CDK2 but not CDK1 Phosphorylates SAMHD1 in Primary Macrophages

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ABSTRACT

Background: SAMHD1 is an HIV-1 restriction factor present in lymphocytes and macrophages that is deactivated by phosphorylation. The mitotic cyclin-dependent kinase (CDK) 1 has been proposed as responsible for phosphorylation-dependent regulation of SAMHD1 activity in immortalized cells. We wanted to study whether other CDK, different from CDK1, may play a role in regulating SAMHD1 in primary monocyte-derived macrophages (MDM).

Methods: CD14+ monocytes were isolated and transfected with small interfering RNA (siRNA) against different CDK. Transfected monocytes were differentiated in macrophages using M-CSF. RNA interference was assessed by quantitative PCR (qPCR) and western blot. MDM were infected with a VSV-pseudotyped NL4-3 GFP-expressing virus or full-replicative R5 strain BaL. Proviral DNA formation was quantified by qPCR and HIV replication measured by flow cytometry. SAMHD1 phosphorylation and CDK2 activation were analyzed by immunoblotting. Recombinant CDK1, CDK2 and CDK6 were used in an *in vitro* kinase assay using HA-purified SAMHD1 in the presence of radioactive ATP.

Results: We effectively downregulated (>80 %) CDK1, CDK2, CDK4, CDK5 and CDK6 mRNA compared to mock-transfected cells or cells transfected with a non-targeting siRNA (siNT) in primary monocyte-derived macrophages (MDM). Protein downregulation of CDK1, CDK2 and CDK6 was further confirmed by western blot. siRNA-mediated knockdown of CDK2 and CDK6 but not CDK1, CDK4 or CDK5 led to reduced SAMHD1 phosphorylation measured by western blot. Similarly, siRNA knockdown of CDK2 and CDK6 but not CDK1, CDK4 or CDK5 inhibited proviral DNA formation (~60% and ~80%, respectively) in macrophages infected with a VSV-pseudotyped NL4-3 GFP-expressing virus or the fully replicative R5-tropic strain BaL. Moreover, knockdown of CDK2 and CDK6 significantly reduced HIV-1 replication (p<0.001) quantified by flow cytometry. Confirmatory siRNA sequences targeting CDK2 and CDK6 showed similar effects on infection and proviral DNA formation after HIV-1 infection. We also found that SAMHD1 is a substrate of CDK2 *in vitro*, being even more efficient than CDK1 in phosphorylating it. In contrast, we could not detect CDK6-mediated phosphorylation of SAMHD1.
However, CDK6 siRNA-mediated downregulation led to reduced activation of CDK2, suggesting that CDK6 signals upstream CDK2.

RNA INTERFERENCE of CDK2 and CDK6 INHIBITS SAMHD1 PHOSPHORYLATION

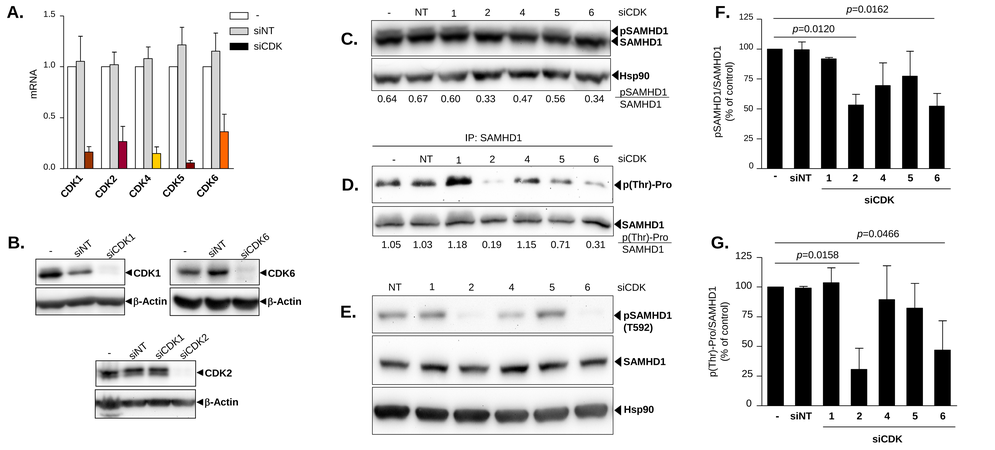


Figure 1. (A) mRNA of CDK1, 2, 4, 5 and 6 was quantified in samples of macrophages treated with siRNA targeting each of the *CDK* and normalized to samples transfected without siRNA (-). Macrophages treated with a non-targeting siRNA (siNT) were also included. (B) Lysates of the siRNA-treated macrophages were subjected to SDS-PAGE and blotted with an anti-CDK1, anti-CDK2, anti-CDK6 or anti-β-actin antibodies. (C) Lysates of macrophages treated with siRNA targeting *CDK* (siCDK), a non-targeting siRNA (siNT) or mock-trasfected (-) were run, transferred and blotted with SAMHD1 and Hsp90 antibodies. The ratio between the density of the phosphorylated band of SAMHD1 and the unphosphorylated form is indicated in the figure (pSAMHD1/SAMHD1). (D) Lysates were subjected to overnight immunoprecipitation of SAMHD1 followed by blotting with and anti-phospho-threonine followed by proline antibody (p(Thr)-Pro) and SAMHD1 antibody. The ratio between the density of the phosphorylated form of SAMHD1 and total immunoprecipitated SAMHD1 is indicated in the figure [(p(Thr)-Pro)/SAMHD1]. (E) Lysates were run, transferred and blotted with the phospho-specific Thr592 antibody (pSAMHD1(T592)), total SAMHD1 and Hsp90 antibodies. (F) Quantification of SAMHD1 phosphorylation relative to the mock-transfected control (-) as measured by the ratio between the density of the phosphorylated band and the unphosphorylated SAMHD1. (G) Quantification of SAMHD1 phosphorylation relative to the mock-transfected control (-) as measured by the ratio between (p(Thr)-Pro) antibody and total immunoprecipitated SAMHD1.

CDK2 and CDK6 INTERFERENCE INHIBITS HIV-1 DURING REVERSE TRANSCRIPTION

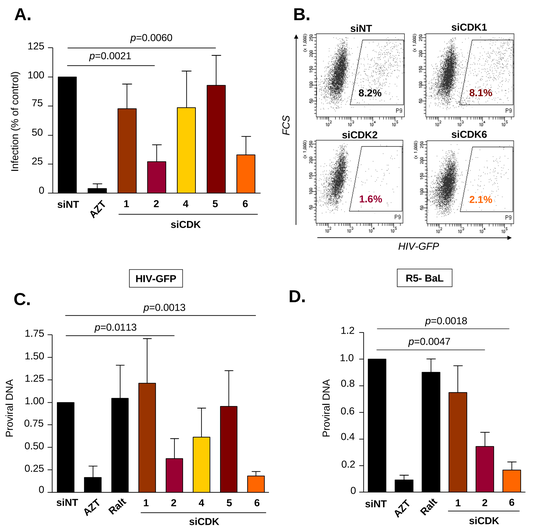


Figure 2. (A) Macrophages transfected with the indicated siRNA were infected. HIV-1 infection was measured as the percentage of GFP positive macrophages in siRNA-treated macrophages and expressed as the percentage to siNT-treated cells. AZT (3 μM) was also used as a control. (B) Representative flow cytometry dot plots showing the percentage of infected, GFP+ cells. (C and D) Transfected macrophages were infected with VSV-pseudotyped NL4-3-GFP virus (C) or R5-tropic HIV-1 BaL strain (D) and proviral DNA was measured 16h post-infection. Proviral DNA was normalized to the sample treated with non-targeting siRNA (siNT). As a control, non-transfected cells were treated with 3 μM of AZT and 2 μM Raltegravir (Ralt). Statistical significance was calculated by a one-sample t test. P values>0.05 are not shown.

CDK6 ACTS UPSTREAM CDK2 WHICH IN TURN DIRECTLY PHOSPHORYLATES SAMHD1

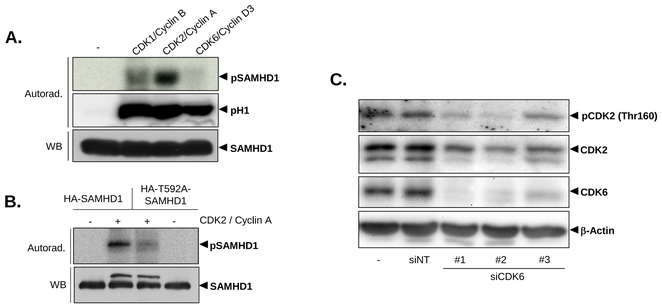


Figure 4. (A) CDK2 phosphorylates SAMHD1 *in vitro*. HA-tagged SAMHD1 immunoprecipitated from HEK293T transfected cells was subjected to an *in vitro* kinase assay with recombinant complexes cdk1/cyclin B, cdk2/cyclin A and cdk6/cyclin D3 in the presence of radioactive ATP. SAMHD1 phosphorylation was monitored by autoradiography of the incorporated radioactive ATP. Histone H1, a known substrate of CDK, was used to monitor total CDK activity. An anti-HA antibody was used to blot for total SAMHD1 in the experiment. (B) HA-tagged wild-type SAMHD1 and mutant Thr592Ala SAMHD1 were immunoprecipitated from HEK293T transfected cells and subjected to an *in vitro* kinase assay with recombinant complex cdk2/cyclin A in the presence of radioactive ATP. SAMHD1 phosphorylation was monitored by autoradiography of the incorporated radioactive ATP. An anti-HA antibody was used to blot for total SAMHD1 in the experiment. (C) Western blot of lysates of untreated macrophages (-), macrophages transfected with a non-targeting siRNA (siNT) and treated with different siRNA targeting CDK6 (siCDK6#1, 2 and 3). Membranes were blotted with an anti phospho-CDK2 antibody raised against phospho-Thr160 (pCDK2(Thr160)). Anti-CDK2, anti-CDK6 and anti-β-actin antibodies were also used as a control.

CONFIRMATORY CDK2 and CDK6 siRNA INHIBIT HIV-1 REPLICATION and PROVIRAL DNA

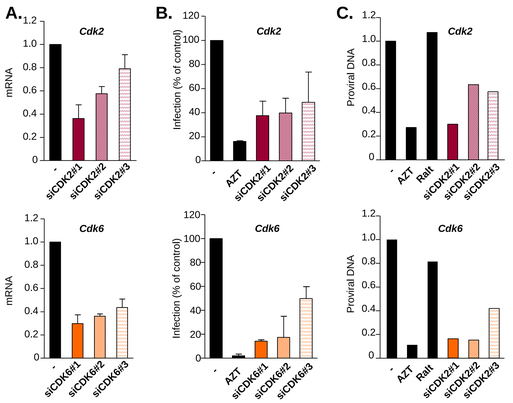


Figure 3. (A) mRNA of CDK2 and CDK6 was quantified in samples of macrophages treated with confirmatory siRNA and normalized to samples transfected without siRNA (-). (B) Percentage of infection of siRNA-treated MDM quantified by percentage of GFP positive cells. (C) Proviral DNA measured after 16 hr of BaL infection of macrophages treated with indicated siRNA and normalized to MDM transfected in the absence of siRNA (-). 3 μM of AZT and 2 μM raltegravir (Ralt) were used as control.

CDK2 and CDK6 KNOCKDOWN in MACROPHAGES LED TO A REDUCTION in THE Ki67+ STAINING

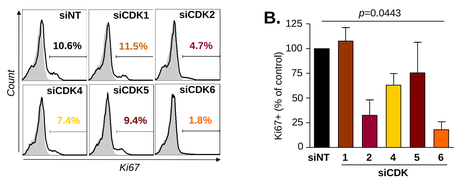


Figure 5. Representative histograms quantifying the percentage of Ki67 positive macrophages (solid black line) transfected with the indicated siRNA. Cells transfected with a non-targeting siRNA (siNT) were used as a control. Isotype staining of the siNT-treated cells was used as a control (grey histogram). (B) Percentage of Ki67+ cells normalized to the siNT cells.

MODEL FOR SAMHD1 PHOSPHORYLATION

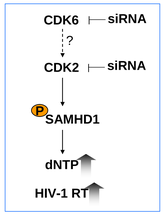


Figure 6. CDK6 controls CDK2 activity, which controls cell cycle progression and SAMHD1 restriction of HIV-1 replication.

CONCLUSIONS

- **Our results provide additional support to the role of CDK-dependent phosphorylation of SAMHD1 in mediating restriction of HIV-1, but argue against the *in vivo* relevance of CDK1 as the single kinase responsible for SAMHD1 phosphorylation.**
- **CDK2 regulates SAMHD1 phosphorylation in primary cells, leading to inhibition of proviral DNA formation and reduced HIV-1 replication.**

See posters #167 and #168