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Low dNTP levels are necessary but may not be sufficient for lentiviral restriction by SAMHD1

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ABSTRACT

SAMHD1 is a cellular dNTPase that restricts lentiviral infection presumably by lowering cellular dNTP levels to below a critical threshold required for reverse transcription; however, lowering cellular dNTP levels may not be the sole mechanism of restriction. In particular, an exonuclease activity of SAMHD1 was reported to contribute to virus restriction. We further investigated the requirements for SAMHD1 restriction activity in both differentiated U937 and cycling HeLa cells. Using hydroxyurea treatment to lower baseline dNTP levels in HeLa cells, we were able to document efficient SAMHD1 restriction without significant further reduction in dNTP levels by SAMHD1. These results argue against a requirement for additional myeloid-specific host factors for SAMHD1 function but further support the notion that SAMHD1 possesses an additional dNTP-independent function contributing to lentiviral restriction. However, our own experiments failed to associate this presumed additional SAMHD1 antiviral activity with a reported nuclease activity.

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Introduction

Sterile alpha motif and HD domain protein 1 (SAMHD1) is a host factor contributing to the inefficient replication of HIV-1 in cells of myeloid lineage and other non-dividing cell types (Baldauf et al., 2012; Berger et al., 2011; Descours et al., 2012; Hrecka et al., 2011; Laguette et al., 2011). The SIVsm/HIV-2 Vpx proteins are however able to counteract SAMHD1 by targeting it for proteasomal degradation and viruses lacking this protein are restricted at the reverse transcription step in susceptible cell types (Goujon et al., 2007; Hrecka et al., 2011; Laguette et al., 2011; Sharova et al., 2008).

Early work on the SAMHD1 protein suggested it to be involved in regulating the innate immune response as mutations in SAMHD1 have been associated with Acardi-Goutieres Syndrome (AGS), a syndrome associated with increased production of interferon alpha (Dussaix et al., 1985; Rice et al., 2009). Accordingly, SAMHD1 knockout mice, while developmentally healthy, show increased expression of interferon stimulated genes (Behrendt et al., 2013; Rehwinkel et al., 2013). The main catalytic activity ascribed to SAMHD1 is its (d)GTP-dependent dNTPase activity with an active site located in the protein's HD domain (Amie et al., 2013; Goldstone et al., 2011; Hansen et al., 2014; Ji et al., 2014, 2013; Koharudin et al., 2014; Powell et al., 2011; Zhu et al., 2013). This enzymatic activity allows SAMHD1 to degrade dNTPs to component nucleosides and free triphosphate in a single step. Therefore, dNTP degradation by SAMHD1 provides a counterpart to dNTP synthesis by ribonucleotide reductase (RNR) with both these proteins being carefully regulated to control the delicate dNTP balance in cells (Franzolin et al., 2013). It was thus suggested that SAMHD1 uses its dNTPase activity to restrict lentivirus (and other dNTP-dependent virus) infection by decreasing dNTP levels in susceptible cells to below the levels required for reverse transcription/replication (Hollenbaugh et al., 2013; Kim et al., 2012, 2013; Lahouassa et al., 2012).

Interestingly, SAMHD1 also possesses nucleic acid binding capability and has been reported in some studies to possess 3′–5′ exonuclease activity against ssRNA and viral genomes (Beloglazova et al., 2013; Goncalves et al., 2012; Ryoo et al., 2014; Tungler et al., 2013; White et al., 2013a). Indeed, several recent reports employing mutagenesis experiments to genetically separate the dNTPase and nuclease activities of the protein suggested that nuclease activity may be the main contributor to SAMHD1 restriction of lentiviruses (Choi et al., 2015; Ryoo et al., 2014). However, other studies have failed to detect nuclease activity associated with the SAMHD1 active site (Goldstone et al., 2011; Seamon et al., 2015) thus, leaving the question of the functional importance of a SAMHD1 exonuclease activity up for future investigations. Furthermore, while phosphorylation has been shown to negatively regulate SAMHD1 restriction ability (Cribier et al., 2013; Welbourn et al., 2013; White et al., 2013b) it is still unclear whether this modification might affect dNTPase activity, nuclease activity or

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possibly another characteristic of SAMHD1 not yet described (Arnold et al., 2015; Ryoo et al., 2014; Tang et al., 2015; Welbourn et al., 2013; White et al., 2013b; Yan et al., 2015). Therefore, whether lowering cellular dNTP levels is the sole mechanism of restriction used by SAMHD1 and whether or not this function is even necessary requires further investigation.

In this study we investigated the importance of cellular dNTP levels for virus restriction by SAMHD1. Unlike other restriction factors such as APOBEC3G that renders normally permissive HeLa cells restrictive for HIV-1, exogenous expression of SAMHD1 in HeLa cells shows little to no restrictive phenotype. It is possible that the continued synthesis of dNTPs in dividing HeLa does not allow SAMHD1 dNTPase activity to sufficiently lower the cellular dNTP pool for lentiviral restriction to occur. Alternatively, it cannot be ruled out that SAMHD1 exerts its antiviral effect in conjunction with additional host factor(s) not expressed in non-myeloid or dividing cell types. To address these questions, we employed SAMHD1 variants together with hydroxyurea treatment to modulate dNTP levels in HeLa cells. Hydroxyurea inhibits ribonucleotide reductase and thus reduces the cellular dNTP pool at the synthesis step (Nordlund and Reichard, 2006). Determination of cellular dATP levels confirmed that hydroxyurea dramatically reduced the baseline dNTP pool in treated HeLa cells with SAMHD1 exhibiting negligible additional effects on the dNTP pool. Using the hydroxyurea strategy of lowering baseline dNTP levels we were able to demonstrate SAMHD1 antiviral activity in HeLa cells whereas no additional antiviral activity was demonstrable in untreated cells. These results suggest that the lack of antiviral activity of SAMHD1 in normal HeLa cells is not due to the lack of additional cellular proteins but are due to the high dNTP levels in these cells. Thus, low cellular dNTP levels appear to be necessary for SAMHD1 restriction activity. However, our results also provide further evidence that SAMHD1 may possess an additional dNTP-independent function that contributes to lentiviral restriction but a contribution of a possible exonuclease activity could not be confirmed.

Results and discussion

SAMHD1 T592E decreases dATP levels in cells without causing significant restriction

We and others have shown that SAMHD1 phosphomimetics (T592E/T592D) were unable to restrict HIV infection in PMA-differentiated U937 cells yet still retained dNTPase activity *in vitro* (Welbourn et al., 2013; White et al., 2013b). While other recent studies have suggested phosphorylation (or phosphomimetics) might modulate dNTPase activity of SAMHD1 under certain conditions using recombinant protein (Arnold et al., 2015; Tang et al., 2015; Yan et al., 2015), only one group has reported cellular dNTP levels measured in cells under conditions where the SAMHD1 restrictive phenotype is lost due to phosphorylation (White et al., 2013b). We therefore wanted to confirm if the dNTPase activity we observed *in vitro* translated into a cellular effect and independently confirm the decreased cellular dNTP levels seen by others using a phosphomimetic mutant (White et al., 2013b). U937 cells were therefore transduced with lentiviral particles expressing SAMHD1 variants or empty vector, selected with puromycin, and differentiated with PMA. Parallel samples were used for western blot analysis, reporter virus infection, or dNTP isolation. All SAMHD1 variants were efficiently expressed (Fig. 1A) and SAMHD1 WT and T592A were able to efficiently restrict HIV-1-GFP infection as compared to cells expressing the active site mutant (H206R/D207N [HD/RN]) or the empty vector control (Fig. 1B). As reported previously, the T592E protein was impaired in its ability to restrict HIV-1 infection (Fig. 1B). Cellular dNTP extraction at time of infection showed that SAMHD1 T592E was indeed able to decrease cellular dATP levels to a similar extent as the wildtype and phosphoablative (T592A) proteins as compared to the much higher levels observed with the active site mutant or empty vector controls (Fig. 1C). These results therefore independently confirm that SAMHD1 T592E is able to decrease cellular dNTP levels as efficiently as the WT protein, suggesting an additional mechanism of restriction by SAMHD1 may exist beyond nucleotide depletion.

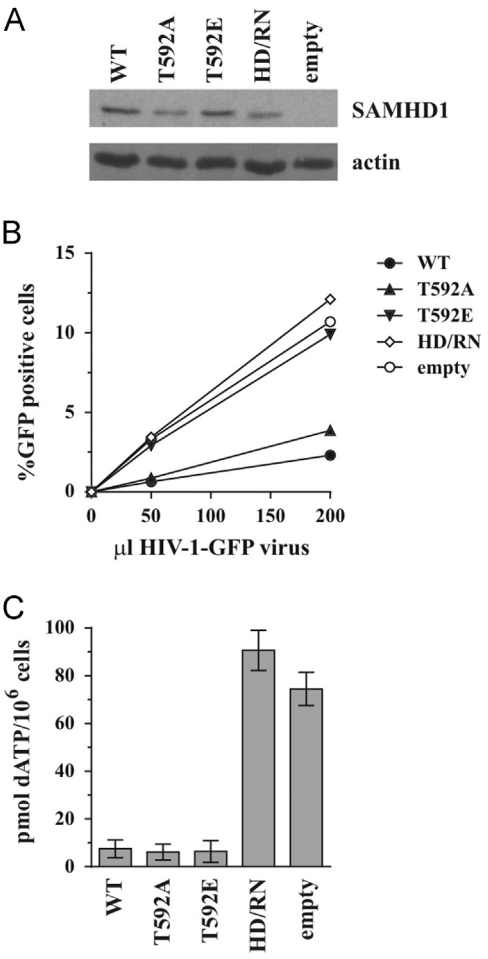

Fig. 1. Effect of SAMHD1 T592 phosphorylation on cellular dATP levels. U937 cells were transduced with lentiviral particles encoding either WT SAMHD1, the indicated mutants, or an empty vector. Following puromycin selection, cells were differentiated overnight with 10 ng/ml PMA. (A) Total cell extracts from differentiated U937 stable cell lines were separated by SDS-PAGE and subjected to immunoblotting for SAMHD1 and actin as indicated. (B) Differentiated cells were infected with increasing amounts of VSV-G pseudotyped HIV-1-GFP (as described in (Welbourn et al., 2013)) and the percent infection (% GFP – positive cells) was determined by flow cytometry 48 h later. (C) Cellular dNTP levels were isolated at time of infection and amounts of dATP present per million cells were determined using a polymerase based assay described in the Methods section. Results in panels A and B are representative of at least 3 independent experiments. Error bars in panel C represent the mean and standard deviation of quantitation from at least 3 independently generated cell lines.

Lack of SAMHD1 restriction activity in dividing cells with high dNTP levels

Although SAMHD1 is present in many actively dividing cell types, the restrictive effect has generally only been observed in differentiated/non-dividing cells (Baldauf et al., 2012; Berger et al., 2011; Descours et al., 2012; Hrecka et al., 2011; Laguette et al., 2011; St Gelais et al., 2012). There are several possible explanations, including a lowering of set point dNTP levels upon cell differentiation, the presence of a cell-type specific co-factor, or modulation of SAMHD1 restriction activity *via* phosphorylation at T592 in dividing cells (Cribier et al., 2013; Welbourn et al., 2013; White et al., 2013b). We