_E152A. Hence, phosphorylation of p53 depended neither on formation of the complex of integrase with Ku70, nor on the capability of integrase to insert viral cDNA into the cell genome; however, phosphorylation of p53 was not observed cells were transduced with the HIV_F185 vector, which is incapable of reverse transcription. It could be suggested based on these data that the presence of linear double-stranded DNA in the transduced cells is the signal for phosphorylation of Ser15 in p53.

Kinetics of γH2AX and pSer15-p53 accumulation during cell transduction with HIV_wt, HIV_mut, and HIV_E152A in the time period from 10 to 18.5 h after transduction, when maximum efficiency of PIR is observed, was also examined [12]. In the case of HIV_wt pseudovirus high level of H2AX phosphorylation was observed already 10 h after transduction, and it was maintained over entire PIR period up to 18.5 h (Fig. 3). Accumulation of γH2AX was also observed during cell

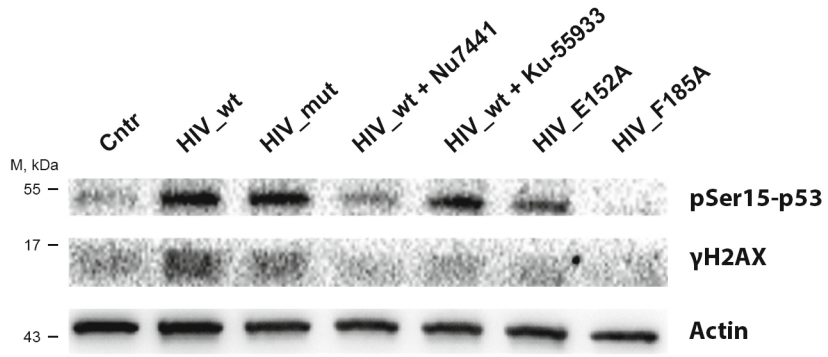

Fig. 2. Western blot analysis of the phosphorylated forms of p53 (pSer15) and H2AX (pSer139). Cntr – non-transduced cells; HIV_wt, HIV_mut, HIV_E152A, HIV_F185A – cells transduced with the HIV-1 based vectors with natural form of integrase or with the corresponding mutants forms; samples HIV_wt + Nu7441 and HIV_wt + Ku-55933 were additionally treated with inhibitor of DNA-PKcs (Nu7441, 2 μM), or with inhibitor of ATM (Ku-55933, 5 μM) after transduction with the HIV_wt vector.

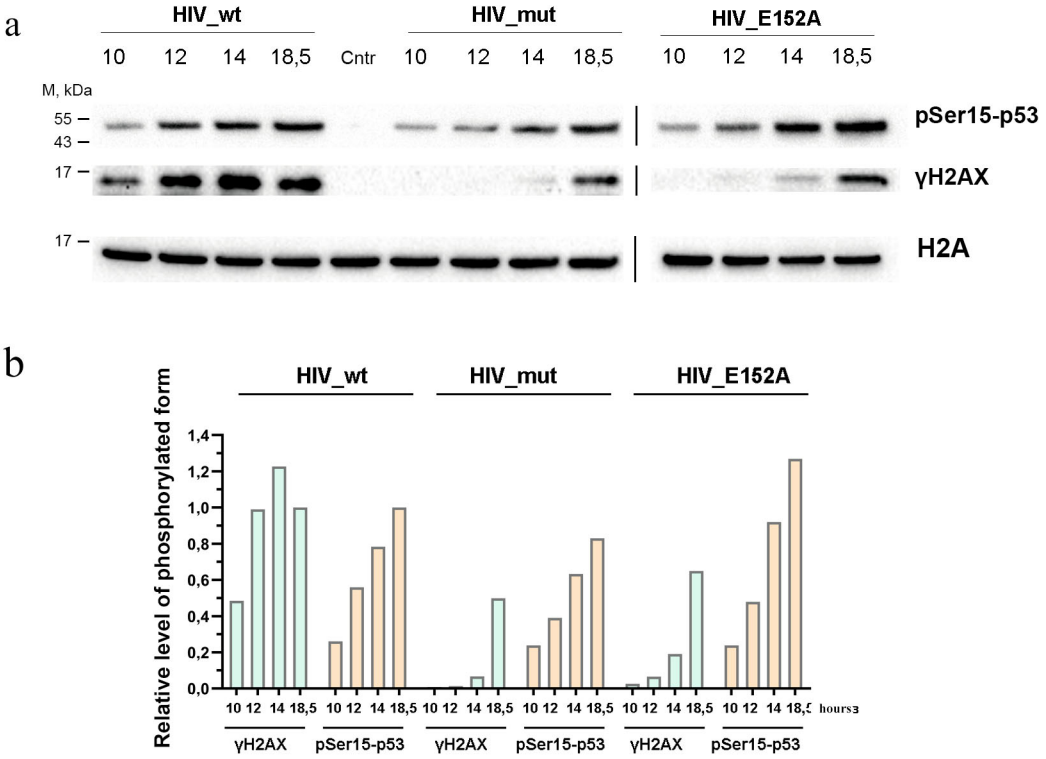

Fig. 3. Kinetics of accumulation of γH2AX and pSer15-p53 in the HEK293T cells transduced with HIV_wt, HIV_mut, or HIV_E152A vectors 10, 12, 14, and 18.5 h after transduction. a) Western blot analysis; b) quantification of the results of Western blotting. Level of γH2AX and pSer15-p53 in the cells transduced with HIV_wt 18.5 h after vector addition was accepted as 1. Control sample (Cntr) was not transduced with the vector.

transduction with the HIV_mut vector, but the profile of its accumulation was significantly different: after 10 h the level of γH2AX slightly exceeded the background level, then it gradually increased, reaching a maximum at 18.5 h. Similar profile of γH2AX accumulation was observed for the HIV_E152 vector (Fig. 3). Phosphorylation of H2AX during cell transduction with HIV_mut and HIV_E152A is likely due to accumulation of the linear cDNA in the nuclei of transduced cells, rather than DNA damage resulting from integration, as in the case of HIV_wt. The profiles of pSer15-p53 accumulation are identical for all three types of the vectors used: 10 h after transduction the level of the phosphorylated form of the protein slightly exceeds control levels, then gradually increases, reaching a maximum at 18.5 h (Fig. 3).

The results presented above demonstrate that phosphorylation of p53 at Ser15 occurs not at the stage of PIR; however, the question of participation of ATM and DNA-PK kinases in this modification remains open. To resolve this issue, accumulation of the

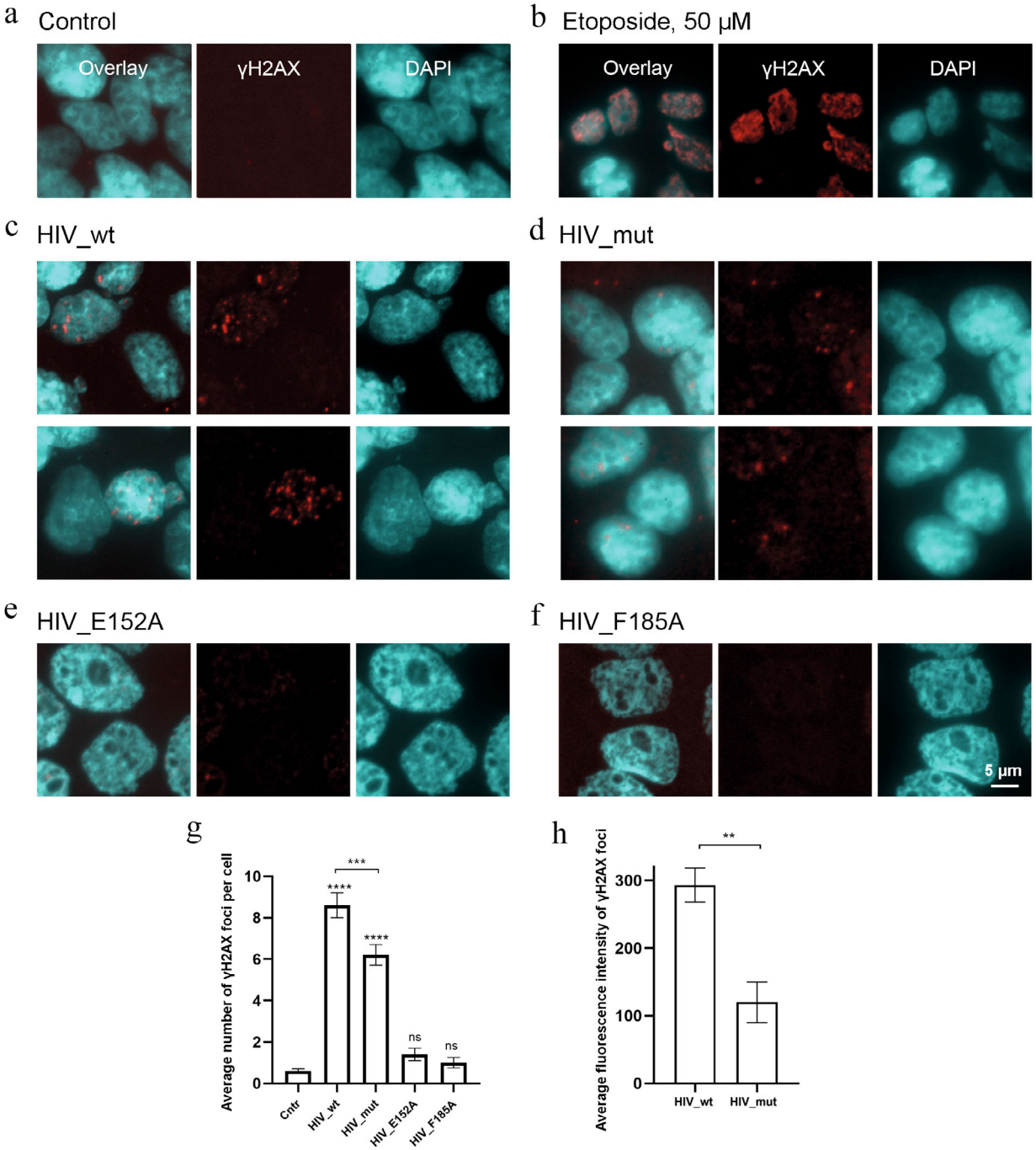

Fig. 4. Accumulation of γH2AX loci in the HEK293T cells transduced with vectors based on the HIV-1 genome. a-f) Confocal images of γH2AX loci in the HEK293T cells: not treated (a), treated with 50 μM etoposide for 1 h prior to fixation (b), transduced with vectors HIV_wt (c), HIV_mut (d), HIV_E152A (e), HIV_F185A (f) at multiplicity of infection (MOI) equal to 10. Fixation was carried out 12 h after transduction. g) Average number of γH2AX loci per one cell (in total 5000 cells was analyzed in three technical repeats for each treatment); statistical significance of differences was evaluated using ANOVA with Tukey test for multiple comparisons; ns, no statistically significant differences observed, *** p-value < 0.001, **** p-value < 0.0001; h) average fluorescence intensity of γH2AX loci; statistical significance of differences was evaluated with the help of t-test, ** p-value < 0.01.

phosphorylated form of p53 in the case of HEK293T cells transduction with the HIV_wt vector in the presence of inhibitors of DNA-PKcs (Nu7441) or ATM (Ku-55933) was examined. Both inhibitors effectively suppressed accumulation of γH2AX (Fig. 2). Phosphorylation of p53 was effectively suppressed by the DNA-PKcs inhibitor, while the ATM inhibitor affected this process to a significantly lower extent (Fig. 2).

Hence, only H2AX is specifically phosphorylated in response to integration of the viral cDNA and PIR.

Phosphorylation of p53 occurs in response to accumulation of the linear cDNA of HIV-1 in the transduced cells, although depends on activity of DNA-PKcs.

Accumulation of γH2AX loci in the nuclei of transduced cells could serve as a marker of PIR efficiency. Treatment of cells with ionizing radiation or agents causing appearance of double-strand breaks in the cellular DNA results in emergence of the γH2AX loci in the cell nuclei. This process is mainly under control of the ATM and DNA-PK protein kinases [17]. Each locus contains at least several hundreds γH2AX molecules, and number of loci correlates with the number of double-strand breaks at least at the early stages of repair [18]. In our case, we observed an increase in the level of phosphorylation of the H2AX histone at Ser139 in response to retroviral integration, so we decided to test whether the γH2AX loci are formed in the nuclei of transduced cells, and whether their formation depends of the ability of integrase to interact with Ku70.

For this purpose, the HEK293T cells were transduced with the HIV_wt vector at MOI = 10 or with the similar amount (based on p24 content) of the HIV_mut, HIV_E152A, and HIV_F185 vectors; 12 h after transduction, amount of the γH2AX loci was analyzed in the cells using immunocytochemical technique and antibodies against pSer139-H2AX followed by visualization of the formed complexes by confocal microscopy. Only in a small fraction of the cells not treated with lentiviral vectors single γH2AX loci were detected (Fig. 4a). To confirm functionality of antibodies used in this study, we analyzed accumulation of γH2AX in the nuclei of cells treated for 1 h prior to fixation with etoposide (50 μM), known inhibitor of topoisomerase II, which induces accumulation of DNA double-strand breaks. Under these conditions practically entire cell nuclei were stained with the antibodies against γH2AX (Fig. 4b), which indicates multiple DNA damages occurring under this etoposide concentration.

Formation of the clearly visible γH2AX histone loci with varying intensity was observed 12 h after cell transduction with the HIV_wt vector (Fig. 4c). In the cases of cell treatment with the HIV_E152A and HIV_F185A vectors, the level of γH2AX was practically undistinguishable from the level in the control cells (Fig. 4, e and f). Transduction of the cells with the HIV_mut vector not capable of initiating efficient PIR also resulted in formation of the γH2AX loci (Fig. 4d), but their average number per cell and average fluorescence intensity of the loci were 1.4- and 2-fold lower, respectively, than in the case of HIV_wt (Fig. 4, g and h).

Hence, the data obtained using confocal microscopy and Western blotting on the differences in accumulation of γH2AX in the cells transduced with pseudoviruses with wild type integrase and integrase defective in Ku70 binding, allow concluding that γH2AX could be considered as a marker of the efficiency of retroviral PIR.

DISCUSSION

Proteinases from the PIKK family (PI3K-related kinases), ATM, DNA-PK, and ATR, are key regulators of the cellular response to DNA damage. ATM and DNA-PK are activated in response to DNA double-stand breaks, and ATR – in response to extended single-strand DNA regions associated with RPA-protein (replication protein A). Recognition of these damages and activation of the corresponding kinases occur with participation of the sensors of indicated DNA damages. DNA double-strand beaks are recognized by the Ku70/Ku80 heterodimer or by the MRN-complex that recruit DNA-PKcs or ATM, respectively, to the damaged sites. ATR is attracted to extended single-strand DNA regions through the ATRIP cofactor [19]. After activation, these kinases phosphorylate a wide spectrum of protein targets, creating optimal conditions for DNA repair [19-22]. Two of these kinases, ATM and DNA-PK, in addition to signalling function, can directly trigger DNA double-strand break repair processes. In particular, the DNA-PK complex initiates the process of non-homologous end joining (NHEJ), and ATM – homologous recombination (HR) [13]. Despite such strict specialization of each of the kinases, current data suggest that all three kinases can mutually affect each other, complementing or modulating each other's activities depending on the context in which the DNA damage occurs [13, 17, 23-28].

When double-stranded DNA breaks occur in a cell and the DNA-PK complex is attracted to them, its catalytic subunit, DNA-PKcs, undergoes autophosphorylation at amino acid residues located in the PQR and ABCDE clusters [29]. Phosphorylation of the S2056 residue located in the PQR-cluster is the most studied DNA-PKcs modification. It is used as a marker of DNA-PK activation [30], but, in addition to this, it regulates the protein conformation and activity [31, 32]. In the case, when ATM kinase is attracted to the site of DNA damage, similarly to DNA-PK, it undergoes autophosphorylation at S1981, and this results in its dimerization and transition into the active state [33, 34]. After activation, DNA-PKcs and ATM are capable of mutual regulation of each other activity through modification of additional sites [13, 28], and also phosphorylate a number of important targets directly or via activation of mediators. Among those, proteins Chk2, p53, and reparative histone H2AX attract most attention; accumulation of the phosphorylated forms of these proteins (pThr68-Chk2, pSer15-p53, and pSer139-H2AX) occurs in response to genotoxic stress [17, 35-38]. While phosphorylation of Chk2 and p53 is required for cell cycle arrest and creation of favorable conditions for DNA repair through regulation of the p53-dependent genes [39], phosphorylation of the histone H2AX at the sites of DNA damage regulates DNA repair directly, because the phosphorylated form of the histone serves

as a scaffold attracting repair factors to the DNA damage sites and retaining them there until the damage is removed [40].

Similarly to ATM and DNA-PK, ATR is autophosphorylated at S428 during its activation in response to the presence of extended single-strand DNA regions [41]. The activated form of ATR phosphorylates Chk1. ATR also is capable to stimulate phosphorylation of H2AX and p53 under oxidative stress, but thorough activation of ATM [42].

Integration of the HIV-1 cDNA into the cellular DNA results in such DNA damages as 5-nucleotide single-stranded regions flanking the inserted viral cDNA and unpaired 5′-AC-3′ dinucleotides at the 5′-ends of the viral cDNA. Successful viral replication is directly dependent on the repair of these DNA damages [11]. We have shown previously that PIR depends on ability of the HIV-1 integrase to interact with the cellular Ku70 protein, which is a part of the DNA-PK-complex, as well as on the activity of two kinases from the PIKK-family, ATM and DNA-PK, but not of ATR [11, 12]. Considering that DNA double-strand breaks are absent in the product of HIV-1 integration, and activation of ATM and DNA-PK depends on formation of the integrase complex with Ku70, we decided to elucidate if the events of phosphorylation of these kinases and their targets described previously for the repair of DNA double-strand breaks, are characteristic for PIR.

In this study we investigated formation of autophosphorylated forms of the kinases, pSer1981-ATM and pSer2056-DNA-PKcs, and of their targets, pSer15-p53, pThr68-Chk2, pSer139-H2AX, in response to transduction of the HEK293T cells with the lentiviral vectors based on the HIV-1 genome. In addition, we analyzed accumulation of the phosphorylated form of ATR (pSer428) and its target Chk1 (pSer345) during cell transduction. We have found out that the phosphorylated forms of ATM, DNA-PK, p53, Chk2, and H2AX, but not of ATR and Chk1, are accumulated in response to retroviral transduction, which once again confirms that ATP does not participate in HIV-1 replication [12].

Using the HIV_mut lentiviral vector not capable of PIR initiation, it was established that autophosphorylation of ATM and DNA-PK as well as phosphorylation of their targets, Chk2 and H2AX, depend on formation of the integrase–Ku70 complex. At the same time, phosphorylation of p53 at Ser15 did not depend on formation of this complex, because its efficiency was the same in the case of cell transduction with the HIV_wt and HIV_mut vectors. Analysis of the accumulation kinetics of pSer15-p53 during cell transduction with the HIV_wt vector, as well as with HIV_mut and HIV_E152A, in which integration is suppressed, demonstrated that the amount of the modified p53 increases gradually over the time interval 10-18.5 h reaching maximum at 18.5 h. We assume that in our system modification of p53 occurs not as a result of PIR, but due to accumulation of the linear non-integrated HIV-1 cDNA in the transduced cells. This assumption is supported by the absence of p53 phosphorylation when using the HIV_F185 vector, in which reverse transcription does not occur.

Under similar conditions, formation of γH2AX occurred efficiently only in the case of the vector capable of initiating PIR (HIV_wt); and a high level of γH2AX was observed throughout the entire period of PIR (10-18.5 h). Under cell transduction with HIV_mut and HIV_E152A γH2AX was also detected, but kinetics of its accumulation differed significantly from the samples transduced with the HIV_wt. It is likely that accumulation of γH2AX in the cases of vectors incapable of PIR or integration is caused by appearance in the cells of linear double-stranded DNA, similarly to the case of p53 modification. We could also demonstrate that γH2AX in the cells transduced with HIV_wt is not distributed diffusely in the nuclei, but forms loci typical for the process of DNA double-stand breaks repair [18]. Differences in the kinetics of accumulation of γH2AX during the cell transduction with different HIV-1-based vectors allowed suggesting that formation of γH2AX (number and average intensity of the loci) could serve as an indicator of the HIV-1 PIR efficiency.

Based on the obtained data it could be concluded that although integration of HIV-1 cDNA does not lead to formation of DNA double-strand breaks, the repair of integration-mediated damages of the cell genome

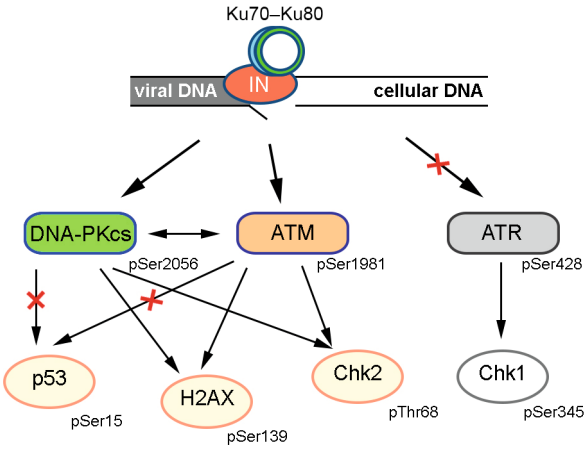

Fig. 5. Phosphorylation of the proteins from the system of cell response to DNA double-strand breaks during HIV-1 postintegrational repair. HIV-1 integrase located at the sites of integration of the HIV-1 genome and, thus marking DNA damages, attracts heterodimeric Ku70/Ku80 complex. This complex is strictly required for recruiting DNA-PK and ATM and followed activation of these kinases at the sites of DNA damages caused by HIV-1 integration. DNA-PK and ATM are autophosphorylated at pSer2056 and pSer1981, respectively. The kinases activated during PIR phosphorylate their targets, H2AX and Chk2, but not p53. ATR does not participate in PIR and is not activated.

is very similar to the DNA double-strand beak repair, at least in the initial stages. The same PIKK-kinases, ATM and DNA-PK, participate in PIR as in the NHEJ and HR processes, they initiate PIR process via autophosphorylation and phosphorylation of their targets, Chk2 and H2AX proteins, as in the cases of NHEJ and HR (Fig. 5). However, these processes exhibit significant differences. While DNA-PKcs or ATM are recruited to DNA double-strand breaks by binding of the Ku70/Ku80 heterodimer or MRN-complex, respectively, to DNA, in the case of PIR both of these protein kinases are attracted to the DNA damage sites due to binding of the Ku70 protein to the viral integrase (Fig. 5). Moreover, phosphorylation of p53 occurs during DNA double-strand break repair, while p53 is not phosphorylated during PIR. We observed production of the p53 phosphorylated form in response to accumulation of linear HIV-1 cDNA in the cells transduced with the lentiviral vectors, furthermore, DNA-PK, but not ATM, participates in formation of this phosphorylated form of p53.

CONCLUSIONS

Postintegrational repair of HIV-1 is an important step in the virus cell cycle, which is absolutely required for effective production of new viral particles. We were able to demonstrate previously that the PIR process is initiated by protein kinases from the PIKK family, ATM and DNA-PK, that usually participate in the repair of DNA double-strand breaks in the cell. However, no DNA double-strand breaks are produced during HIV-1 integration, and these kinases are recruited to the DNA damage sites due to binding of the viral integrase to the cellular Ku70 protein, which is a part of DNA-PK.

In this study we investigated status of autophosphorylation of the kinases from the PIKK family (ATM, DNA-PK, and ATR), as well as phosphorylation status of their targets (Chk1, Chk2, H2AX, and p53) during HIV-1 PIR. It has been found out that neither protein kinase ATR, nor its target Chk1 are modified, while both ATM and DNA-PKcs undergo autophosphorylation. Targets of the latter two kinases, i.e., Chk2, H2AX, and p53, are also phosphorylated when cells were transduced with lentiviral vectors based of the HIV-1 genome. However, while ATM, DNA-PK, Chk2, and H2AX were modified only in the case of successful integration followed by formation of the complex of HIV-1 integrase with Ku70; p53 was modified in response to accumulation of cDNA in the transduced cells, which did not depend on the ability of integrase to interact with Ku70. Based on the obtained data it could be concluded that although integration of the HIV-1 cDNA does not result in DNA double-strand breaks, the repair of genome damages caused by integration, at least in the early stages, is very similar to the repair of DNA double-strand breaks, while being different with respect to p53 phosphorylation.

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