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Cell Cycle Control and HIV-1 Susceptibility Are Linked by CDK6-Dependent CDK2 Phosphorylation of SAMHD1 in Myeloid and Lymphoid Cells

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Proliferating cells are preferentially susceptible to infection by retroviruses. Sterile α motif and HD domain–containing protein-1 (SAMHD1) is a recently described deoxynucleotide phosphohydrolase controlling the size of the intracellular deoxynucleotide triphosphate (dNTP) pool, a limiting factor for retroviral reverse transcription in noncycling cells. Proliferating (Ki67+) primary CD4+ T cells or macrophages express a phosphorylated form of SAMHD1 that corresponds with susceptibility to infection in cell culture. We identified cyclin-dependent kinase (CDK) 6 as an upstream regulator of CDK2 controlling SAMHD1 phosphorylation in primary T cells and macrophages susceptible to infection by HIV-1. In turn, CDK2 was strongly linked to cell cycle progression and coordinated SAMHD1 phosphorylation and inactivation. CDK inhibitors specifically blocked HIV-1 infection at the reverse transcription step in a SAMHD1-dependent manner, reducing the intracellular dNTP pool. Our findings identify a direct relationship between control of the cell cycle by CDK6 and SAMHD1 activity, which is important for replication of lentiviruses, as well as other viruses whose replication may be regulated by intracellular dNTP availability. The Journal of Immunology, 2014, 193: 1988–1997.

Sterile α motif and HD domain–containing protein-1 (SAMHD1) is a deoxynucleotide triphosphate (dNTP) triphosphohydrolase that regulates the size of the intracellular dNTP pool (1), reducing the availability of dNTP for the reverse transcriptase (RT) of incoming virus and, therefore, limiting infection (2–6). SAMHD1 was shown to block HIV-1 RT in cell culture (2, 3, 6, 7) and, recently, in samhd1-knockout mice (8, 9). HIV-2 and some SIV strains have acquired a function for the accessory protein X (Vpx) that leads to the degradation of SAMHD1 by the proteasomal machinery, increasing the dNTP pool and eliminating the cellular restriction to lentiviral RT (10).

SAMHD1 is a key element in the restriction of HIV-1 infection in myeloid cells, and suppression of SAMHD1 in quiescent CD4+ T lymphocytes enables HIV-1 infection (3). Nevertheless, SAMHD1 expression levels are similar in HIV-1–resistant cells, such as monocyes or quiescent lymphocytes, compared with HIV-1–susceptible cells, such as monocye-derived macrophages (MDMs) or activated lymphocytes (3), suggesting that SAMHD1 expression is controlled by a posttranscriptional mechanism. Phosphorylation of SAMHD1 by cyclin-dependent kinase (CDK) 6 was postulated as the regulatory mechanism for its restriction of HIV-1 infection in stable cell lines (11, 12); more recently, CDK2 and the CDK2 inhibitor p21 were shown to be regulators of SAMHD1 phosphorylation (13, 14). However, it is unclear how SAMHD1 phosphorylation is regulated and linked to cell activation and the cell cycle in primary cells, and the relative contributions of the different CDKs to phosphorylation-mediated SAMHD1 control are not known.

CDK activation is tightly controlled, depending on the moment in the cell cycle. Mammalian cell cycle progression throughout the G1 phase is controlled by signaling pathways that regulate the activities of G1, CDK4/6–cyclin D and CDK2–cyclin E/A, which are responsible for modulating the expression, activity, and stability of many cell cycle regulatory proteins, particularly in spe-
cialized, including the hematopoietic lineage (15, 16). According to the classical model of cell cycle control, CDK4 or CDK6 regulates events in the early G_{0} to G_{1} phase, CDK2 triggers the S phase, CDK2/CDK1 regulate the completion of the S phase, and CDK1 is responsible for mitosis. CDK1 was shown to block the cell cycle at the G_{2}–M phase (17), and it may be the only CDK that is required to execute the general mitotic cell cycle program. Thus, deregulation of the cell cycle is a hallmark of most human tumors and laboratory-adapted cell lines in which CDK1 may play a preponderant role in controlling the cell cycle; other CDKs may have a tissue-specific role in driving the cell cycle and cell differentiation (18).

The cellular dNTP pool, required for cell division and HIV-1 infection, is tightly controlled during the different steps of the cell cycle. In turn, HIV-1 replication is influenced by the stage of the cell cycle at the time of infection (19). It is known that cell cycle control plays a major role in determining susceptibility to HIV-1 infection (20, 21). Indeed, it was previously shown that limiting dNTP synthesis (e.g., through ribonucleotide reductase inhibition by hydroxyurea) restrains HIV-1 replication (22). In this article, we show that CDK6-dependent CDK2 phosphorylation of SAMHD1 but not CDK1, CDK4, or CDK5, is responsible for SAMHD1 deactivation, bypassing its restriction activity on HIV-1 replication in primary cells. Thus, we demonstrate that the concerted action of CDK2 and CDK6 during the cell cycle plays a major role in determining susceptibility to HIV-1 infection modulating viral dNTP access through SAMHD1.

Materials and Methods

Cells

PBMCs were obtained by Ficoll-Paque density gradient centrifugation and used for fresh purification of CD^{+} T lymphocytes by negative selection (STEMCELL Technologies) or for purification of monocytes using a negative selection 

Ab mixture (STEMCELL Technologies). Purity of the populations was confirmed by flow cytometry. CD^{+} T lymphocytes and monocytes were stained using fluorochrome-conjugated Abs against CD4 (10\%) or CD14 (>95\%) or CD14 (>80\%), respectively (1:20; BD Biosciences). CD^{+} T lymphocytes were kept in complete RPMI 1640 (Life Technologies), with or without IL-2 (16 U/ml) and PHA (4 \mu g/ml; Sigma-Aldrich). Monocytes were resuspended in complete culture medium: RPMI 1640 medium supplemented with 10% heat-inactivated FBS, penicillin, and streptomycin (all from Life Technologies). Monocytes were differentiated for 4–5 d in the presence of M-CSF (100 ng/ml; PeproTech), HEK293T cells were maintained in DMEM (Life Technologies) with 10% FBS, penicillin, and streptomycin (all from Life Technologies). Monocytes were differentiated to macrophages, as described above.

Compounds

3′-azido-3′-deoxythymidine (zidovudine; AZT) was purchased from Sigma-Aldrich. Raltegravir (Ralt) was obtained from the National Institutes of Health AIDS Reagent Program. CDK inhibitor AT7591 and CDK4/6 inhibitor 0332991 (23) were purchased from Selleckchem. All compounds received were totally anonymous and untraceable, and the only information performed for their use in assays of total viral DNA formation.

Viral fusion

The virus–cell membrane fusion was quantified as described before (28). Briefly, HEK293T cells were cotransfected with NL4-3 HIV provirus plasmid and a plasmid carrying the vpr gene fused with β-lactamase (National Institutes of Health AIDS Reagents Program) and cocultured overnight with primary CD4^{+} T lymphocytes. Cells were loaded with the CCF2-AM loading kit (Invitrogen), following the protocol provided by the manufacturer. Cells were incubated for 1 h at room temperature, washed, and immediately fixed. The change in emission of the cleaved CCF2 generated by the β-lactamase–Vpr chimera was measured by flow cytometry.

Virus infection

MDMs were pretreated with drugs or VLP_{Vpx} 24 h before infection and subsequently infected with NL4-3–GFP, as previously described (26); HIV-1 infection was measured 2 d later by flow cytometry (LSR II; BD Biosciences). Infection range showed variability among donors, ranging from 2 to 35\% GFP^{+} cells in the absence of treatment. For quantification of total viral DNA, a primer and probe set that is able to amplify both uni-integrated and integrated viral DNA was used, as described previously (29). Infections were stopped at 16 h to measure early events of viral infection. DNA was extracted using a DNA extraction kit (QIAGEN), and total viral DNA was quantified. Ct values for total viral DNA were normalized using rRNA and RT reactions were used to serve as a housekeeping gene to normalize for the ΔΔCt method. Infections were normalized to an untreated control. For total viral DNA quantification, samples treated with the RT inhibitor AZT (3 \mu M) were run in parallel to ensure that the total viral DNA measured was the product of productive infection and not a result of DNA contamination of the viral stocks. Also, Ralt (2 \mu M) was used to ensure that no post-RT steps were being quantified by the assay. CD^{+} T lymphocytes were preincubated with different inhibitors and spinoculated in the presence of the virus for 90 min at 1200 \times g. Samples for total viral DNA quantification were harvested 4 h later.

mRNA quantification

For relative mRNA quantification, RNA was extracted using an RNeasy Mini Extraction kit (QIAGEN), as recommended by the manufacturer, including the DNAse I treatment step. RT was performed using a High Capacity cDNA Reverse Transcription Kit (Life Technologies). Relative mRNA levels of different genes were measured by two-step quantitative RT-PCR and normalized to GAPDH mRNA expression using the ΔΔCt method. Primers and DNA probes were purchased from Life Technologies (TaqMan Gene Expression Assays).

Flow cytometry

For intracellular Ki-67 staining, cells were fixed for 3 min with fixation buffer (FIX & PERM; Life Technologies) before adding precooled 50\% methanol for 10 min at 4°C. Cells were washed in PBS with 5\% FBS and incubated for 30 min with the Ki-67 FITC (1:10; clone B56; BD Biosciences) Ab diluted in permeabilization buffer. For cell cycle–sorting experiments, CD^{+} T cells were incubated in 10 \mu g/ml Hoechst 33342 (Life Technologies) for 45 min at 37°C. Pyronin Y (Sigma-Aldrich) was added to a final concentration of 1.5 \mu g/ml, and cells were incubated at 37°C for an additional 45 min. The cell cycle subpopulations were identified by FACS and immediately sorted using a FACS Aria II (BD Biosciences). Cell cycle subpopulations were suspended in 0.03% saponin (Sigma-Aldrich) in PBS and then incubated in 20 \mu M 7-aminoactinomycin D (7AAD; Sigma-Aldrich) for 30 min at room temperature in the dark. Cells were kept on ice for 5 min before the addition of pyronin Y (Sigma-Aldrich) to a final concentration of 5 \mu M. After incubation for 10 min on ice, cells were directly analyzed by flow cytometry. The data were analyzed using FlowJo software.
Immunoprecipitation and immunoblotting

Treated cells were rinsed in ice-cold PBS, and extracts were prepared in lysis buffer (50 mM Tris HCl [pH 7.5], 1 mM EDTA, 1 mM EGTA, 1 mM Na3VO4, 10 mM Na β-glycerophosphate, 50 mM NaF, 5 mM sodium pyrophosphate, 270 mM sucrose, and 1% Triton X-100) supplemented with protease inhi- bitors and 1 mM PMSE. Immunoprecipitation of lysates were incubated with anti-SAMHD1 Ab (ab67820; Abcam) overnight at 4°C. The next day, lysates were incubated with Sepharose Fast Flow (Sigma-Aldrich) for 1 h, and beads were washed three times with lysis buffer and dematernalized with Læmmlini buffer or incubated for 30 min at 30°C with 80 U a- phosphatase (New England Biolabs) in the presence or absence of phosphatase inhibitors. Lysates or beads were subjected to SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Immobilon-P; Thermo Scientific). The following Abs were used for immunoblotting: anti-rabbit and phosphatase inhibitors. Lysates or beads were subjected to SDS-PAGE and Sigma-Aldrich). Anti–phospho-SAMHD1 Thr 592 (pSAMHD1 T592) was 1:1000; Cell Signaling Technologies); and anti

Determination of dNTP intracellular levels

MDMs were rinsed and lysed with trichloroacetic acid (0.5 M). Cellular proteins were cleared by centrifugation, and the supernatant was neutralized with 0.5 M Tri-n-octylamine in 1:1.2-trichlorotri fluoroethane (Sigma-Aldrich). The recovered aqueous phase was recovered and dried in a SpeedVac. Pellets were resuspended in Tris-HCl buffer (40 mM [pH 7.4]), and the dNTP content was determined using a polymerase-based method (30), with minor modifications. Briefly, 20 µl reaction mixture contained 5 µl dNTP extract in 40 mM Tris-HCl (pH 7.4), 10 mM MgCl2, 5 mM DTT, 0.25 µM oligomer, 0.75 µM [α-32P]deoxyadenosine triphosphate (dATP), 12–21 Ci/mmol (or [methyl-3H] deoxythymidine triphosphate (dTPP) for the dATP assay) and 1.7 U Thermo Sequenase DNA Polymerase (GE Healthcare). Reaction mixtures with aqueous dNTP standards were processed in parallel. After incubation at 48°C for 60 min, 18 µl of the reaction mixture was spotted on a Whatman DE81 paper and left to dry. The filters were washed three times for 10 min with 5% Na2HPO4, once with water, and once with absolute ethanol and left to dry again. The retained radioactivity was determined by scintillation counting, and dNTP amounts were calculated from interpolation on the calibration curves. To ensure the reliability of the results, triplicates of two dilutions of each dNTP extract (usually undiluted and 1:3 water diluted) were processed in each independent experiment.

Kinase assays

Recombinant His-cdk1/GST-CycB, His-cdk2/GST-CycA, and His-cdk6/GST-CycD3 complexes, purified from S213 insect cells, were used (Milli- pore). HA-SAMHD1 was immunoprecipitated from HEK293T transfected cells. A total of 0.5 µg the indicated CDK/cyclin complex was incubated with HA-SAMHD1 or 1 µg histone H1 (Roche Diagnostics). Phosphorylation reactions were carried out in kinase assay buffer (50 mM Tris-HCl [pH 7.5], 10 mM MgCl2, 2 mM MgCl2) in the presence of 50 µM cold ATP and 1 µCi/assay radiolabeled [32P]γ-ATP (3000 Ci/mmol; PerkinElmer) in a final volume of 40 µl/assay for 15 min at 30°C. Reactions were stopped by adding SBSX5 (250 mM Tris-HCl [pH 6.8], 0.5 M DTT, 10% SDS, 20% glycerol, 0.5% bromophenol blue) and boiling at 100°C for 5 min. Phosphorylated proteins were subjected to SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and exposed to Biomax XAR films (Kodak).

Statistical methods

Data were analyzed with the Prism statistical package. If not stated otherwise, all data were normally distributed and expressed as mean ± SD; p values were calculated using an unpaired, two-tailed, Student t test. For normalized data from different donors, a one-sample t test against a hypotheitical value of 1 or 100 was applied.

Results

SAMHD1 is phosphorylated in proliferating cells that are susceptible to HIV-1

Resting CD4+ T lymphocytes are resistant to HIV-1 infection by a mechanism independent of viral fusion but prior to total viral DNA formation, contrasting with PHA/IL-2–activated T CD4+ lymphocytes (Fig. 1A, Supplemental Fig. 1A). However, resting CD4+ T lymphocytes can be infected by HIV-1 carrying Vpx, which is able to degrade SAMHD1 (3) (Supplemental Fig. 1B). Only a percentage of the activated CD4+ lymphocytes enters at a cell-dividing stage, which is characterized by increased levels of total RNA and DNA content, as measured by 7AAD and pyronin staining (data not shown), and correlating with high levels of Ki67 staining, whereas quiescent (G0) CD4+ T lymphocytes are Ki67− (Fig. 1B). Although the expression of SAMHD1 in resting and activated lymphocytes is not significantly different, slow migrating forms of SAMHD1 can be observed in 6% acrylamide gels (Fig. 1C). We found that SAMHD1 has several phosphorylation sites that are responsible for the observed protein shift, which were further confirmed by a specific phospho–threonine-proline (pThr)-Pro Ab (Fig. 1C, Supplemental Fig. 2, data not shown). Lysates of activated CD4+ T lymphocytes were also positive for immunoblotting with a specific Ab recognizing the phospho-threonine at position 592 of SAMHD1 [pSAMHD1(T592)] (Fig. 1D). In addition, HIV-1–susceptible MDMs were Ki67+, as previously suggested (31), confirmed by the original population of monocyties (Fig. 1E).

DNA interference of CDK2 and CDK6 regulates SAMHD1 phosphorylation and HIV-1 RT

To better understand the effect of SAMHD1 on HIV-1 infection, the phosphorylation-dependent regulation of SAMHD1 activity was further investigated using siRNAs against well-characterized CDKs known to regulate cell cycle progression. We effectively downregulated (>60%) CDK1, CDK2, CDK4, CDK5, and CDK6 mRNAs in primary MDMs (Fig. 2A). Protein downregulation was further confirmed by Western blot for CDK1, CDK2, and CDK6 (Fig. 2B). siRNA-mediated knockdown of CDK2 and CDK6, and, to a lesser (not significant) degree, of CDK4 or CDK5, but not CDK1, led to reduced SAMHD1 phosphorylation, as measured by the disappearance of the slow-migrating form of SAMHD1 and the reduction in the pThr-Pro signal after SAMHD1 immunoprecipitation and confirmed using the specific pSAMHD1(T592) Ab (Fig. 2C–E).

CDK2 and CDK6 knockdown in MDMs led to a reduction in Ki67+ staining (Fig. 3A), suggesting that CDK2 and CDK6 control cell cycle progression and SAMHD1 deactivation, linking cell cycle control to SAMHD1 deactivation. Moreover, inhibition of SAMHD1 phosphorylation correlated with an effect on viral replication, because knockdown of CDK2 and CDK6 significantly reduced HIV-1 infection with a VSV-pseudotyped NL4–3–GFP-expressing virus (p = 0.0021 and p = 0.0060, respectively) (Fig. 3B). However, inhibition of CDK1, CDK4, and CDK5 had a minor (not significant) effect.

To identify the viral-replication step affected by CDK2 and CDK6 inhibition, total viral DNA formation after overnight in- fection was measured. Knockdown of CDK2 (p = 0.0113) and CDK6 (p = 0.0013) and, to a lesser extent, CDK4 and CDK5 (p = 0.05), but not CDK1, significantly inhibited total viral DNA formation (60% and 80%, for CDK2 and CDK6, respectively) in MDMs infected with a VSV-pseudotyped NL4–3–GFP-expressing virus (Fig. 3C) or the fully replicative HIV-1 R5-tropic strain BaL (Fig. 3D). As expected, the HIV-1 RT inhibitor AZT completely blocked total viral DNA formation, whereas the HIV-1 integrase inhibitor Ralt did not have an effect. Importantly, confirmatory siRNA sequences targeting CDK2 and CDK6 showed similar inhibitory effects on total viral DNA formation (data not shown) and HIV-1 infection (Fig. 3E).
CDK inhibitors block SAMHD1 phosphorylation and HIV-1 replication

To confirm the role of CDK in the regulation of SAMHD1 phosphorylation and HIV replication, we used a highly specific inhibitor of CDK6 and CDK4 (CDK6 inhibitor) (23) and a pan-CDK inhibitor with potent activity against CDK2 and CDK6 (CDK2/6 inhibitor) (32). Treatment of MDMs with CDK2/6 or CDK6 inhibitors led to the disappearance of SAMHD1 phosphorylation (Fig. 4A) and inhibition of HIV-1 replication (Fig. 4C) at subtoxic concentrations (data not shown). Importantly, when SAMHD1 was degraded using VLP Vpx (Fig. 4B), the inhibitory effect of CDK2/6 or CDK6 inhibitors was completely lost, indicating that their effect was mediated by SAMHD1 (Fig. 4C). This observation was confirmed at the level of total viral DNA formation when both inhibitors were used in the absence or presence of VLP Vpx (Fig. 4D). In activated CD4+ T cells, a short exposure (6 h) to CDK2/6 or CDK6 inhibitors was sufficient to restrict the formation of total viral DNA when challenged with the HIV-1 NL4-3 strain, recapitulating the phenotype of unstimulated, resting CD4+ T lymphocytes (Fig. 4E).

CDK2 is expressed and activated upon lymphocyte activation, correlating with SAMHD1 phosphorylation

We showed that SAMHD1 is fully active in resting, quiescent CD4+ T lymphocytes but is partially inactive in activated CD4+ T cells. To address the role of CDK2 in this process, we obtained four populations of CD4+ T lymphocytes consisting of resting CD4+ (Fig. 5A, upper left panel), PHA/IL-2–activated CD4+ (Fig. 5A, upper right panel), sorted quiescent CD4+ from the PHA/IL-2–activated population (G0, Fig. 5A, lower left panel), and CD4+ at the G1, M, or G2 cell cycle stage (Fig. 5A, lower right panel), as identified by RNA (pyronin A) and DNA (7AAD) staining. Activation status of the populations correlated with the percentage of Ki67+ cells (Fig. 5B, bar graph). Correspondingly, CDK2 expression was barely detectable by Western blot in both unstimulated, resting CD4+ T lymphocytes and in G0 cells sorted from the stimulated CD4+ T lymphocytes (Fig. 5B, “R” and “G0” columns). Upon activation, CDK2 was expressed and activated (seen as phosphorylation at Thr160 in the T-loop of the ATP binding site of CDK2) in cells that had entered the cell cycle (G1/M/G2) (Fig. 5B, G1/M/G2 column). Notably, expression and activation of CDK2 correlated with the phosphorylation of SAMHD1, indicating that CDK2 links cell cycle progression to SAMHD1 deactivation. Only cells that had entered the cell cycle, showing >80% Ki67+ staining (Fig. 5C), were susceptible to HIV-1 infection, measured as the formation of total viral DNA (Fig. 5D), in contrast to G0, Ki67+ cells (Fig. 5C) sorted from the same population of PHA/IL-2–activated CD4+ T lymphocytes.

CDK6 acts upstream of CDK2, which directly phosphorlyates SAMHD1

In light of the results shown above, we addressed whether SAMHD1 was a substrate of CDK2 in vitro. We found that CDK1 and CDK2 were able to phosphorylate SAMHD1. As expected, both kinases phosphorylated histone H1 (Fig. 6A, lanes 2 and 3). Phosphorylation by CDK2 was drastically reduced, but not completely abolished, when the SAMHD1(T592A) mutant was evaluated, indicating a prominent role for T592 (Fig. 6B). Nevertheless, we also observed that SAMHD1 has several phosphorylation sites that are responsible for the protein shift, which were confirmed by a specific p(Thr)-Pro Ab (Supplemental Fig. 3). Moreover, CDK2 coimmunoprecipitated with SAMHD1 when an HA-tagged SAMHD1 construct was overexpressed in 293T cells (Fig. 6C).
observed that CDK6 siRNA knockdown or blockade with the CDK6 inhibitor led to a slight reduction in CDK2 expression and a marked reduction in the activation of CDK2, measured as phosphorylation at Thr160 (Fig. 6D), indicating that CDK6 function affects CDK2.

Finally, to further confirm the direct control of CDK6 over the dNTP pool, we addressed the effect of the CDK6 inhibitor on the dNTP pool. Treatment of MDMs in the presence of the CDK6 inhibitor for 24 h led to a strong reduction in the intracellular levels of dNTPs (Fig. 6E). dATP and dTTP showed the strongest reduction upon treatment, which is consistent with the fact that their levels are tightly controlled during the cell cycle (33). Our results indicate that dTNP levels and HIV-1 replication are tightly controlled by CDK6 and CDK2 as depicted in Fig. 7.

Discussion

HIV-1 requires a coordinated action of the cell machinery to provide dNTPs for RT. In mammalian cells, the cell cycle regulation of the enzymes controlling dNTP pool sizes, ribonucleotide reductases and SAMHD1, is adjusted to the requirements of DNA replication (1). Both dNTP synthesis and destruction regulate the replication of both cell and virus genomes (34). The virus replicative cycle is tightly linked to the cell cycle control of dNTP availability. The cell cycle, in turn, is tightly controlled by the differential expression, activation, and function of CDKs. In this study, we show that, in primary macrophages and T cells, CDK6-dependent CDK2 phosphorylation of SAMHD1 appears to control SAMHD1 restriction of HIV-1.

Although CDK1 was initially suggested as the kinase responsible for SAMHD1 phosphorylation in immortalized cell lines (11, 12), CDK1 and CDK2 are closely related kinases that share many in vitro substrates (35, 36), suggesting that CDKs other than CDK1 may play a role in regulating SAMHD1 phosphorylation. SAMHD1 was reported to interact with CDK2 in a large-scale mass spectrometry approach (37) and, more recently, in small-scale experiments using lysates of stable cell lines (13), in agreement with our results (Fig. 6C). CDK dominant-negative mutants, such as those used by White et al. (12), may be problematic if kinases share the same activating partner, because expression of defective CDK1 can inhibit CDK2 by titration of S-phase cyclins (12, 17). When CDK2 is completely absent, these cyclins are available to activate CDK1 and enable it to replace CDK2 (17).
Immortalized cell lines exhibit deregulated cell cycles and abnormally rapid proliferation, so we found it relevant to address the role of SAMHD1 phosphorylation in nonstable primary human cells susceptible to HIV infection. siRNA-mediated knockdown of CDK2 and CDK6, but not CDK1, CDK4, or CDK5, in primary MDMs led to inhibition of SAMHD1 phosphorylation, total viral DNA formation, and viral replication. In addition, CDK2 was able to phosphorylate SAMHD1 in vitro more efficiently than was CDK1 (Fig. 6A), arguing against CDK1 being the only kinase responsible for SAMHD1 phosphorylation in vivo. Nevertheless, our observation that siRNA targeting CDK6 and CDK2, but not CDK1, CDK4, or CDK5, abrogated SAMHD1 phosphorylation in primary cells may provide sufficient evidence to suspect that early arrest of the cell cycle by CDK6 and CDK2 controls SAMHD1 restriction of HIV-1 replication.

Importantly, the identification of CDK6 modulation of SAMHD1 was further confirmed by the use of a selective CDK4 and CDK6 inhibitor (38). Treatment of primary cells with this CDK6 inhibitor or a pan-CDK inhibitor, active against CDK2 and CDK6, led to inhibition of SAMHD1 phosphorylation, total viral DNA formation, and virus replication. Pan-CDK inhibitors were shown to have anti–HIV-1 activity that generally has been attributed to an effect on viral transcription (39). However, we show that, inde-
CDK6-DEPENDENT CDK2 PHOSPHORYLATION OF SAMHD1

**FIGURE 5.** CDK2 links cell cycle progression to G1 and SAMHD1 phosphorylation in primary CD4+ T cells. (A) Representative flow cytometry dot plots of DNA and RNA content of resting (upper left panel) or activated (upper right panel) CD4+ T lymphocytes stained with 7AAD and pyronin. Cells at the G0 stage of the cell cycle (gray square) or at the G1, G2, or M stage (dotted square) were sorted by flow cytometry and analyzed for DNA/RNA content (lower panels). (B) Percentage of Ki67+ cells (bar graph) and Western blot analysis of the populations depicted in (A). Resting (R), activated (A), and sorted (G0, G1/M/S) populations were stained with anti-Ki67 Ab and analyzed by flow cytometry. Cell lysates were subjected to SDS-PAGE and immunoblotted with anti–phospho-CDK2 Ab raised against phospho-Thr160 [pCDK2(Thr160)], anti-SAMHD1, anti-CDK2, anti-HSP90 and anti-β-actin Abs. Representative results for one donor of three are depicted. (C) Graphs showing the percentage of Ki67+ cells in G0 (left panel) and G1/M/S populations (right panel) sorted from PHA/IL-2–activated CD4+ T lymphocytes, as indicated in (A). Isotype staining was used as a control (shaded graph) for each population. Representative graphs for one experiment of three are shown. (D) Cells shown in (C) were infected with NL4-3, and total viral DNA was measured by quantitative PCR. Data were normalized to the total viral DNA detected in untreated Ki67+ (black bars) lymphocytes. AZT (3 μM) was used as control. Data represent mean ± SD of two donors.

Pendently of additional anti-HIV activity, CDK blockade leads to inhibition of early total viral DNA formation attributed to an effect on SAMHD1 phosphorylation. Moreover, the observation that CDK inhibitors lost their effect when SAMHD1 was absent (after Vpx-induced degradation) strongly indicates the specificity of their effect. In view of our results, we suggest that SAMHD1 restriction of the RT step of HIV-1 replication in primary macrophages or T cell lymphocytes may be controlled by a pathway that is tightly linked to cell cycle control and progression, as proposed in the model in Fig. 7.

Of note, Vpx-mediated degradation of SAMHD1 induced an ~2-fold increase in the HIV-1 infection of MDMs, observed at the level of viral replication (Fig. 4C) or viral DNA formation (Fig. 4D). This is consistent with the observation that MDMs differentiated with M-CSF are not quiescent cells (Fig. 1E), show partial phosphorylation of SAMHD1 (Fig. 1F) and, therefore, are not completely resistant to HIV-1 infection, showing significant basal levels of infection (Fig. 4C). As a consequence, SAMHD1 degradation showed a 2–3-fold increase in viral replication, in contrast to the stronger effect observed between quiescent lymphocytes, which are resistant to HIV-1 infection (Figs. 1A, 4E), and activated lymphocytes (Fig. 4E).

It was suggested that phosphorylation at residue T592 of SAMHD1 does not significantly affect the catalytic dNTPase activity of SAMHD1 (12, 40). However, it is possible that SAMHD1 dNTPase activity is regulated through additional factors or requires phosphorylation of multiple phosphosites. Our work indicates that phosphorylation at T592 correlates with the overall status of SAMHD1 phosphorylation, measured as the appearance of slow migrating forms or by staining with the specific p(Thr)-Pro Ab, suggesting that this is a good indicator of SAMHD1 restriction. However, a complete study of all of the phosphorylation sites may be required to address the role of phosphorylation in the function of SAMHD1. Moreover, it was shown that the C terminus of SAMHD1 (aa 596–626) was not required for dNTPase activity in vitro but was required for full depletion of dNTPs in vivo, suggesting that in vitro catalytic activity of SAMHD1 may not always correlate directly with its ability to decrease cellular dNTP pools (41). Previous observations indicated that SAMHD1 blocks HIV-1 replication by decreasing the intracellular pool of dNTPs (2, 6, 12, 42) and, importantly, we provide evidence that a drug capable of inhibiting SAMHD1 phosphorylation decreases the intracellular dNTP pool (Fig. 6E).

It is intuitive to think that, during the cell cycle, SAMHD1 deactivation must occur prior to DNA synthesis (S phase of the cell cycle) when an elevated dNTP pool is required. Progression to the early G1 phase is required for completion of HIV-1 RT and viral replication in T cells (19). Moreover, Ki67+ effector T cells, susceptible to virus infection, accumulate in the G1 phase of the cell cycle but not in the S and G2/M phases in HIV+ individuals (43), suggesting that SAMHD1 restriction must be suppressed when cells enter the G1 phase. According to the classical model of cell cycle control, cell cycle transition from G0 to G1 by CDK4/CDK6 leads to CDK2 activation during the G1 phase that triggers the S phase (15, 17). Correspondingly, we observed that, upon activation, CDK2 is expressed and activated when resting, quiescent CD4+ T cells (Ki67−) enter the G1 phase and start proliferating (Ki67+); this correlates with the phosphorylation of
SAMHD1. Moreover, CDK2 and CDK6 knockdown led to a reduction in Ki67 staining, providing further evidence that CDK2 and CDK6 control cell cycle progression and SAMHD1 deactivation, linking cell cycle control to viral replication through SAMHD1 deactivation.

Although in vitro phosphorylation experiments (Fig. 6A) do not exclude that CDK1 complexed with other cyclins (e.g., cyclin A) may be more active and might phosphorylate SAMHD1, they clearly indicate that CDK2 also phosphorylates SAMHD1. Our results also suggest a novel function for CDK6 upstream of CDK2, consistent with the lack of in vitro phosphorylation of SAMHD1 by CDK6, providing an explanation for the effect of CDK6 knockdown by RNA interference and CDK6 inhibition on the phosphorylation of SAMHD1 and HIV-1 infection. CDK6 knockdown or treatment with a CDK6 inhibitor led to inactivation of CDK2, measured as phosphorylation at Thr160, suggesting that CDK6 is an upstream regulator of CDK2. This observation matches the cell cycle model in which CDK6, together with CDK4, is the kinase involved in the first steps of the sequential cell cycle progression from G0 to G1, which subsequently activates CDK2 through the induction of cyclin E expression (15, 18), leading to SAMHD1 phosphorylation and upregulation of the dNTP pool.

Mutations in SAMHD1 in humans cause Aicardi-Goutières syndrome, which is consistently associated with an IFN signature (44, 45), suggesting a link between SAMHD1 and the innate immune-driven IFN response. Moreover, a recent report suggested that SAMHD1 restriction triggers activation of the type-I IFN response postinfection with the retrovirus human T cell leukemia virus type 1 (46). It will be interesting to assess the possible link between innate immunity and cell cycle control or progression, maybe in response to HIV-1 infection.

Previous studies analyzing the transcriptome of cells from long-term nonprogressor HIV-1 patients found changes in several clusters of genes, including those controlling the cell cycle (47–49), suggesting that differences in how the cell cycle is regulated may play a role in the natural control of HIV infection. The observation that cdk2−/− lymphocytes may show similar proliferating capacity (15, 50) suggests that CDK2 or its upstream control by CDK6 could represent a potential target for antiviral treatment. The present data provide insight into the molecular mechanism of SAMHD1 activation and offer possible approaches for how these could be used for pharmacological intervention.
progression throughout the G1 phase is controlled by signaling pathways regulated, depending on the moment in the cell cycle. Mammalian cell cycle regulated by G1 CDK4/6-cyclin D and CDK2-cyclin E/A activity. The cell nucleotide pool required for cell division and HIV-1 infection is tightly controlled during the cell cycle. Phosphorylation of SAMHD1 by CDK2 is responsible for deactivation of the restriction activity on HIV-1 replication in primary myeloid and lymphoid cells. CDK6 may directly or indirectly regulate CDK2 activity. RNA interference or pharmacological inhibition of CDK6 or CDK2 prevents SAMHD1 phosphorylation, leading to increased dNTPs available for RT and viral replication.

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Disclosures

The authors have no financial conflicts of interest.

References


Supplementary figure 1. (A) Representative dot plots of a fusion experiment performed as indicated in Materials and methods section using resting (top panels) and activated (bottom panels) CD4+ T lymphocytes treated (top panels) or not (No drug; ND) with 1 µM CXCR4 inhibitor AMD3100. Percentage of cells in which fusion (cleavage of CCF2) is detected are shown. One representative experiment of three is shown. (B) Resting CD4+ T lymphocytes were infected with NL4-3*GFP virus which carries (NL4-3*GFP + Vpx) or not Vpx. Representative dot plots of one experiment are shown (left panel). Fold increase infection of resting CD4+ T lymphocytes using NL4-3*GFP carrying Vpx (+Vpx) compared to the same virus in the absence of Vpx was calculated for four different donors. Figure represents mean ±SD.
Supplementary figure 2. SAMHD1 was immunoprecipitated from lysates of PHA/IL-2 activated CD4+ T cells or THP-1 cells and treated with λ-phosphatase in the presence or absence of phosphatase inhibitors as indicated. Samples were run and blotted using pThr-Pro and SAMHD1 antibodies. One representative blot of three independent experiments is shown.
Supplementary figure 3. Phosphorylation of SAMHD1 (A) Schematic representation of human SAMHD1 indicating previously identified phosphorylation sites. Aminoacid sequences were obtained from UniProtKB database. Potential phospho- serines (S) or threonines (T) are shown in the graph indicating the aminoacid position (Q9Y3Z3) (B) HEK293T cells were transfected with an empty expression plasmid (empty) or the plasmid encoding the wild-type form of SAMHD1 (WT). Serines at position 18 and 33 and threonines at position 21, 25 and 592 were mutated to alanines (S18A, T21A, T25A, S33A and T592A). A plasmid with a combined mutation of S18A, T21A, T25A and S33A (S18A/T21A/T25A/S33A) was also transfected. SAMHD1 was immunoprecipitated from cell lysates and blotted using a generic phospho-threonine followed by proline antibody (pThr-Pro; upper blot). Thr-to-Ala mutation in Thr21 and Thr592 contributed to the overall signalling detected by the pThr-Pro antibody. Total SAMHD1 was used as control of immunoprecipitation. Cell lysates were also run in 6% polyacrylamide gels and SAMHD1 phosphorylation estimated by the appearance of slow migrating bands when immunoblotting with a specific anti-SAMHD1 antibody (bottom blot). Combined mutations in the N-terminal cluster of SAMHD1 led to the total disappearance of the slow migrating forms, suggesting a major contribution to the SAMHD1 phosphorylation. Hsp90 immunoblotting was used as loading control. One representative blot of three is shown.