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Restriction of Virus Infection but Not Catalytic dNTPase Activity Is Regulated by Phosphorylation of SAMHD1

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SAMHD1 is a host protein responsible, at least in part, for the inefficient infection of dendritic, myeloid, and resting T cells by HIV-1. Interestingly, HIV-2 and SIVsm viruses are able to counteract SAMHD1 by targeting it for proteasomal degradation using their Vpx proteins. It has been proposed that SAMHD1 is a dGTP-dependent deoxynucleoside triphosphohydrolase (dNTPase) that restricts HIV-1 by reducing cellular dNTP levels to below that required for reverse transcription. However, nothing is known about SAMHD1 posttranslational modifications and their potential role in regulating SAMHD1 function. We used ^{32}P labeling and immunoblotting with phospho-specific antibodies to identify SAMHD1 as a phosphoprotein. Several amino acids in SAMHD1 were identified to be sites of phosphorylation using direct mass spectrometry. Mutation of these residues to alanine to prevent phosphorylation or to glutamic acid to mimic phosphorylation had no effect on the nuclear localization of SAMHD1 or its sensitivity to Vpx-mediated degradation. Furthermore, neither alanine nor glutamic acid substitutions had a significant effect on SAMHD1 dNTPase activity in an *in vitro* assay. Interestingly, however, we found that a T592E mutation, mimicking constitutive phosphorylation at a main phosphorylation site, severely affected the ability of SAMHD1 to restrict HIV-1 in a U937 cell-based restriction assay. In contrast, a T592A mutant was still capable of restricting HIV-1. These results indicate that SAMHD1 phosphorylation may be a negative regulator of SAMHD1 restriction activity. This conclusion is supported by our finding that SAMHD1 is hyperphosphorylated in monocytoid THP-1 cells under nonrestrictive conditions.

Lentiviruses, such as HIV and SIV, encode several accessory proteins that function to counteract host cell restriction factors (reviewed in reference 1). Sterile alpha motif and HD domain protein 1 (SAMHD1) is a recently identified host cell factor targeted by the HIV-2 and SIVsm encoded Vpx protein to allow replication of these viruses in myeloid cells (2–4). Interestingly, while HIV-1 does not possess a Vpx protein, Vpx also enhances infection of myeloid and dendritic cells, as well as resting $CD4^+$ T cells by this virus (5–10). In susceptible cell types, SAMHD1 has been shown to restrict infection of these lentiviruses at the reverse transcription step, and Vpx counteracts this restriction by binding to and causing the proteasomal degradation of SAMHD1 via interaction with a Cul4/DDB1/DCAF1 ubiquitin-ligase complex (2, 3, 11). Similarly, without Vpx, the same enhancement of HIV-1 infection in these cell types can therefore be achieved by the knockdown of SAMHD1 (2–4, 9).

SAMHD1 consists of an N-terminal SAM domain and a C-terminal HD domain and mutations in SAMHD1 have been associated with Aicardi-Goutieres Syndrome (AGS) (12). This syndrome is associated with increased production of interferon alpha and therefore mimics congenital infections (13). Mutations in two other proteins (TREX1 and RNaseH2) have also been associated with AGS, and it has therefore been suggested that all three of these proteins may be involved in regulating the innate immune response (14). While SAMHD1 has recently been shown to possess nucleic acid binding properties (15–18) and in one study was also reported to have exonuclease activity (17), its main catalytic activity described to date is its dGTP-dependent deoxynucleoside triphosphohydrolase (dNTPase) activity that allows it to degrade cellular deoxynucleoside triphosphates (dNTPs) (19, 20). In this way, SAMHD1 is thought to restrict HIV-1 infection by decreasing the levels of cellular dNTP pools to below that required for reverse transcription (19–22).

Interestingly, while SAMHD1 has been shown to reduce HIV-1 infection of nondividing cell types such as MDMs, dendritic cells, resting CD4 T cells as well as phorbol-12-myristate-13-acetate (PMA)-differentiated THP-1 and U937 cells (the latter requiring exogenous expression of SAMHD1) (2–4, 9, 10, 23), SAMHD1 restriction does not strictly correlate with its expression. Indeed, fully HIV-1 permissive cells, such as activated $CD4^+$ T cells or undifferentiated THP-1 cells, also express high amounts of the SAMHD1 protein (3, 9). Whether other mechanisms exist to keep the dNTP levels high in these dividing cells and/or whether SAMHD1 function might be regulated at the level of posttranslational modifications or interaction with cell specific cofactors remains to be determined.

Here, we report that SAMHD1 can be phosphorylated at several sites, and this suggests a mechanism to regulate its cellular function. We show that phosphorylation of SAMHD1 at any of the four identified positions did not significantly affect protein stability, localization, or sensitivity to Vpx-mediated degradation. Mutation of any of the phosphorylation sites also had no significant effect on dNTPase catalytic activity of SAMHD1 *in vitro*. Using orthophosphate labeling, we identified T592 as a major site of SAMHD1 phosphorylation. Mutating residue 592 to alanine (T592A) had no effect on SAMHD1 restriction activity. In contrast, replacing residue 592 by the phosphomimetic glutamic acid (T592E) abolished SAMHD1 restriction activity in a single-round

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infectivity assay. Mutation of any of the other phosphorylation sites identified did not modulate the ability of SAMHD1 to restrict HIV-1. Thus, phosphorylation at T592 appears to act as a negative regulator of SAMHD1 restriction activity. Consistent with this, phosphorylation of endogenous SAMHD1 in THP-1 cells was reduced under conditions where the protein can restrict HIV-1 replication, further implicating SAMHD1 phosphorylation as a mechanism to regulate anti-HIV SAMHD1 function.

MATERIALS AND METHODS

Cell culture and transfections. HeLa cells were propagated in Dulbecco modified Eagle medium containing 10% fetal bovine serum. THP-1 and U937 cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum. Undifferentiated batches of THP-1 cells were tested for susceptibility to HIV-1 infection prior to use in the present study. For differentiation, THP-1 cells were treated with 100 nM PMA for 24 h, washed, and left an additional 24 h in culture prior to infection or harvest. U937 cells were differentiated using 10 ng of PMA/ml for 24 h and then immediately infected or harvested for Western blot analysis. For transfections, HeLa cells were grown in 25-cm^2 flasks to ca. 80% confluence ($\sim 3 \times 10^6$ cells). Cells were transfected using Lipofectamine PLUS (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's recommendations. A total of 5 μg of plasmid DNA per 25-cm^2 flask was used. Where appropriate, empty vector DNA was used to adjust total DNA amounts. For the generation of pCDH-SAMHD1 lentiviral transduction particles, 293TN cells (Systems Biosciences) were transfected using Lipofectamine PLUS. For each 25-cm^2 flask, 0.67 μg of pCDH plasmid was used, together with 6.7 μl of pPACKH-1 packaging mix (Systems Biosciences, Mountain View, CA). Lentiviral particle-containing supernatants were collected after 48 h, clarified by filtration through a 0.45-μm-pore-size filter, and frozen at −80°C. VSV-G-pseudotyped HIV-1-green fluorescent protein (GFP) reporter viruses were produced from 293T cells as described previously (15).

Antibodies. Polyclonal antibodies to human SAMHD1 (SAM416) and SIV_{mac239} Vpx were described previously (24). A polyclonal antibody to actin was purchased from Sigma-Aldrich (St. Louis, MO; catalog no. A-5060) and used as a loading control. A mouse monoclonal antibody to human lamin B was purchased from Accute Chemical and Scientific Corp. (Westbury, NY; catalog no. BMDV3002) and used to stain the nuclear lamina. Fluorescently conjugated secondary antibodies used for immunofluorescence were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

Plasmids. The mammalian expression constructs pcDNA-SAMHD1 wild type (WT), pcDNA-HA-SAMHD1 WT, and pCMV-Vpx_{mac239} have been described previously (24). Mutation of SAMHD1 phosphorylation sites at residues S6, T21, S33, and T592 was performed using QuikChange (Stratagene, La Jolla, CA) mutagenesis with wild-type pcDNA constructs as a template. For the generation of pCDH-SAMHD1 constructs used to generate lentiviral transduction particles, SAMHD1 (WT or mutant) was amplified by PCR using primers containing Bmt1 (forward, 5′-AAT AAG CTA GCG CCA CCA TGC AGC GAG CCG ATT CCG AG-3′) and BamHI (reverse, 5′-CGC GGA TCC TTA CAT TGG GTC ATC TTT AAA AAG CTG GAC-3′) restriction sites. These fragments were then inserted into the BmtI/BamHI sites of pCDH-CMV-MCS-EF1-puro (Systems Biosciences, Mountain View, CA). For amplification of the S6A and S6E mutants, the forward primer was modified to reflect the mutated sequence of these constructs in the N terminus of SAMHD1.

Immunoblotting. For immunoblot analysis of cell-associated proteins, whole-cell lysates were prepared as follows. Cells were washed once with phosphate-buffered saline (PBS), suspended in PBS, and mixed with an equal volume of sample buffer (4% sodium dodecyl sulfate, 125 mM Tris-HCl [pH 6.8], 10% glycerol, 0.002% bromophenol blue). Proteins were solubilized by heating 10 to 15 min at 95°C with occasional vortexing. Cell lysates were subjected to SDS-PAGE; proteins were transferred to polyvinylidene difluoride (PVDF) membranes and reacted with appropriate antibodies as described in the text. Membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (GE Healthcare, Piscataway, NJ), and proteins were visualized by enhanced chemiluminescence (GE Healthcare).

[^{32}P]orthophosphate labeling. For orthophosphate labeling of SAMHD1, cells were preincubated at 37°C for 20 min in 5 ml of phosphate-free RPMI medium to deplete the endogenous pool of phosphate. The cells were then pelleted in a tabletop centrifuge and suspended in 450 μl of phosphate-free RPMI medium. Then, 150 μl of the cell suspension was used to prepare whole-cell extracts for protein analysis by immunoblotting. Next, 100 μl of [^{32}P]orthophosphate (2 mCi/ml; Perkin-Elmer, Waltham, MA) was added to the remaining 300 μl of cells, and the samples were incubated for 90 min at 37°C. Cells were pelleted, and the supernatant containing unincorporated isotope was discarded. Cells were lysed in 200 μl of 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 1% Triton X-100 at 4°C for 5 min, and the clarified supernatants were subjected to immunoprecipitation by SAMHD1-specific antibody as described previously (24).

^{35}S labeling of SAMHD1. Cells were suspended in 5 ml of RPMI medium lacking methionine and cysteine (MP Biomedical, Solon, OH) and incubated for 20 min at 37°C to deplete the intracellular methionine-cysteine pool. Cells were then labeled for 90 min at 37°C in 200 μl of methionine-free RPMI containing 5% fetal calf serum supplemented with 150 μCi of [^{35}S]Expre^{35}S^{35}S protein labeling mix (Perkin-Elmer; catalog no. NEG072). After the labeling period, cells were pelleted in a microfuge, and the unincorporated isotope was removed. Cell lysates were immunoprecipitated with SAMHD1-specific antibodies in parallel with the ^{32}P-labeled samples.

Phospho-specific (anti-T*P) Immunoblotting. For detection of phosphorylated SAMHD1 by phospho-specific Western blot, transfected HeLa cells were lysed in lysis buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1% Triton X-100, 1 mM Na_3VO_4, 1 mM NaF) at 4°C for 20 min and clarified at 10,000 × *g* for 10 min at 4°C. Cleared lysates were then incubated for 1.5 h at 4°C with antibody (SAM416)-conjugated protein A-Sepharose beads. Beads were washed three times with wash buffer (50 mM Tris [pH 7.5], 300 mM NaCl, 0.1% Triton X-100, 1 mM Na_3VO_4, 1 mM NaF). Bound proteins were eluted in sample buffer for 10 min at 95°C, separated by SDS-PAGE, transferred to a PVDF membrane, and analyzed by immunoblotting with Phospho-(Thr) MAPK/CDK substrate mouse MAb (Cell Signaling Technologies, Boston, MA; catalog no. 2321). This antibody recognizes any phosphorylated threonine (and some serines) when followed by a proline. For phosphatase treatment, samples were immunoprecipitated as described above. Beads were then washed twice with wash buffer without phosphatase inhibitors and incubated for 30 min at 30°C in the presence or absence of 200 U of lambda phosphatase (New England BioLabs, Ipswich, MA) in buffer supplied by the manufacturer (50 mM HEPES [pH 7.5], 100 mM NaCl, 2 mM dithiothreitol [DTT], 0.01% Brij 35, 1 mM $MnCl_2$). Beads were then washed one more time in wash buffer and processed for SDS-PAGE as described above.

***In vitro* dNTPase assay.** HA-SAMHD1 proteins were isolated from HeLa cells and used in a dNTPase assay as described previously (24). In brief, HeLa cells transfected with HA-SAMHD1 constructs were lysed in 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 1% Triton X-100 at 4°C for 20 min and then clarified by centrifugation at 10,000 × *g* for 10 min at 4°C. Cleared cell lysates were mixed with anti-hemagglutinin (HA) antibody-conjugated agarose beads (Sigma-Aldrich) and incubated at 4°C for 2 h. The samples were then washed three times with 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.1% Triton X-100 and once with assay buffer A (50 mM Tris-HCl [pH 8.0], 50 mM KCl, 5 mM $MgCl_2$, 0.1% Triton X-100). An equal volume of assay buffer A was then added to the bead pellet, 15% of the bead slurry was reserved as an input control, and the rest was mixed in a 1:1 ratio with assay buffer B (50 mM Tris-HCl [pH 8.0], 50 mM KCl, 5 mM $MgCl_2$, 0.1% Triton X-100, 400 μM dTTP, 0.5 μCi of [α-^{32}P]dTTP, and 400 μM dGTP) in duplicate reactions. The reactions were incubated at 37°C for 3 h with occasional mixing and stopped by

heating to 70°C for 5 min. A portion of the reaction products were separated out on polyethyleneimine-cellulose thin-layer chromatography plates (Sigma-Aldrich) using 0.8 M LiCl as the mobile phase.

U937 cell-based HIV-1 restriction assay. HIV-1 restriction assays were performed essentially as described previously (15). Monocytic U937 cells were transduced with pCDH-SAMHD1 lentiviral particles and selected with 0.4 μg of puromycin/ml for ~1 week. A total of 6×10^4 cells were then differentiated overnight in a 24-well plate using 10 ng of PMA/ml. The next day, the differentiated cells were washed and infected with increasing amounts of VSV-G-pseudotyped HIV-1–GFP as indicated above. Infection of cells was determined as GFP expression 48 h later using flow cytometry.

Infection of THP-1 cells with HIV-1–GFP. A total of 6×10^4 THP-1 cells were treated with 100 nM PMA for 24 h in a 24-well plate, washed, and incubated for an additional 24 h before infection. Differentiated cells or an equivalent number of untreated cells were then infected with increasing amounts of VSV-G-pseudotyped HIV-1–GFP as indicated in the text. Infection of cells was determined as the percent GFP positive 48 h later using flow cytometry.

Protein preparation for mass spectrometry. For analysis of SAMHD1 phosphorylation by mass spectrometry, large-scale purification of SAMHD1 from transfected HeLa cells was performed. Transfected cells were lysed in lysis buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1% Triton X-100, 1 mM Na_3VO_4, 1 mM NaF) at 4°C for 20 min and clarified at 10,000 × *g* for 10 min at 4°C. Cleared lysates were then incubated for 2 h at 4°C with SAMHD1-specific antibody (SAM416) conjugated to protein A-Sepharose beads. Beads were washed three times with wash buffer (50 mM Tris [pH 7.5], 300 mM NaCl, 0.1% Triton X-100, 1 mM Na_3VO_4, 1 mM NaF). Bound proteins were eluted in sample buffer for 10 min at 95°C, and separated by SDS-PAGE. The gel was then stained with PageBlue protein staining solution (Thermo Scientific, Rockford, IL), and the SAMHD1 band was cut out of the gel for mass spectrometry analysis.

SAMHD1 in-gel reduction, alkylation, denaturation, and trypsin digestion. PageBlue-stained gels were destained with washing buffer (50 mM ammonium bicarbonate, 50% acetonitrile, and 80% acetonitrile). The gel-bound proteins were reduced with 1 ml of 40 mM DTT for 25 min at 56°C and alkylated with 1 ml of 50 mM iodoacetamide for 30 min at 25°C in the dark with constant mixing. The iodoacetamide was discarded, and the gel-bound proteins were digested with 0.5 ml of trypsin (20 ng/μl; Promega, Madison, WI) in 50 mM ammonium bicarbonate buffer at 37°C with constant mixing for 12 h. The tryptic peptides were collected and dried using a SpeedVac apparatus (Thermo Fisher Scientific, San Jose, CA) and stored at 4°C prior to mass spectrometric analysis.

Peptide/protein identifications by ESI-MS/MS analysis. The dried samples were dissolved with 20 μl of 0.1% formic acid-water. Then, 2 μl of each sample was analyzed by liquid chromatography/electrospray ionization-tandem mass spectrometry (LC/ESI-MS/MS) using a Q-Exactive (Thermo Fisher Scientific) mass spectrometer with an Easy NanoLC-1000 system with data-dependent acquisition with dynamic exclusion (DE = 1) settings. The data-dependent acquisition settings used were a top 12 higher-energy collision-induced dissociation (HCD) for the Q-Exactive MS. The resolving power for Q-Exactive was set at 70,000 for the full MS scan and at 17,500 for the MS/MS scan at *m/z* 200. LC/ESI-MS/MS analyses were conducted using a C_{18} column (75 μm by 150 mm). The solvents used for the reversed-phase chromatography separation were 0.1% HCOOH-water (solvent A) and 0.1% HCOOH (solvent B) in acetonitrile. A four-step linear gradient was used for the LC separation (2 to 30% solvent B in the first 47 min, followed by 80% solvent B in the next 1 min and holding at 80% solvent B for 12 min).

The Sequest algorithm was used to identify peptides from the resulting MS/MS spectra by searching against the combined human protein database (a total of 22,673 proteins) extracted from Swiss-Prot (v.57) using taxonomy "homo sapiens" using Proteome Discoverer (version 1.3; Thermo Scientific). Searching parameters for parent and fragment ion tolerances were set as 20 ppm and 30 milli-mass units (mmu) for the Q-Exactive MS. Other parameters used were a fixed modification of carbamidomethylation (Cys), variable modifications of phosphorylation (S, T, and Y), and oxidation (Met). Trypsin was set as the protease, with a maximum of two missed cleavages. Raw files were searched against SAMHD1 protein sequence (along with 500 other random proteins and reversed proteins as decoys) using Byonic (25) with a peptide tolerance of 15 ppm, an MS/MS tolerance of 20 ppm for HCD data, the carbamidomethylated cysteine as a fixed modification, and the oxidation of methionine and the phosphorylation of serine, threonine, and tyrosine as variable modifications. Byonic scoring gives an indication of whether modifications are confidently localized.

Immunofluorescence and confocal microscopy. HeLa cells were transfected as indicated in the text. Transfected cells were treated with trypsin 3 h later, and single-cell suspensions were distributed into 12-well plates containing 0.13-mm coverslips. Cells were grown overnight at 37°C and then fixed in 10% methanol for 10 min at −20°C. Coverslips were blocked with 1% bovine serum albumin (BSA) in PBS for 30 min. For antibody staining, coverslips were incubated with appropriate primary antibodies in 1% BSA in PBS for 45 min at 37°C. Coverslips were washed with PBS and incubated with appropriate secondary antibodies for 45 min at 37°C. Cells were then washed twice with PBS and mounted onto microscope slides with glycerol gelatin (Sigma-Aldrich) containing 0.1 M *N*-propyl gallate (Sigma-Aldrich) to prevent photo-bleaching. Samples were analyzed on a Zeiss LSM410 inverted laser scanning microscope equipped with a krypton/argon mixed-gas laser. Images were acquired with a Plan-Apochromat 63−/1.4 oil immersion objective (Zeiss).

RESULTS

SAMHD1 is a phosphoprotein. In order to investigate whether SAMHD1 contains phosphorylation sites that could potentially regulate its function, we first determined the phosphorylation status of SAMHD1 using a [^{32}P]phosphate incorporation assay. To do so, SAMHD1 was exogenously expressed in HeLa cells, a cell line that contains very little endogenous SAMHD1. Transfected cells were labeled with either [^{35}S]methionine-cysteine as a control for SAMHD1 expression or [^{32}P]orthophosphate to check the SAMHD1 phosphorylation status. As can be seen in Fig. 1A, SAMHD1 is labeled with radioactive phosphate, indicating that it contains one or more phosphorylation sites. Full-length SAMHD1 is a 626-residue 72-kDa protein that contains multiple serine, threonine, and tyrosine residues as potential phosphoacceptor sites. Of particular interest, SAMHD1 contains several threonine and serine residues that are followed by proline and could be part of a CDK/MAPK kinase recognition sequence (26). We therefore probed SAMHD1 isolated from transfected HeLa cells with a CDK/MAPK substrate antibody that recognizes phosphorylated threonines (and some serines) that are followed by a proline. Figure 1B shows that SAMHD1 is efficiently recognized by this antibody and that treatment with lambda phosphatase results in loss of this phospho-specific signal (Fig. 1B, α-T*P). This therefore confirms the orthophosphate labeling result that SAMHD1 can be phosphorylated and indicates that at least one site in SAMHD1 is subject to phosphorylation by a member of the cyclin-dependent kinase (CDK) or mitogen-activated protein kinase (MAPK) family of kinases.

Identification of SAMHD1 phosphorylation sites. To determine which sites in SAMHD1 are phosphorylated, we performed mass spectrometry. For that purpose, SAMHD1 was enriched from transfected HeLa cells by immunoprecipitation and further purified by gel electrophoresis. SAMHD1 was visualized by Coomassie blue staining (Fig. 2A), extracted from the gel, and processed for mass spectrometry as described in Materials and Meth-

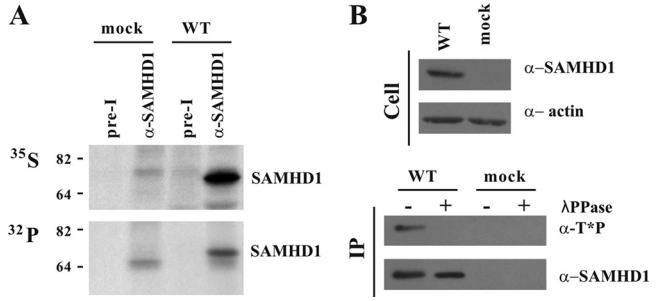

FIG 1 SAMHD1 is a phosphoprotein. (A) HeLa cells were transfected with 2 μg of pcDNA-SAMHD1 WT or mock transfected. At 24 h after transfection, labeling was performed with either [^{35}S]methionine-cysteine or [^{32}P]orthophosphate for 90 min. Labeled SAMHD1 proteins were immunoprecipitated using preimmune (pre-I) or polyclonal anti-SAMHD1 antiserum, separated by SDS-PAGE, and visualized by fluorography or autoradiography. (B) HeLa cells were transfected with 1 μg of pcDNA-SAMHD1 WT or mock transfected. Detergent cell extracts were prepared 24 h after transfection and used for immunoprecipitation using anti-SAMHD1 antiserum. After immunoprecipitation, the samples were treated with or without 200 U of λ protein phosphatase (λPPase) for 30 min at 30°C. The immunoprecipitated proteins were separated by SDS-PAGE and detected by immunoblotting with an anti-phospho (threonine) MAPK/CDK substrate antibody (anti-T*P). The membrane was then reprobed using anti-SAMHD1 antiserum. Cell extracts prior to immunoprecipitation were also subjected to immunoblotting with antibodies against SAMHD1 and actin as indicated.

ods. The four main phosphorylation sites in SAMHD1 were identified as residues serine 6, threonine 21, and serine 33 in the N terminus of SAMHD1, as well as threonine 592 located near the C terminus (Fig. 2B). Of these, T21, S33, and T592 are followed by a proline residue and are likely phosphorylated by a member of the CDK or MAPK family of kinases (26). On the other hand, S6 is part of an RXXS motif that forms a substrate recognition motif for the Arg-directed family of kinases (e.g., protein kinase A [PKA], PKC, and Akt). In order to confirm the contribution of phosphorylation at these sites to the [^{32}P]orthophosphate labeling of SAMHD1, transfected HeLa cells expressing single alanine mutants (S6A, T21A, S33A, and T592A) of SAMHD1 were subjected to [^{32}P]orthophosphate labeling (Fig. 2C, ^{32}P). Comparable protein expression was verified by immunoblotting (Fig. 2C, WB). Consistent with the results from the mass spectrometry, mutation of each of these sites to alanine resulted in a decrease in the phosphorylated level of SAMHD1 compared to the WT protein. Whereas S6 does not appear to contribute significantly to overall SAMHD1 phosphorylation, mutation of T592A consistently had the largest impact on SAMHD1 phosphorylation, suggesting that T592 represents a major target for SAMHD1 phosphorylation. We also created a mutant in which all four identified phosphoac-

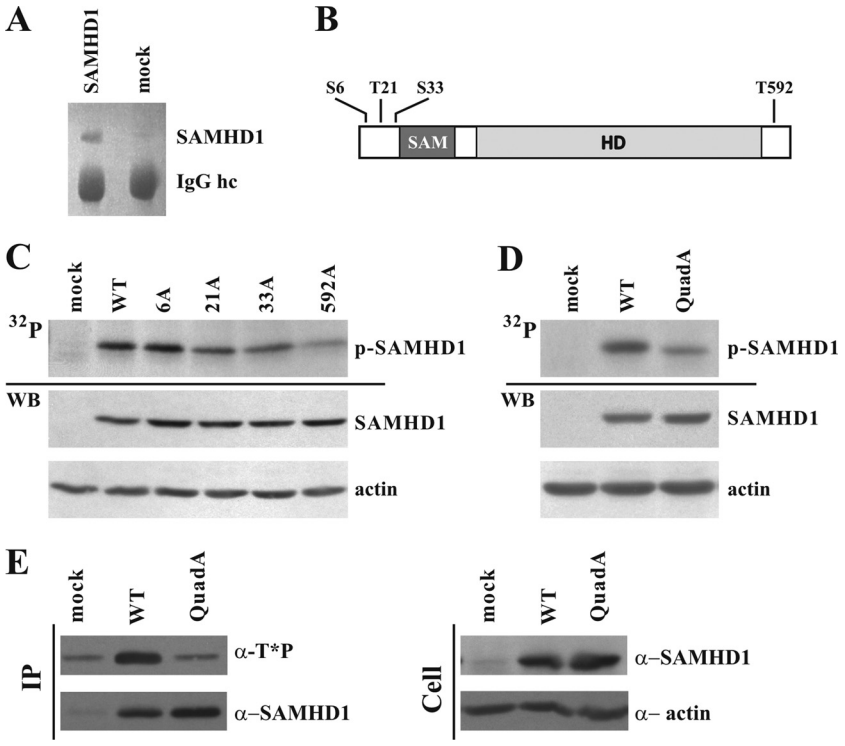

FIG 2 Identification of SAMHD1 phosphorylation sites. (A) Large-scale immunoprecipitation of SAMHD1 from transfected HeLa cells was performed as detailed in Materials and Methods. Immunoprecipitated SAMHD1 was separated by SDS-PAGE and isolated from the Coomassie blue-stained gel for mass spectrometry analysis. (B) Schematic representation of SAMHD1 protein with the phosphorylated amino acids identified by mass spectrometry indicated. (C and D) HeLa cells were transfected with 2 μg of pcDNA-SAMHD1 variants as indicated. QuadA represents a variant in which all four positions (S6, T21, S33, and T592) were changed to alanine. At 24 h after transfection, labeling with [^{32}P]orthophosphate was performed for 90 min. Labeled SAMHD1 proteins were immunoprecipitated with SAMHD1-specific antiserum, separated by SDS-PAGE, and visualized by autoradiography. Aliquots of the transfected cells were also retained prior to labeling, and cell lysates were separated by SDS-PAGE. Immunoblot analysis was then performed using antibodies to SAMHD1 and actin as indicated. (E) HeLa cells were transfected with 1 μg of pcDNA-SAMHD1 WT, QuadA, or mock transfected. Detergent cell extracts were prepared 24 h after transfection and used for immunoprecipitation with anti-SAMHD1 antiserum. The immunoprecipitated proteins were then separated by SDS-PAGE and detected by immunoblotting with an anti-phospho (threonine) MAPK/CDK substrate antibody (anti-T*P). The membrane was then reprobed using anti-SAMHD1 antiserum. Cell extracts prior to immunoprecipitation were also subjected to immunoblotting with antibodies against SAMHD1 and actin as indicated.

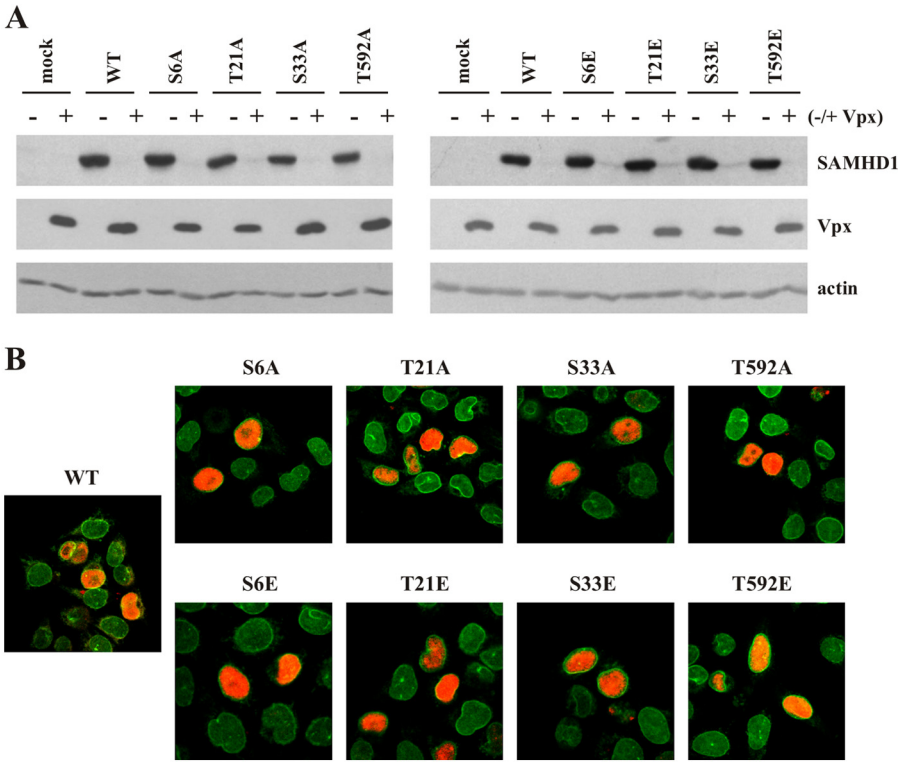

FIG 3 Localization of SAMHD1 phosphorylation site mutants and sensitivity to Vpx-mediated degradation. (A) HeLa cells were transfected with 1 μg of pcDNA-SAMHD1 variants as indicated, in the presence or absence of 0.5 μg of pCMV-Vpxmac239. Whole-cell extracts were prepared 24 h after transfection and separated by SDS-PAGE. Immunoblot analysis was performed with rabbit polyclonal antibodies to SAMHD1, SIV Vpx, and actin, as indicated. (B) HeLa cells were transfected with 1 μg of pcDNA-SAMHD1 variants as indicated, replated onto coverslips, allowed to grow overnight, and fixed with methanol (10 min, −20°C). Cells were then stained with a rabbit polyclonal antiserum to SAMHD1 (red) and a mouse monoclonal antibody to lamin B (green). Confocal microscopy was performed as described in Materials and Methods.

ceptor sites were mutated to alanine (Fig. 2D, QuadA). Interestingly, [^{32}P]orthophosphate labeling of the SAMHD1 quadruple mutant was strongly impaired but was not abolished, indicating that residues other than the four identified by mass spectrometry may act as phosphoacceptor sites. Furthermore, the pT*P phospho-specific antibody only shows background detection of the quadruple mutant protein, indicating one or several of T21, S33, or T592 as the major site(s) phosphorylated by a kinase from the CDK or MAPK family (Fig. 2E). To investigate the role of phosphorylation on SAMHD1 properties and functions, we then performed a mutagenesis study where the four identified phosphorylation sites were mutated to alanine (to prevent phosphorylation) or to glutamic acid (to mimic phosphorylation).

Mutation of phosphorylation sites does not abolish sensitivity to Vpx and does not affect the cellular distribution of SAMHD1. To assess the effect of phosphorylation site mutations in SAMHD1 on protein expression and sensitivity to Vpx-mediated degradation, SAMHD1 WT, as well as mutants carrying alanine or glutamic acid substitutions at positions 6, 21, 33, or 592, was transfected into HeLa cells either in the absence or in the presence of SIVmac239 Vpx (Fig. 3A). We found that all SAMHD1 phosphorylation site mutants remained sensitive to SIVmac239 Vpx-induced degradation. Furthermore, all mutants were found by immunofluorescence to localize to the nucleus, similar to the WT protein (Fig. 3B), suggesting that SAMHD1 phosphorylation at these sites does not influence cellular distribution of SAMHD1. Taken together, these data suggest that the phosphorylation site mutants of SAMHD1 behave similarly to the wild-type protein and that conversion of the respective serine and threonine residues to alanine or glutamic acid does not significantly affect the physicochemical properties of the protein.

SAMHD1 phosphorylation mutants are catalytically active dNTPases. SAMHD1 functions to degrade cellular dNTPs into component nucleosides and inorganic triphosphate (19, 20). It has been postulated that it is this enzymatic activity of SAMHD1 that imposes HIV-1 restriction in susceptible cells by decreasing the dNTP pool available for reverse transcription (21, 22). We therefore wanted to determine whether phosphorylation could modulate the dNTPase activity of SAMHD1. N-terminally HA-tagged SAMHD1 variants were isolated from transfected HeLa cells by immunoprecipitation. An aliquot of the immunoprecipitated SAMHD1 proteins was analyzed by immunoblotting to ascertain comparable protein input (Fig. 4A and B, top panels). The remaining samples were used in an *in vitro* dNTPase assay using [α-^{32}P]dTTP as a substrate. The released radioactive triphosphate was separated by thin-layer chromatography and visualized by autoradiography (Fig. 4A and B, PPP released). A SAMHD1 variant carrying an active-site mutation (H206A/D207A) was included as a negative control and shows background levels of triphosphate release similar to the mock control (Fig. 4A and B, lanes AA). All alanine substitution mutants were able to catalyze the release of triphosphate from dTTP with similar efficiency as

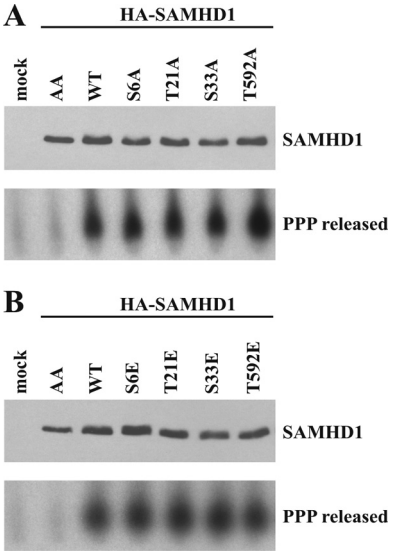

FIG 4 Effect of SAMHD1 phosphorylation on catalytic dNTPase activity. (A and B) HeLa cells were transfected with pcDNA-HA-SAMHD1 WT or the indicated mutants. Cell extracts were prepared 24 h later and used for immunoprecipitation using HA-beads. To control for variations in SAMHD1 protein expression levels, a portion of the immunoprecipitated SAMHD1 was separated by SDS-PAGE and subjected to immunoblotting with SAMHD1-specific antiserum (top panels). The remaining precipitates were used directly on beads in a dNTPase assay using [α-^{32}P]dTTP as described in Materials and Methods. Released triphosphate (PPP) was separated by polyethyleneimine-cellulose thin-layer chromatography using 0.8 M LiCl as the mobile phase and detected by autoradiography.

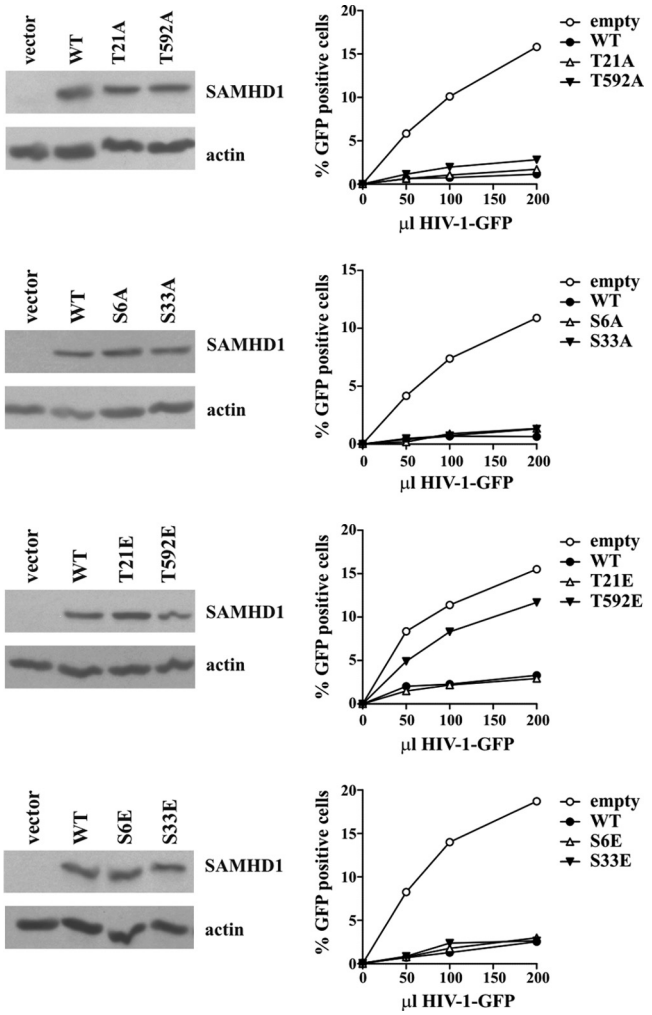

FIG 5 Effect of SAMHD1 phosphorylation on restriction activity against HIV-1. U937 cells were transduced with pCDH lentiviral particles encoding either WT SAMHD1, the indicated SAMHD1 mutants, or an empty vector. After puromycin selection for 1 week, cells were differentiated overnight with 10 ng of PMA/ml. Differentiated cells were infected with increasing volumes of VSV-G-pseudotyped HIV-1–GFP, and the percent infection (% GFP-positive cells) was determined by flow cytometry 48 h later. Total cell extracts from differentiated U937 stable cell lines were separated by SDS-PAGE and subjected to immunoblotting for SAMHD1 and actin as indicated (left panels). Stable cell lines and graphs shown are representative of at least three independent experiments.

SAMHD1 WT (Fig. 4A). This indicates SAMHD1 phosphorylation at the identified positions is not required for dNTPase enzymatic activity, at least *in vitro*. Interestingly, glutamic acid substitutions, which simulate constitutive phosphorylation at the indicated sites, also did not affect the catalytic function of SAMHD1 (Fig. 4B). These results therefore suggest that SAMHD1 catalytic activity is not significantly regulated by phosphorylation. These results also confirm that the mutation of S6, T21, S33, and T592 to either alanine or glutamic acid did not cause any gross structural defects.

SAMHD T592E phosphomimetic mutant is defective for HIV-1 restriction. The SAMHD1-imposed restriction to retrovirus infection is thought to result from the reduction in the available dNTP pool due to SAMHD1's dNTPase activity (21, 23, 27, 28). Based on our results thus far, we would therefore predict all of our phosphorylation site mutants to restrict virus infection. To test this hypothesis, we analyzed our mutants using a U937 cell-based restriction assay. Monocytic U937 cells were transduced with lentiviral particles encoding either WT or phosphorylation-site mutant SAMHD1. After 1 week of selection with puromycin, stable cell lines were differentiated with PMA overnight and then infected with VSV-G-pseudotyped HIV-1 GFP. Two days after infection, the percentage of GFP-positive cells was determined by flow cytometry (Fig. 5, right panels). Immunoblot analysis confirmed that all SAMHD1 mutants were expressed at comparable levels in the differentiated stable cell lines (Fig. 5, left panels). As expected, SAMHD1 WT was able to restrict infection by HIV-1 GFP compared to the cell line transduced with empty vector lentiviral particles (Fig. 5, right panels; compare open and closed circles). Consistent with their ability to degrade dNTPs *in vitro*, mutating S6, T21, S33, and T592 to alanine had no effect on the restriction ability of SAMHD1 (Fig. 5, top two graphs). Similarly, the phosphomimetic mutants S6E, T21E, and S33E could efficiently restrict HIV-1 GFP. Surprisingly, the phosphomimetic mutant T592E only poorly restricted HIV-1 infection despite being an active dNTPase. This result would therefore suggest that phosphorylation of SAMHD1 at T592 negatively regulates its restriction activity toward HIV-1. Importantly, this regulation of SAMHD1 activity appears to be independent of any effect on catalytic activity or cellular localization.

SAMHD1 shows less phosphorylation in cells that restrict HIV-1 infection. The results shown in Fig. 2 indicate that phosphorylation of T592 contributes significantly to the gross phos-

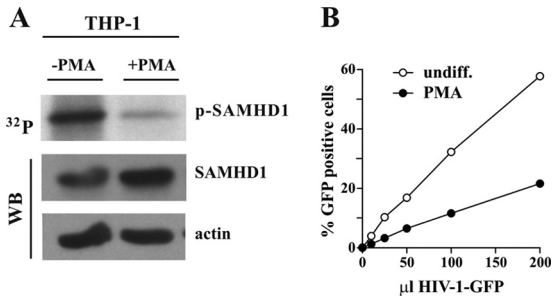

FIG 6 Analysis of phosphorylation levels in undifferentiated and differentiated THP-1 cells. (A) Undifferentiated or PMA-differentiated (100 nM, 24 h as described in Materials and Methods) THP-1 cells were labeled with [^{32}P]orthophosphate for 90 min. Labeled SAMHD1 was immunoprecipitated with SAMHD1-specific antiserum, separated by SDS-PAGE, and visualized by autoradiography or fluorography. A fraction of the cells remained unlabeled and was used to assess SAMHD1 levels relative to cellular actin by immunoblotting (WB). (B) To assess the infectibility of undifferentiated and differentiated THP-1 cells, cells were infected with increasing volumes of VSV-G-pseudotyped HIV-1–GFP as indicated, and the percent infection (% GFP-positive cells) was determined by flow cytometry 48 h later.

phorylation of SAMHD1. Thus, if phosphorylation of SAMHD1 at residue T592 is critical for regulating its restriction activity, we would expect to see a difference in the phosphorylation level of endogenous SAMHD1 in monocytoid THP-1 cells, depending on their differentiation state. It has been reported that undifferentiated THP-1 cells do not restrict HIV-1 infection, in contrast to differentiated THP-1 cells, which cannot be efficiently infected by HIV-1 (5, 15). To test this hypothesis, we subjected both cycling and PMA-differentiated THP-1 cells to [^{32}P]orthophosphate labeling (Fig. 6A, ^{32}P). At the same time, we performed immunoblotting to compare the levels of SAMHD1 in differentiated and undifferentiated THP-1 cells relative to cellular actin (Fig. 6A, WB). We found that SAMHD1 levels were comparable or even slightly elevated in differentiated THP-1 cells. Importantly, the overall phosphorylation level of SAMHD1 in differentiated cells was significantly lower. Consistent with previous reports (5, 15), differentiated THP-1 cells exhibited reduced infectibility by HIV-1 (Fig. 6B). The observation that SAMHD1 appears to be hypophosphorylated in cells that restrict HIV-1 infection is consistent with our results from the U937 cell-based restriction assay (Fig. 5) and fits our model of phosphorylation being a negative regulator of SAMHD1 restriction activity.

DISCUSSION

HIV replication *in vivo* involves a complex interplay of host antiviral restriction mechanisms and viral countermeasures. Apolipoprotein B mRNA-editing enzyme 3G (APOBEC3G), bone marrow stromal cell antigen 2 (BST-2), cyclophilin A, and tripartite motif protein 5α (Trim5α) are among the growing list of host restriction factors that have been identified (for a review, see reference 1). The most recent addition to this list of antiviral restriction factors is SAMHD1, which was identified in a proteomics screen as a major Vpx-interacting factor and is responsible for the Vpx-sensitive restriction of HIV and SIV in myeloid cell types such as macrophages and dendritic cells (2, 3). Intriguingly, SAMHD1 restriction could be alleviated by silencing its expression in restrictive cell types; however, transfer of SAMHD1 into permissive cell types failed to induce a restrictive phenotype even when the protein was overexpressed (23). This distinguishes SAMHD1 from restriction factors such as APOBEC3G or BST-2 whose restrictive phenotype was readily transferred by expression in permissive target cells. The inability of SAMHD1 to restrict viral infection when expressed in HeLa or 293T cells could either suggest that SAMHD1 exerts its effect in conjunction with other thus far unidentified host factor(s) or, alternatively, could indicate that the antiviral activity of SAMHD1 is regulated not at the expression level but at a posttranslational level. Such a regulation of SAMHD1 restriction activity could explain why levels of SAMHD1 observed in undifferentiated nonrestrictive THP-1 cells and in differentiated restrictive THP-1 cells are quite similar. Indeed, while SAMHD1 was first described as a human orthologue of a gamma interferon-induced mouse protein (29), interferon expression was recently shown to only affect SAMHD1 expression levels in cell types that express low levels of SAMHD1 (e.g., HeLa and 293T cells); in cell types that already express high amounts of SAMHD1, the interferon effect was insignificant (23, 30, 31).

Although mass spectrometry of purified SAMHD1 identified four phosphorylation sites, [^{32}P]phosphate labeling of SAMHD1 mutants points to T592 as a major phosphoacceptor site. Indeed, phosphorylation at T592 appears to be a negative regulator of SAMHD1 restriction activity against HIV-1 since a phosphomimetic mutant at this site allowed HIV-1 to infect SAMHD1-transduced U937 cells. T592 is located near the C terminus of SAMHD1, and its surrounding sequence LIT*PQKK conforms well to a CDK consensus phosphorylation target site, S/T*PXR/K (32), suggesting T592 could be phosphorylated *in vivo* by a CDK. CDKs are important proteins that, together with their partner cyclins, promote cell proliferation by driving progression through the cell cycle (33). The suggestion that the regulation of SAMHD1 function is tied into the cell proliferation status is consistent with recent observations, namely, that SAMHD1 has thus far only been shown to restrict HIV-1 in differentiated or nondividing cells. During cell cycle progression, CDKs would be active to phosphorylate SAMHD1 at T592 and therefore render it inactive for HIV-1 restriction. Meanwhile, in nondividing/differentiated cells, CDKs would not be actively stimulating cell cycle progression by phosphorylation of target proteins; SAMHD1 T592 would thus largely be in an unphosphorylated state, and it would therefore be able to restrict HIV-1. This model is supported by our finding that SAMHD1 incorporates less ^{32}P in differentiated THP-1 cells than in their cycling counterparts.

What is the mechanism by which phosphorylation at T592 is able to negatively regulate SAMHD1 restriction of HIV-1? Using a phosphomimetic construct, our data show that phosphorylation at T592 does not significantly affect levels or nuclear localization of the SAMHD1 protein. High levels of dNTPs are required for cellular DNA synthesis during cell cycle progression. It would therefore be tempting to speculate that phosphorylation at T592 impairs the catalytic ability of SAMHD1 to degrade dNTPs. In this scenario, lack of phosphorylation at T592 would activate SAMHD1 dNTPase activity to lower dNTP levels at times when the cell does not undergo DNA replication, as is the case in terminally differentiated nondividing cells. Surprisingly, however, our data indicate that T592 phosphorylation does not significantly affect the catalytic dNTPase activity of SAMHD1. It is important to point out, however, that SAMHD1 dNTPase activity was measured in an *in vitro* assay, and our data do not formally rule out the

possibility that at a cellular level, SAMHD1 dNTPase activity may be regulated through additional factors. Indeed, Yan et al. recently reported that the C terminus of SAMHD1 (amino acids 596 to 626) was not required for dNTPase activity *in vitro* but was required for full depletion of dNTPs *in vivo*, suggesting that the *in vitro* catalytic activity of SAMHD1 may not always directly correlate with its ability to decrease cellular dNTP pools (34).

While the present study was in preparation, two other groups reported similar cell cycle-dependent regulation of SAMHD1 by phosphorylation of T592 by CDK1 (35, 36). Our study supports their conclusions. Cribier et al. and White et al. both show phosphorylation at T592 to negatively correlate with the ability of SAMHD1 to restrict HIV-1 using several cell lines and primary cell types (35, 36). Both reports also demonstrate CDK1 to be the cell cycle-dependent kinase responsible for phosphorylation at T592 in cycling cells (35, 36). These findings fit well with our data showing recognition of SAMHD1 isolated from transfected HeLa cells by an antibody specific to phosphorylated substrates of CDK/MAPK family kinases. Our mutagenesis study is also in agreement with the biochemical analysis of SAMHD1 mutants performed by White et al. showing SAMHD1 phospho-site mutants have no effect on SAMHD1 localization, sensitivity to Vpx-induced degradation, or *in vitro* dNTPase activity (35).

Interestingly, White et al. found that SAMHD1 T592 phosphomimetic proteins were able to decrease dNTP pools in live cells (35), suggesting the negative regulation of SAMHD1 restriction activity by T592 phosphorylation may indeed be independent of its catalytic dNTPase activity. It is therefore possible that phosphorylation at T592 might affect other properties of SAMHD1 that have yet to be well defined. SAMHD1 has been shown to oligomerize through its HD domain; however, this property of SAMHD1 is also required for its dNTPase activity *in vitro* (15, 18, 34). Whether SAMHD1 nucleic acid binding (15, 16, 18) or potential exonuclease activity (17) can contribute to SAMHD1 restriction activity independent of any effect on dNTP levels also remains to be determined. Thus, the possibility that SAMHD1 might require a cellular cofactor and that interaction with this cofactor could depend on the T592 phosphorylation status cannot be discounted and will be subject to future investigations.

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