CyclinA2-Cyclin-dependent Kinase Regulates SAMHD1 Protein Phosphohydrolase Domain*

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Background: Human sterile α motif and histidine-aspartate domain-containing protein 1 (SAMHD1) is a deoxyribonucleoside triphosphohydrolase that is phosphorylated by cyclinA2-dependent kinases.

Results: SAMHD1 mutants defective for cyclinA2 binding disrupt S phase progression, and this is alleviated by Thr-592 phosphomimetic mutation.

Conclusion: CyclinA2-dependent kinases regulate SAMHD1 activity.

Significance: SAMHD1 dNTP phosphohydrolase activity is regulated during the cell cycle.

SAMHD1 is a nuclear deoxyribonucleoside triphosphate (dNTPase) triphosphohydrolase that contributes to the control of cellular deoxyribonucleoside triphosphate (dNTP) pool sizes through dNTP hydrolysis and modulates the innate immune response to viruses. CyclinA2-CDK1/2 phosphorylates SAMHD1 at Thr-592, but how this modification controls SAMHD1 functions in proliferating cells is not known. Here, we show that SAMHD1 levels remain relatively unchanged during the cell division cycle in primary human T lymphocytes and in monocytic cell lines. Inactivation of the bipartite cyclinA2-CDK-binding site in the SAMHD1 C terminus described herein abolished SAMHD1 phosphorylation on Thr-592 during S and G2 phases thus interfering with DNA replication and progression of cells through S phase. The effects exerted by Thr-592 phosphorylation-defective SAMHD1 mutants were associated with activation of DNA damage checkpoint and depletion of dNTP concentrations to levels lower than those seen upon expression of wild type SAMHD1 protein. These disruptive effects were relieved by either mutation of the catalytic residues of the SAMHD1 phosphohydrolase domain or by a Thr-592 phosphomimetic mutation, thus linking the Thr-592 phosphorylation state to the control of SAMHD1 dNTPase activity. Our findings support a model in which phosphorylation of Thr-592 by cyclinA2-CDK down-modulates, but does not inactivate, SAMHD1 dNTPase in S phase, thereby fine-tuning SAMHD1 control of dNTP levels during DNA replication.

Sterile α motif domain and HD domain-containing protein 1 (SAMHD1) is a nuclear deoxyribonucleoside triphosphohydrolase (dNTPase) having important roles in cellular homeostasis and innate immunity (1, 2). Loss-of-function SAMHD1 mutations in humans are associated with Aicardi-Goutières syndrome (AGS), an auto-inflammatory genetically inherited interferonopathy, whose clinical presentation resembles congenital viral infection (3). In mice, SAMHD1 gene knock-out triggers spontaneous production of type I interferon (IFN) and up-regulation of type I IFN-stimulated genes (4, 5). SAMHD1 also plays important roles in innate immunity to viral infections. It inhibits replication of viruses that require dNTPs to synthesize their genomes. In particular, SAMHD1 strongly inhibits reverse transcription of retroviruses, including HIV-1 in non-dividing monocyte-derived macrophages and dendritic cells, and in quiescent T cells (6–12).

The anti-inflammatory effect of SAMHD1 likely reflects an important role of this enzyme in regulating cellular deoxyribonucleoside triphosphate (dNTP) concentrations through their hydrolysis. Loss of SAMHD1 function in individuals with AGS and transgenic models results in an increase in cellular dNTP concentrations (4, 5, 13). This, in turn, is thought to lead to inappropriate synthesis and/or presentation of cellular DNA to cytoplasmic sensors, thereby triggering production of type I IFN. The antiviral effect of SAMHD1 also involves the dNTPase function. SAMHD1 inhibits HIV replication in nondividing cells by lowering the dNTP concentrations below the threshold necessary for the viral reverse transcriptase to synthesize proviral DNA (14, 15). Notably, HIV-2 and related simian lentiviruses possess an accessory virulence factor termed Vpx, which targets SAMHD1 for proteasome-dependent degradation in infected cells (6, 7, 16–18). This results in an increase in cellular dNTP concentrations and thereby relieves the inhibition of reverse transcription of the incoming viral genomes (14).

Cellular dNTP concentrations are tightly controlled through the cell division cycle by balancing ribonucleotide reductase
Cells and Retrovirus Transduction—Human embryonic kidney cells (HEK 293T) were maintained in DMEM supplemented with 10% fetal bovine serum and antibiotics. THP-1 and U937 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics. Stable U937 cell lines expressing the doxycycline-inducible Tet trans-activator were established by transduction with the plLVX-3G lentiviral vector followed by G418 selection (Clontech). Cells were then infected with VSV-G-pseudotyped plLVX-TRE3G viruses expressing wild type or mutant forms of hfa-tagged SAMHD1. 48 h after infection, cells were selected with and then cultured in the continuous presence of puromycin (2 μg/ml). SAMHD1 expression in cells transduced with plLVX-TRE3G viruses was induced with 100 ng/ml doxycycline for 16 h. THP-1 cells stably expressing SAMHD1 variants were constructed by transduction with retroviral MSCV(puro) vectors and selected with puromycin. CD4⁺ T lymphocytes were isolated from peripheral blood of healthy donors using the human CD4⁺ T cell enrichment kit (StemCell Technologies), activated using human T-activator CD3/CD28 Dynabeads (Invitrogen) and expanded with IL-2 according to the product manual (R&D Systems).

Immunoprecipitation, Immunoblotting, and Antibodies—Typically, detergent extracts were prepared from 10⁶ cells, and protein complexes were immunoprecipitated via FLAG or HA epitope tag as described previously (6, 32). Cell extracts were separated by SDS-PAGE and transferred to PVDF membrane for immunoblotting. Proteins were detected with appropriate primary antibodies, and immune complexes were revealed with HRP-conjugated antibodies specific for the Fc fragment of mouse or rabbit immunoglobulin G (1:5000, Jackson ImmunoResearch) and enhanced chemiluminescence (GE Healthcare), or with fluorescent antibodies to mouse or rabbit immunoglobulin G (Kirkegaard & Perry Laboratories) and Odyssey Infrared Imager (LiCor). The following antibodies were used: α-SAMHD1 C terminus (33); α-SAMHD1 peptide residues 366–380 (SAB1101454, Sigma); α-cyclin-A2 (H432, Santa Cruz Biotechnology); α-CHK1(S345) (133D3, Cell Signaling); α-FLAG epitope (M2, Sigma); α-HA epitope (12CA5); and α-splicing factor 2 (gift of A. Krainer).

The antibody specific for Thr-592-phosphorylated SAMHD1 was raised in rabbits to CIAPL1(pT)PQKKE peptide (Covance) and purified by affinity chromatography on the immunizing peptide. Blotting with the affinity-purified antibody was performed in the presence of an unphosphorylated competitor peptide at 10 μg/ml.

Multidimensional Protein Identification Technology (MudPIT) Analysis—Protein complexes were purified from THP-1 cells stably expressing hfa-tagged human SAMHD1 protein, by sequential immunoprecipitations via HA and then FLAG epitope tags, each followed by competitive elution with the respective epitope peptide (34). MudPIT analyses of purified protein complexes were performed as described previously (34, 35). Distributed normalized spectral abundance factors were calculated for each detected protein as described (36).

Cell Cycle Analysis—Aliquots of U937 cells (1 × 10⁶) were transduced with MSCV(puro) viruses expressing epitope-tagged wild type or variant forms of SAMHD1. Three days later,
cells were pulse-labeled with 5-ethyl-2'-deoxyuridine (EdU, 10 μM) for 60 min, and the incorporated EdU was detected using Click-IT® Plus EdU Alexa Fluor® 647 flow cytometry assay kit (Life Technologies, Inc.), following the manufacturer’s protocol. DNA content was measured by staining with 2 μg/ml 7-AAD (Life Technologies, Inc.). Cell cycle distributions of the stained populations were recorded with FACSCalibur flow cytometer and analyzed using FlowJo software.

Isolation of G1, S, and G2/M Cells by Cell Sorting—4 × 10⁶ cells were stained with 10 μM Vybrant® DyeCycle™ Green stain (Life Technologies, Inc.) in 8 ml RPMI Media at 37°C for 30 min and G1, S and G2/M, or populations (~3 × 10⁶ cell for each population) were isolated based on DNA content. Aliquots of 10⁵ of the sorted cell populations were then stained with 7-aminoactinomycin D and re-analyzed to assess their purity. The cells were lysed in Laemmli sample buffer (37), and whole cell extracts were analyzed by immunoblotting.

Quantification of dNTPs—dNTPs were extracted from cells (1 × 10⁶), evaporated under vacuum at 70°C, and the lyophilized extracts stored at ~80°C. The dried material was resuspended in H₂O, and dATP was quantified using an HIV-1 reverse transcriptase-based single nucleotide incorporation assay, essentially as described previously (38). Standard curves prepared with known amounts of dATP were used to calculate dATP amounts in test samples based on the extent of primer extension.

Recombinant Proteins and Purification of CyclinA2-CDK Complex—The cDNAs encoding cyclinA2 (CycA2) residues 173–432 and full-length CDK2(T160E) were cloned into the pET28 vector (EMD4Biosciences) and pET21 vector (EMD4Biosciences), respectively. The CycA2-CDK2 complex was purified following co-expression in E. coli (DE3) and cultured in Luria-Bertani medium using 0.4 mM isopropyl-1-thio-β-D-galactopyranoside for induction at 18°C for 16 h. Proteins were purified using a 5-ml nickel-nitriilotriacetic acid column (GE Healthcare), followed by gel filtration column chromatography (Hi-Load Superdex 200 16/60, GE Health care) equilibrated with a buffer containing 25 mM sodium phosphate, pH 7.5, 150 mM NaCl, 1 mM DTT, 10% glycerol, and 0.02% sodium azide. Human SAMHD1 wild type and mutants were expressed in E. coli and purified as described previously (25).

SAMHD1 Tetramerization and dNTPase Assays—Mixtures of SAMHD1 (0.5 μM), GTP, and dATP, as indicated, were injected into an analytical Superdex200 column at a flow rate of 0.5 ml/min, equilibrated with 20 mM Tris-HCl, pH 7.8, 50 mM NaCl, 5 mM MgCl₂, 5% glycerol, and 0.02% sodium azide. The proteins were detected using an in-line fluorometer with excitation at 282 nm and emission at 313 nm. The peak areas of tetramer and dimer/monomer were integrated, and the percent fraction of tetramer was calculated as described previously (25, 28). dNTPase activity of SAMHD1 and its variants (0.1 μM) was measured in an assay mixture containing 25 mM Tris-HCl, pH 7.8, 50 mM NaCl, 2 mM MgCl₂, 5% glycerol, and appropriate concentrations of GTP and dNTPs. Reactions were stopped with 20 mM EDTA at 4, 8, 16, and 32 min and subjected to HPLC for quantification of deoxyribonucleosides (24, 28).

| TABLE 1 | CyclinA2-CDK Regulates SAMHD1 HD Domain |
|---------|------------------------------------------|
| NCBI_gene | IP: SAMHD1 | IP: mock |
| CCNA2 | 57; 0.00182855⁶ | 0; 0 |
| CCNB1 | 4; 0.00012802 | 0; 0 |
| CDK1 | 65; 0.00303300 | 0; 0 |
| CDK2 | 52; 0.00241826 | 0; 0 |
| CDK5 | 4; 0.00021221 | 0; 0 |
| CDK6 | 3; 0.00012753 | 0; 0 |
| CKS1B | 1; 0.00017542 | 0; 0 |
| SAMHD1 | 13742; 0.30422208 | 0; 0 |

⁶ Spectral count indicates the number of mass spectrometry spectra matching peptides from the indicated protein.

SAMHD1 in Vitro Phosphorylation Assay—In a typical assay with monomeric/dimeric SAMHD1 as a substrate, 1 μg of aliquots of recombinant SAMHD1 protein was incubated with 250 ng of cyclinA2-CDK2 (T160E) and 250 μM ATP in the presence of 5 mM MOPS, pH 7.2, 2.5 mM β-glycerophosphate, 5 mM MgCl₂, 1 mM EDTA, 0.4 mM EDTA, and 0.05 mM DTT, at 30°C for 30 min. In assays using tetrameric SAMHD1 substrate, 1 μg of recombinant SAMHD1 was preincubated with 150 μM of each GTP/dATP for 30 min on ice, to assemble SAMHD1 tetramer, and the phosphorylation reaction was carried out as described above. Reactions were terminated by addition of 0.25% volume of 5X Laemmli sample buffer containing phosphatase inhibitors. The reactions were resolved by SDS-PAGE, and SAMHD1 was revealed by immunoblotting.

Results
CyclinA2 Associates with SAMHD1 C-terminal Domain—SAMHD1 antiviral function was reported to be modulated by mutations in the C-terminal region, including a phosphomimetic substitution for Thr-592 (25, 29, 30, 39). This threonine was shown to be a substrate for CDK1 and/or CDK2 kinase in complex with S phase cyclinA2 (13, 29). We searched for additional candidate regulators of SAMHD1 in monocytes, via Thr-592 phosphorylation, by purifying SAMHD1 immune complexes from THP-1 cell extracts and characterizing their composition by multidimensional protein identification technology (MudPIT). CDK1, CDK2, and cyclinA2 were the most abundant serine/threonine kinases and cyclins that we found associated with SAMHD1, in agreement with previous observations (see Table 1).

Cyclin-CDK complexes bind their substrates via bipartite recognition sequences comprising the phosphoacceptor site and a downstream cyclin-binding motif (40, 41). To further characterize the interaction between the SAMHD1 and cyclinA2-CDK complex, we mapped the cyclinA2-binding site in SAMHD1. Initially, we constructed a small set of SAMHD1 C-terminal deletion and point mutants, shown in Fig. 1A, and expressed them in U937 cells by retrovirus transduction. The proteins were tagged with tandem HA-FLAG-AU1 (hfa) epitope tag at their N termini. SAMHD1 immune complexes were precipitated via FLAG tag from whole cell extracts pre-
pared from the transduced cells and analyzed by immunoblotting for cyclinA2. Whereas cyclinA2 association with full-length SAMHD1 (residues 1–626) was readily detectable, deletion of the SAMHD1 C terminus abolished the association (Fig. 1, B and C, compare 1–626 with 1–595 and 1–617). Further mapping revealed that an alanine substitution for the di-hydrophobic amino acid residues leucine and phenylalanine (L620A/F621A) was sufficient to disrupt stable association with cyclinA2 (Fig. 1C). Alanine substitution for the neighboring valine and glutamine residues (V618A/Q619A) weakened but did not abolish the binding. We conclude that the SAMHD1 C terminus is required for the formation of a stable complex with cyclinA2.

The catalytically active form of SAMHD1 is a tetramer assembled from dimers upon GTP and dNTP binding to two distinct allosteric sites (24, 28). To assess whether SAMHD1 ternary state affects cyclinA2 binding, we tested the effect of a mutation in the SAMHD1 dimer-dimer interface (K534A, V537A, L540A) and of a mutation that prevents GTP/dGTP binding to allosteric site 1, which prevents dimer formation, on association with cyclinA2 in U937 cells (24, 28). We found that neither of the two mutations prevented cyclinA2 binding, although the protein with the D137A mutation was expressed at much lower levels in cells (Fig. 1D). Thus, the cyclinA2-docking site appears accessible for binding in all ternary forms of SAMHD1.

**Di-hydrophobic CyclinA2-CDK-binding Motif in the SAMHD1 C Terminus Mediates Thr-592 Phosphorylation**—In silico analysis of the amino acid sequence context of SAMHD1 Thr-592 with the GPS 2.0 tool raised the possibility that this residue can be phosphorylated by protein kinases other than CDKs, such as p38 MAPK and GSK, among others (42). To address this issue and to corroborate the key role of the SAMHD1 C-terminal cyclinA2-binding element identified above for Thr-592 phosphorylation, we analyzed the effect of alanine scan mutations in the SAMHD1 C terminus on Thr-592 phosphorylation in vivo. A panel of variant SAMHD1 proteins with alanine substitutions in the C terminus was expressed in U937 cells, and the Thr-592-phosphorylated form of SAMHD1 was detected by immunoblotting of cell extracts with an antibody specific for the Thr-592-phosphorylated C-terminal peptide. In parallel, total SAMHD1 levels were revealed with an antibody specific for an epitope located in the HD domain several hundred amino acids N-proximal to Thr-592. As shown in Fig. 2A, two of the mutant proteins resisted Thr-592 phosphorylation. One mutant carried an alanine substitution for the last positively charged residue in the consensus TPX(K/R) cyclin-dependent kinase/MAPK target sequence (K595A in Q594A/K595A/K596A). The other was mutated in the di-hydrophobic cyclinA2-binding motif (L620A/F621A). We thus conclude that cyclinA2-CDK1/2 are the main kinases for Thr-592 in proliferating U937 cells.
To corroborate the results from the \textit{in vivo} experiments, we next investigated the effect of mutations in the SAMHD1 C terminus on Thr-592 phosphorylation by the cyclinA2-CDK2 complex \textit{in vitro}, using recombinant proteins. Wild type and mutant SAMHD1 proteins were incubated with purified cyclinA2-CDK2 complex in reaction buffer with or without GTP and dATP, and the SAMHD1 Thr-592 phosphorylation status was assessed by immunoblotting. As shown in Fig. 2B, deletion of the C-terminal residues 618 – 626 (1 – 617) and alanine substitutions for leucine and phenylalanine LF620 (L620A/F621A) abolished Thr-592 phosphorylation, in agreement with the results from \textit{in vivo} experiments shown in Fig. 2A.

\textbf{Di-hydrophobic LF Motif Is Conserved in Vertebrate SAMHD1 Proteins}—The key role played by the di-hydrophobic LF620 motif for Thr-592 phosphorylation in human SAMHD1 suggested that this motif is likely shared among SAMHD1 proteins. Alignment of amino acid sequences of SAMHD1 proteins from several diverse vertebrate species revealed strict conservation of this motif at locations $– 21 – 40$ residues C-proximal from the phosphoacceptor threonine and $3 – 10$ amino acid residues from the C terminus, in otherwise quite divergent amino acid sequences.
sequence contexts. This, together with the above findings, supports a model in which cyclinA2-CDK1/2 complexes are the major kinases that phosphorylate SAMHD1 Thr-592.

**SAMHD1 Levels Are Unchanged during the Cell Division Cycle in Leukocytes**—dNTP concentrations vary across the cell cycle being highest during nuclear DNA synthesis (43, 44). Because the dNTP pool sizes reflect a steady state between dNTP synthesis by RNR and their degradation by SAMHD1, it is expected that SAMHD1 levels and/or activity be down-regulated in S phase (21, 45). As cyclinA2-CDK is activated at G1/S transition and responsible for actual initiation of nuclear DNA synthesis, SAMHD1 Thr-592 phosphorylation by this kinase is a plausible candidate event triggering suppression of SAMHD1 in S phase (45).

To begin addressing this possibility, we first asked whether SAMHD1 levels change according to cell cycle phase in leukocytes. Experiments were performed with primary human CD4-positive T lymphocytes and two monocytic cell lines, THP-1 and U937. THP-1 monocytes express endogenous SAMHD1 at high levels comparable with those found in major populations of primary human leukocytes (6, 7). U937 cells do not express endogenous SAMHD1 but can efficiently express ectopic SAMHD1 following transduction with retroviral expression vectors (7, 25).

CD4-positive T lymphocytes were purified from peripheral blood of a healthy donor, induced to proliferate by cross-linking T cell receptor and CD28 co-stimulatory receptor with antibodies, a treatment that mimics physiological antigen-mediated T cell activation, and then expanded in the presence of IL-2. Eight days later live cells were stained with a Vybrant® DyeCycle™ Green vital stain, and were sorted based on their DNA content. The purity of the isolated cell populations was characterized by analyzing the DNA profiles following staining with 7-aminoactinomycin D. DNA profile of the initial asynchronously growing THP-1 cells is also shown. The peaks of the histograms were adjusted to the same heights, for the purpose of comparison. B, levels of total and Thr-592-phosphorylated SAMHD1 during cell division cycle. Total cell extracts prepared from 2 × 10^5 asynchronously dividing lanes A, G1, S, and G2/M populations of THP-1 monocytes, proliferating human primary CD4^+ T lymphocytes, and U937 cells expressing retrovirally transduced wild type SAMHD1 were analyzed by immunoblotting with the antibodies reacting with cyclinA2, SAMHD1 HD domain and G2/M phase populations. Whole cell extracts prepared from the sorted cells were immunoblotted for cyclinA2, total SAMHD1, and phospho-Thr-592 SAMHD1. CyclinA2 was detected only in the S and G2/M extracts, as expected, thus confirming the purity of the sorted cell populations (Fig. 3B). SAMHD1 was detected in G1, S, as well as G2/M extracts at approximately similar levels, in CD4-positive T lymphocytes and THP-1 monocytes. In U937 cells, the levels of the ectopically expressed SAMHD1 were highest in the S phase population, probably due to expression directed by a heterologous retroviral promoter. The phospho-Thr-592 SAMHD1 was readily detectable in the S and G2, but not in G1 phase cells, and its levels were well correlated with those of cyclinA2, as expected. These observations again support the notion that cyclinA2-CDK is the main kinase phosphorylating Thr-592, and they demonstrate that SAMHD1 levels are not post-translationally down-regulated during the S phase in primary T lymphocytes and transformed monocytes.

**Thr-592 and Control of SAMHD1 Levels in Cell Cycle**—To further explore whether Thr-592 phosphorylation has a role in regulating SAMHD1 levels, we next characterized the effect of the L620A/F621A cyclinA2-binding site mutation and Thr-592 phosphomimetic mutation on SAMHD1 levels during the cell cycle. The hfa epitope-tagged wild type and variant SAMHD1 proteins were expressed in THP-1 cells by retroviral transduction, and their steady state levels in G1, S, and G2 cells, along with those for the endogenously expressed SAMHD1, were characterized as described above. The ectopic (epitope-tagged slower migrating) and the endogenous (faster migrating) SAMHD1 proteins were readily separated by SDS-PAGE (Fig. 4A, lanes 1–4). Notably, the relative levels of the ectopic wild type SAMHD1 in G1, S, and G2 phases tracked closely those of the endogenous protein, except for being perhaps slightly lower in G1 phase cells.

We then tested the effect of the L620A/F621A mutation in SAMHD1. This mutation did not appear to alter the relative SAMHD1 levels in S versus G1 phase cells, compared with those of the ectopically expressed wild type SAMHD1 (Fig. 4A, compare lanes 6–8 with 2–4). Similarly, the T592D phosphomimetic mutation lacked an apparent effect on SAMHD1 levels during the cell cycle. These data confirmed that SAMHD1 levels are relatively constant through the THP-1 cell cycle.
CyclinA2-CDK Regulates SAMHD1 HD Domain

SAMHD1(L620A/F621A) Variant Perturbs S Phase Progression—Unchanged SAMHD1 levels upon S phase entry suggested that Thr-592 phosphorylation suppresses SAMHD1 function by a different mechanism. To address this, we characterized the effect of SAMHD1 mutations that disrupt the binding to the cyclinA2-CDK complex and, consequently, Thr-592 phosphorylation on cell cycle progression in U937 monocytes ectopically expressing the mutant SAMHD1 proteins (7, 25).

Wild type and variant SAMHD1 proteins were expressed in U937 cells by retroviral transduction, and cell cycle distributions of the transduced populations were characterized by flow cytometry. In brief, cells were infected with retroviral vectors expressing SAMHD1 at three different multiplicities of infection in a dose-response experiment, and SAMHD1 expression levels were assessed by immunoblotting 3 days later. As shown in Fig. 5A, this approach resulted in comparable dose-dependent expression levels of various SAMHD1 proteins.

To assess the effect exerted by SAMHD1 on DNA synthesis and cell cycle progression, cells were pulse-labeled with EdU, a “click-able” thymidine analogue, to mark cells actively synthesizing nuclear DNA, and the extent of DNA synthesis and DNA content was determined by flow cytometry. Expression of wild type SAMHD1 or the SAMHD1(VQ618AA) variant, which are good substrates for cyclinA2-CDK kinases, did not perturb cell cycle profiles, except for a slight reduction in the proportion of cells in S phase seen for the cell populations with the highest levels of SAMHD1 expression (Fig. 5B, EdU incorporation histograms). In contrast, expression of the SAMHD1(L620A/F621A) and SAMHD1(1–617) variants that fail to bind cyclinA2-CDK led to reduced EdU incorporation. This reduction is revealed by a downward shift of EdU fluorescence on bivariate plots as well as by a broadening of the S phase peak seen on EdU incorporation histograms. The perturbation in DNA synthesis in cells expressing the SAMHD1(1–617) variant was associated with an increase in Chk1(Ser-345) phosphorylation (Fig. 5C), indicative of the activation of a DNA damage checkpoint. As a positive control, U937 cells were cultured in the presence of hydroxyurea, which inhibits RNR and dNTP supply leading to cell cycle arrest at DNA damage checkpoint in the S phase.

The above results did not support the possibility that the Thr-592 phosphorylation by cyclinA2-CDK down-regulates SAMHD1 levels in the S phase. The possibility remained, however, that the T592D phosphomimetic mutation’s ability to direct SAMHD1 for degradation was masked in heterotetramers formed with endogenously expressed wild type SAMHD1. To exclude this possibility, we expressed the SAMHD1(T592D) variant in U937 cells, which lack endogenous SAMHD1, and analyzed its levels in G1 and S phase cells.

U937 cells were transduced to express SAMHD1(1–617) without or with the T592D phosphomimetic mutation, and G1 and S phase cells were isolated by sorting. SAMHD1 levels in lysates prepared from the sorted cells were then analyzed by immunoblotting. As shown in Fig. 4B, the phosphomimetic mutation had little if any effect on SAMHD1 levels in S phase cells. Together, these findings do not support the possibility that Thr-592 phosphorylation drives a rapid turnover of SAMHD1 molecules during S phase.

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CyclinA2-CDK Regulates SAMHD1 HD Domain

A

SAMHD1
α-tubulin

1-617
HD206RN
1-626
QF618AA
LF620AA
1-626

B

EdU
edU incorporation
DNA content
rel cell #

1-626

VQ618AA

1-617, HD206RN
HU

EdU
edU incorporation
DNA content
rel cell #

LF620AA

1-617

EdU
edU incorporation
DNA content
rel cell #

1-617, HD206RN

EdU
edU incorporation
DNA content
rel cell #

C

Chk1(S345)
Chk1
SAMHD1
Chk1P:Chk1
SAMHD1

MIP
1-626
1-617
1-617, HD206RN
HU

58.1
60.7
55

58.1
60.8
61.4

61.4
60.9
47.5

56.1
51.7
47.5

51.7
47.5
44.6

down-regulates SAMHD1 phosphohydrolase activity, most likely its dNTPase activity.

T592D Phosphorylation Mimetic Relieves Cell Cycle Effects of CyclinA2-binding Deficient SAMHD1—To further understand how the Thr-592 phosphorylation modulates SAMHD1 function, we investigated dNTP concentrations in U937 cells expressing the variant SAMHD1 proteins.

U937 cells expressing wild type or variant forms of SAMHD1 were harvested for the determination of SAMHD1 expression levels or were pulse-labeled with EdU to assess their progression through S phase. In parallel, to gauge SAMHD1’s ability to lower dNTP concentrations, we determined dATP pool sizes for all cell populations by single nucleotide primer extension assay (38). As expected, expression of SAMHD1 with L620A/F621A or T592D substitutions, both of which abolish Thr-592 phosphorylation, interfered with EdU incorporation in S phase cells. In contrast, expression of the SAMHD1 variant with a T592D phosphomimetic mutation, at comparable levels, did not exert such an effect (Fig. 6A). Similarly, the SAMHD1 protein with combined L620A/F621A and T592D mutations did not perturb DNA replication, even though it was expressed at a higher level than that with the L620A/F621A alone (Fig. 6B). Thus, the T592D phosphomimetic mutation compensated for the lack of cyclinA2-CDK kinase recruitment, which phosphorylates Thr-592, and moderated SAMHD1 function in S phase.

We quantified dATP levels as the readout for the ability of SAMHD1 variants to lower dNTP concentrations. Examination of dATP pool sizes in asynchronously dividing cell populations revealed that all variant SAMHD1 proteins retained the ability to deplete dATP levels. However, dATP concentrations in cells expressing SAMHD1(L620A/F621A) and SAMHD1(T592D) variants and showing abnormal EdU incorporation were 2–4-fold lower than those in cell populations displaying no S phase abnormalities and expressing SAMHD1 wild type or the T592D phosphomimetic mutant (Fig. 6B, compare bars 3 of all constructs). Conversely, the T592D phosphomimetic variant depleted dATP to levels similar to or slightly higher than those seen with wild type SAMHD1 protein (Fig. 6B, compare 1–626 lanes/bars 2 and 3 with T592D bars 2 and 3). Of note, wild type SAMHD1 is phosphorylated on Thr-592 in U937 cells (Figs. 3 and 4). The attenuating effect of this T592D mutation was best seen when T592D was combined with the L620A/F621A mutation disrupting cyclinA2-binding (compare L620A/F621A, lanes/bars 2 and 3 with L620A/F621A, T592D, lanes 2 and 3, and T592D, lanes 2 and 3). Together, the above genetic data provide compelling evidence that Thr-592 phosphorylation regulates SAMHD1 HD phosphohydrolase function and, in particular, its dNTPase activity.

T592D Controls SAMHD1 Tetramerization and Catalytic Activity in Vitro—The catalytically active form of SAMHD1 dNTPase is the tetramer (25). To assess whether Thr-592 controls directly SAMHD1 dNTPase activity, we analyzed the effect of Thr-592 substitutions on SAMHD1 tetramerization and catalytic activity. Recombinant SAMHD1 without or with Thr-592 substitution was incubated in the presence of GTP and dATP allosteric activators over a wide range of dATP concentrations, and the quaternary state of SAMHD1 was determined by analytical size exclusion chromatography (25, 28). As shown in Fig. 7A, wild type SAMHD1 tetramerized efficiently with ~50% of protein in the tetramer form, even at the lowest dATP concentration tested in this assay. In contrast, the T592D variant formed tetramers much less efficiently, and this was especially well pronounced at the lower end of the range of dATP concentration tested. The SAMHD1(T592D) variant was also less catalytically active than the wild type SAMHD1 (Fig. 7B).

Together, these data indicate that the T592D phosphorylation mimic negatively regulated both SAMHD1 tetramerization and catalytic activity.

We then tested whether the T592A mutation affects SAMHD1 function and found a slight negative effect on tetramer formation/stability (Fig. 7C). Surprisingly, the dATPase activity of SAMHD1(T592A) variant was found to be diminished and similar to that conferred by the T592D phosphomimetic substitution (Fig. 7D). Of note, dATPase activities of the wild type SAMHD1 and the Thr-592 variants were not dependent on the particular concentrations of GTP we used in our experiments. This is an expected result, as the apparent binding constant of GTP at the primary allosteric site is less than 100 μM, both for the wild type SAMHD1 and its variants (data not shown). The dNTPase activities of SAMHD1(T592D) and SAMHD1(T592A) toward other dNTPs were much lower than those of the wild type enzyme, similar to that seen with the dATP substrate (data not shown). Together, the above findings support the key role of Thr-592 in regulating SAMHD1 catalytic activity but do not provide a biochemical explanation for the effect of the T592A substitution on SAMHD1 function during S phase.

Discussion

Cellular dNTP concentrations fluctuate during cell division cycle, being the lowest in G1 phase and the highest during S phase. Recent studies revealed that in mammalian cells dNTP

FIGURE 5. Perturbed DNA synthesis and checkpoint activation in cells expressing SAMHD1 with cyclinA2-binding site mutations. U937 cells were transduced with increasing doses of MSCV(puro) viruses expressing FLAG-tagged wild type or variant SAMHD1 proteins. A, SAMHD1 levels in extracts prepared from the transduced cell populations were revealed by immunoblotting with anti-FLAG antibody. B, cell cycle analysis of U937 cells expressing wild type and variant SAMHD1 proteins. 3 days after infection, cells were pulse-labeled with 10 μM EdU for 1 h, and EdU incorporation and DNA content were analyzed by flow cytometry. Bivariate distributions of EdU incorporation versus DNA content (upper panels) and histograms of EdU incorporation (lower panels) are shown for each SAMHD1 variant characterized. The percent fraction of cells in G1, S, and G2/M cell cycle phases is indicated in lower left, upper, and lower right sides of each panel. C, checkpoint activation in cells expressing SAMHD1 variant with deleted cyclinA2-binding site (1–617). Whole cell extracts prepared from U937 cells expressing wild type (1–626), cyclinA2-binding site deleted (1–617), or cyclinA2-binding site deleted and catalytically inactive (HD206RM) SAMHD1 proteins were analyzed by immunoblotting with antibodies reacting with Chk1, Ser-345-phosphorylated form of Chk1, or FLAG tag (SAMHD1), followed by fluorescently labeled secondary antibodies. Cells transduced with an empty vector (MIP) or treated with 300 μM hydroxyurea (HU) for 18 h to induce S phase DNA damage checkpoint provided negative and positive controls, respectively. Fluorescent signals were quantified with Odyssey imager. The extents of Chk1 Ser-345 phosphorylation, expressed as a ratio of Chk1(Ser-345) signal to total Chk1 signal and normalized to that for U937 infected with an empty MSCV(puro) at the multiplicity of infection equivalent to the highest dose of MSCV(puro) expressing the 1–617 SAMHD1 variant, is shown below the image. The relative levels of SAMHD1 expression are also shown below.
levels are controlled by a combined action of RNR and SAMHD1 (21). Whereas the elaborate control of dNTP synthesis by RNR in the cell cycle is understood in great detail, little is known about how SAMHD1 function is regulated in proliferating cells. Studies in nondividing innate immune cells and fibroblasts expressing loss-of-function SAMHD1 mutants revealed that SAMHD1 is required for low dNTP concentrations in G1 phase, when RNR activity is relatively low (14, 21). Intuitively, one would expect SAMHD1 to be down-regulated in S phase as dNTP pools are relatively small (although higher than in G1), and dNTPs need to be synthesized continuously to support uninterrupted DNA replication. Because human SAMHD1 Thr-592 phosphorylation by cyclin-CDKs could be the switch that controls SAMHD1 function in proliferating cells, we characterized this event in more detail and assessed the consequences of disrupting Thr-592 phosphorylation on SAMHD1 function in the cell cycle. Our data confirm that Thr-592 is phosphorylated in S phase by cyclinA2-CDK1/2 and further show that this enzyme is the main kinase for phosphorylation of Thr-592. Our studies also show that Thr-592 phosphorylation controls SAMHD1 function in S phase, without down-regulating SAMHD1 levels. Our findings indicate that cyclinA2-CDK phosphorylation of Thr-592 regulates SAMHD1 activity linked to the catalytic residues in the HD domain, and they further demonstrate that this modification suppresses SAMHD1’s ability to lower dNTP concentrations in S phase, without having an overt inhibitory effect on its dNTPase activity.

It is evident that the cyclinA2-CDK complex is the main kinase phosphorylating Thr-592 in proliferating leukocytes in S/G2 phases of the cell cycle. Our cell-based and in vitro structure-function studies show that SAMHD1 is recruited to cyclinA2-CDK complexes via a bipartite binding site comprising a short peptide with an embedded cyclin-dependent kinase phospho-acceptor site and a distal di-hydrophobic LF motif, reminiscent of a generic bipartite cyclin-CDK substrate recognition site (40). We also show that mutation of the distal LF motif is sufficient to prevent SAMHD1 binding to cyclinA2-CDK.
CDK and/or Thr-592 phosphorylation both in vivo and in vitro and that the levels of SAMHD1 phospho-Thr-592 peak in S and G2/M phase while being virtually undetectable in G1 phase cells, thus mirroring cyclinA2 levels.

Our studies reveal that SAMHD1 levels are relatively unchanged during the cell cycle in T lymphocytes and monocytes. In contrast, previous studies with primary human fibroblasts suggested that SAMHD1 levels fluctuate significantly, the highest being in G1 quiescent cells and the lowest in S phase (21). Several explanations seem possible. In our experiments G1 and S phase cells were isolated directly by cell sorting of asynchronously dividing cell populations, without employing any synchronization protocols that could provoke checkpoint activation and thereby unintentionally modulate SAMHD1 levels. In contrast, the previous study used a serum starvation protocol to drive the fibroblast cultures into quiescence. Another explanation is suggested by previous observations that SAMHD1 levels in leukocytes are naturally relatively high compared with those in fibroblasts (6, 7, 21). These observations raise an intriguing possibility that SAMHD1 levels are regulated in distinct modes in different cell types, possibly depending on whether SAMHD1 exerts its innate immune function.

Our data show that in leukocytes, phosphorylation of Thr-592 controls SAMHD1 function in S phase, without modulating its levels. It can be clearly seen that expression of SAMHD1 variants that cannot be Thr-592-phosphorylated, either due to mutations in the cyclinA2-binding site or alanine substitution for Thr-592, interfered with DNA replication. Disruption of DNA replication was associated with activation of DNA damage checkpoint and depletion of dNTP to levels lower than those seen upon expression of wild type SAMHD1 protein. Significantly, two types of mutations in SAMHD1 relieved these effects. One was a substitution of the catalytic residues of the phosphohydrolase domain (HD206RN), which effectively inactivates the dNTP hydrolase activity (24, 46). The other was the T592D substitution, which mimics Thr-592 phosphorylation by the cyclin2-CDK complex. Together these data link the phosphorylation state of Thr-592 to the control of SAMHD1 dNTPase activity and suggest a model in which Thr-592 phosphorylation by cyclinA2-CDK down-regulates dNTPase activity in the S phase. In the absence of this modification, excessive dNTP hydrolysis in S phase disrupts DNA synthesis.

The catalytic His-206 and/or Asp-207 residues have also been implicated in two additional enzymatic activities of the SAMHD1 HD domain, nuclease and RNase (47, 48). Whereas potential contributions from these activities to S phase perturbations induced by SAMHD1 variants should be considered, the RNase activity was found to be insensitive to the T592D phosphomimetic mutation (48). Whether the SAMHD1 nuclease activity is modulated by Thr-592 is not known.

The in vivo and in vitro studies reported here with recombinant SAMHD1 proteins reveal that the T592D phosphomimetic variant is an active dNTPase, consistent with previous reports (30, 39). It is evident, however, that the T592D substitution weakens the in vitro dNTPase activity of the recombinant enzyme as well as its ability to lower cellular dNTP levels. The former effect was especially well pronounced at relatively low concentrations of the dATP allosteric activator, which is consistent with the observed higher dNTP concentrations in cells expressing the phosphomimetic variant. Together, our findings suggest a tentative model for S phase SAMHD1 regulation, in which Thr-592 phosphorylation leads to an increase in the threshold concentration of an allosteric dNTP regulator that is required for the activation of dNTP hydrolysis. The finding that alanine substitution for Thr-592 lowers dNTPase activity of the recombinant enzyme in vitro, but does not interfere with the ability of SAMHD1 to deplete cellular dNTP pools, is intriguing. On the one hand, it supports the key role of Thr-592 in controlling SAMHD1 dNTPase activity. On the other hand, the apparent discordance points to a more complex mode of SAMHD1 dNTPase regulation in vivo, possibly by an additional post-translational modification(s) and/or other modality(ies), which are absent in our in vitro assay. Nevertheless, although the exact mode of SAMHD1 regulation in vivo is not fully understood, it is evident from our genetic and biochemical studies that Thr-592 controls SAMHD1 dNTPase activity and its ability to deplete cellular dNTP levels.

It is intriguing why the presence of a constitutively active dNTPase, such as Thr-592-phosphorylated SAMHD1, during genome replication would be beneficial to a cell. One possible explanation is that SAMHD1 dNTPase activity needs to be fine-tuned in a spatiotemporal manner for seamless progression of DNA replication in S phase while preventing aberrant DNA synthesis on illegitimate templates, the failure of which could lead to an autoinflammatory condition. This possibility will need to be addressed in future studies.

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