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Acta Med. Nagasaki 58: 103 – 111

SAM domain-containing N-terminal region of SAMHD1 plays a crucial role in its stabilization and restriction of HIV-1 infection

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SAMHD1 restricts human immunodeficiency virus type 1 (HIV-1) infection in a cell-type specific manner. Other than primary monocyte derived cells and resting CD4+ T cells, the SAMHD1-mediated HIV-1 block was reported only in phorbol 12-myristate 13-acetate (PMA)-differentiated THP-1 and U937 monocyte cell lines. We previously reported that SAMHD1 restricted HIV-1 infection in TE671 rhabdomyosarcoma cells in addition to these cell lines. In this study, we compared the amounts of the full-length SAMHD1 and its deletion mutants, SAM domain containing N-terminal fragment (residues 1-119, SAMHD1n) and HD domain containing C-terminal fragment (120-626, SAMHD1c) in U937, TE671, and HeLa cells. The results showed that the full-length SAMHD1 and SAMHD1n proteins were significantly more abundant than the SAMHD1c protein in TE671 and differentiated U937 cells. The proteasome inhibitor MG132 increased the amount of the SAMHD1c and the SAMHD1c-fused GFP proteins. In contrast, the fusion of the SAMHD1n to the APOBEC3G protein inhibited Vif-induced proteasomal degradation in TE671 and in differentiated U937 cells. These results indicated that the SAMHD1 C-terminal HD domain-containing region leads the SAMHD1 to proteasomal degradation, and the SAMHD1 N-terminal SAM domain-containing region stabilizes the protein. Our study showed that the SAMHD1 protein expression is post-translationally regulated and the significance of SAM and HD domains for the full-length SAMHD1 protein stability. Further, we suggest that the SAM domain-containing N-terminal region participate in the cell-type specific restrictive function of SAMHD1 against HIV-1 infection, by protein stabilization.

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Introduction

SAMHD1 (1-626 aa), composed of a tandemly linked-SAM domain (45-110 aa) and HD domain (164-319 aa), has recently been identified as a restriction factor against HIV-1. SAMHD1 was originally identified as a human homolog of mouse *Mg11* isolated from IFNγ-stimulated mouse dendritic cells [1,2], suggesting that SAMHD1 is an IFN-stimulated gene [3,4] though it depends on cell-type [3,5].

In parallel to the hosts obtaining restriction factors, viruses have developed mechanisms to overcome these restriction factors. Vpx, encoded by HIV-2 and simian immunodeficiency viruses (SIVs) but not by HIV-1 [6,7,8,9,10], is a potent counterpart of SAMHD1. These Vpx proteins bind to the SAMHD1 C-terminal motif (606-626 aa) and deliver the complex to the $CRL4^{DCAF1}$ ubiquitin E3 ligase [11] for subsequent proteasome-dependent degradation.

HD domain contains a catalytic core for hydrolysis activity and the several reports showed that the HD domain-containing C-terminal region of SAMHD1 (120-626 aa) inhibits HIV-1 reverse transcription (RT) by its dGTP dependent deoxynucleoside triphosphate-triphosphatase (dGTP triphosphatase)

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activity [12,13]. In addition, recombinant SAMHD1 protein possesses 3' to 5' exonuclease activities against DNAs and RNAs, as well as nucleic acids and viral genome binding abilities in which the SAM domain containing N-terminal region (1-118 aa) plays a crucial role [14].

Interestingly, various cell lines endogenously express the SAMHD1 [1,6,7], but SAMHD1 can inhibit HIV-1 infection only in terminally differentiated myeloid cells, such as PMA-differentiated THP-1 [6,9,15], monocyte-derived macrophages (MDMs) [13] and monocyte-derived dendritic cells (MD-DCs) [6,7,13], and resting $CD4^+$ T cells [8,16] but not in undifferentiated THP-1 and HEK293T cells [7]. Exogenously over-expressed SAMHD1 restricted the infection in PMA-differentiated U937 macrophage cells but not in undifferentiated U937 and HeLa cells [3]. Thus, there are unknown mechanism(s) contributing to the cell type-specific HIV-1 restriction by SAMHD1.

Meanwhile, the protection of innate immunity-associated proteins from ubiquitination and degradation, *i.e.*, protein stabilization, is one of the mechanisms to induce innate immune signaling. For example, Lee *et al.* [17] recently showed that the TBK1 protein, a key mediator in type I IFN expression, is stabilized by CDC37 in IFN-stimulated DNA and retrovirus sensing. Korczeniewska *et al.* [18] showed that the COP9 signalosome stabilizes the IFN regulatory factor 5 (IRF5) protein, an important role player in the induction of type I IFNs and proinflammatory cytokines. Thus, protein stabilization is a critical event in innate immunity.

To understand the involvement of SAMHD1 stabilization in the SAMHD1-induced cell-type specific HIV-1 infection restriction, we analyzed the expression levels and stability of the full-length SAMHD1 protein and its deletion mutants in U937, TE671, and HeLa cells. We describe supporting evidences for SAMHD1 is post-translationally regulated and the significance of SAM domain-containing and HD domain-containing region for the SAMHD1 protein stability.

Materials and Methods

Cells. THP-1 and U937 cells were grown in RPMI medium (Wako) at 37 °C in a 5% CO_2 incubator. HeLa and TE671 cells were grown in Dulbecco's modified Eagle medium (D-MEM) (Wako). Both media were supplemented with 8% heat-inactivated fetal bovine serum (FBS) (PAA Laboratories GmbH) and 1 % penicillin-streptomycin (Sigma-Aldrich). Unless indicated, all cells were seeded at $1x10^6$ cells per 6 cm culture-dish. At 24 hrs after transfection, U937 cells were stimulated by phorbol 12-myristate 13-acetate (PMA) at various concentrations, to promote differentiation into macrophages.

Real-time PCR

THP-1 cells were seeded in 24-well dishes (1x105/well), and total RNA samples were prepared by Trizol Reagent (Ambicon) after differentiation with 30, 50, and 500 ng/ml PMA for 48 hrs. Semi-quantitative RT-PCR was performed with a high fidelity RT-PCR kit (TAKARA). Real-time PCR was performed with M-MLV reverse transcriptase (NEB). A SYBR Green PCR kit (TOYOBO) was used to quantitate the PCR products on the ABI PRISM 7900HT real-time PCR detection system. The amounts of the SAMHD1 PCR products were normalized by those of the GAPDH PCR products. The nucleotide sequences of the primers for the SAMHD1 mRNA were 5'-AGCGATTGGTTCAAATCCAC-3' and 5'-TCGATTGTGTGAAGCTCCTG-3', and for the GAPDH mRNA were 5'-GAGTCAACGGATTTGGTCGT-3' and 5'-TTGATTTTGGAGGGATCTCG-3'.

Plasmids. The C-terminally HA epitope-tagged full-length SAMHD1 (1-626 aa), SAMHD1n (1-119 aa), and SAMHD1c (120-626 aa) expression plasmids were constructed by amplifying each sequence from the full-length SAMHD1 expression plasmid (OriGene Technologies), as a template. The pcDNA3.1 vector was purchased from Invitrogen.

The PCR primers for amplifying the full-length HA-tagged SAMHD1 sequence were a forward primer 5'-GTGTAGCCATGCAGCGA-3' (SAMHD1 F) and a reverse primer, 5'-TCATGCGTAATCCGGAACATCGTACGGGTACATTGGGTCATCTTTAAAAAGCTG-3' (SAMHD1-HA-R); for HA-tagged SAMHD1n sequence, the SAMHD1 F primer and a reverse primer, 5'-TCATGCGTAATCCGGAACATCGTACGGGTAATTAATTACCTTCATTGTATC-3'; and for HA-tagged SAMHD1c sequence, a forward primer, 5'-GTAGCCATGGATCCTATCCATGGCCAC-3', and the SAMHD1-HA-R primer. Each PCR product was inserted into the pcDNA3.3-TOPO vector (Invitrogen).

An enhanced green fluorescent protein (EGFP) expression plasmid was generated in our laboratory. The EGFP stop codon was replaced by an *Eco*RI sequence in the EGFP expression plasmid (EGFP-EcoRI). To construct the C-terminally SAMHD1c-fused EGFP expression plasmid (GFP-HD), the HD region was amplified by PCR, using the SAMHD1c-HA expression plasmid as the template, with the *Eco*RI sequence-containing forward primer, 5'-TTATTAGAATTCGATCCTATCCATCATCAC-3', and the *Xho*I

sequence-containing reverse primer, 5'-TTATTACTCGAGT-CACATTGGGTCATCTTT-3'. The PCR product was digested with the *Eco*RI and *Xho*I restriction enzymes and inserted into the EGFP-EcoRI plasmid at the *Eco*RI and *Xho*I sites.

The expression plasmid for C-terminally Myc-tagged APOBEC3G was a kind gift from Dr. Yasumasa Iwatani (National Hospital Organization Nagoya Medical Center, Nagoya, Japan) [19]. The Vif expression plasmid was obtained from Dr. Klaus Strebel (National Institutes of Health, Bethesda, MD, United States of America) [20], through Dr. Yasumasa Iwatani. The N-terminally SAMHD1n-fused APOBEC3G expression plasmid (SA3G) was generated as follows. The SAMHD1n-*Sal*I sequence was produced by PCR with the SAMHD1 F primer and the *Sal*I sequence-containing reverse primer 5'-CCCGTCGACATTAATTAC-CTTCAT-3', and cloned into the pCR 2.1 TOPO-vector (Invitrogen). The SalI-APOBEC3G-Myc plasmid was generated by introducing a *Sal*I sequence in frame at the 5' side of the start codon of the APOBEC3G-Myc expression plasmid with the *Sal*I sequence-containing forward primer, 5'-CTG-CAGAATGTCGACATGAAGCCTCACTT -3', and a reverse primer, 5'-AGGCTTCATGTCGACATTCTGCAGATATCC-3'. The SAMHD1n-SalI plasmid was digested with the *Eco*RI and *Sal*I restriction enzymes. The SalI-APOBEC3G-Myc plasmid was digested with the *Sal*I and *Hind*III restriction enzymes. The Myc-tagged APOBEC3G expression plasmid was digested with the *Eco*RI and *Hind*III restriction enzymes and used as the vector. The *Eco*RI/*Sal*I-digested SAMHD1n fragment, the *Sal*I/*Hind*III-digested APOBEC3G fragment, and the *Eco*RI/*Hind*III-digested vector fragment were ligated.

All plasmids described above are driven by the CMV promoter. The nucleotide sequences of the plasmid DNAs constructed in this study were confirmed by sequencing (Applied Biosystems).

Transfection. 1x10^6 cells per 6cm culture-dish was seeded, and incubated at 37 °C in a 5% CO_2 incubator for 24 hrs. Two μg of plasmid DNA were mixed with 10 μl of FuGENE HD transfection reagent (Promega), in 100 μl of D-MEM medium without FBS and antibiotics. The transfection mixture was added to the cell supernatant.

SDS-PAGE and western blotting analysis. A 10 mM MG132 (Sigma) stock solution was prepared in dimethyl sulfoxide. At 24 hrs after transfection, the cell culture medium was replaced with fresh medium, and MG132 was added to a 5 μM final concentration. Cell lysates were prepared 24 hrs after with 1x sample buffer (10% glycerol, 1.5% SDS, 0.005% bromophenol blue, 50 mM Tris/HCl, pH 6.8), and equal quantities of total protein from each sample were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

For western blot (WB) analyses, the proteins were transferred to a PVDF membrane (Millipore) in a wet blotter (Bio-Rad). The membranes were incubated with the corresponding primary antibodies overnight at 4 °C, and then incubated with HRP-conjugated anti-mouse IgG (Bio-Rad) or with protein G (Bio-Rad) for 1 hr at 4 °C. The primary antibody-bound proteins were visualized by enhanced chemiluminescence reagents (Bio-Rad). Immunoblotting images were captured using Fluor Chem Imaging System (IS-8800, Alpha Innotech). The relative intensity levels of each protein compared to β-actin were calculated with the equation, protein intensity/β-actin protein intensity by using Alpha Ease software.

Primary antibodies. Monoclonal antibodies against β-actin (Santa Cruz Biotechnology), HA (COVANCE), GFP (Nacalai Tesque), and Myc (Cell Signaling) were used. The anti-Vif antibody was obtained through the AIDS Research and Reference Reagent Program, NIAID, NIH, from Dr. Michael H. Malim [21,22]. Polyclonal antibody against human SAMHD1 (residues 88-337) was purchased from Proteintech (12586-1-AP).

Results

The SAMHD1 C-terminal HD domain-containing region is degraded in the proteasome, and the N-terminal SAM domain-containing region inhibits the degradation. THP-1 monocyte and U937 monocyte cells express and do not express endogenous SAMHD1 protein, respectively. However, the transduction of U937 cells with SAMHD1, followed by the PMA-induced differentiation dramatically restricts the HIV-1 infection similar extent to the PMA-differentiated THP-1 cells [6,9,15,23]. Why SAMHD1 becomes functional as an anti-HIV-1 factor after the PMA-induced differentiation in monocyte cells? To address this query, Lahouassa *et al.* showed that the expression of exogenous SAMHD1 protein was elevated after the PMA-induced differentiation in U937 cells [13]. This led us to speculate that the SAMHD1 protein level may be regulated at certain stage. Thus, we compared the endogenous SAMHD1 protein expression in THP-1 cells treated or untreated with the PMA stimulation. As shown in Figure 1A, the

SAMHD1 protein level was significantly enhanced by PMA stimulation in THP-1 cells. To know if these protein expression levels reflect a quantitative difference of SAMHD1 mRNA before and after the PMA stimulation, we performed the real-time PCR by using specific primers for SAMHD1 on total RNAs extracted from THP-1 cells treated with different PMA quantity (0, 10, 50, and 500 ng/mL). To our surprise, there was no difference in SAMHD1 mRNA level before and after the stimulation, regardless of increased quantity of the PMA (Fig. 1B). There are two possible ways to regulate protein abundance: post-transcriptional control of transcripts and post-translational control of protein. However, in many cases, protein abundance is post-translationally regulated by proteasomal degradation. In fact, the HD region contains many putative ubiquitination sites, as determined with the CKSAAP_ UbSite program (data not shown). Thus, it is highly speculated that endogenous SAMHD1 protein expression was post-translationally regulated in THP-1 cells.

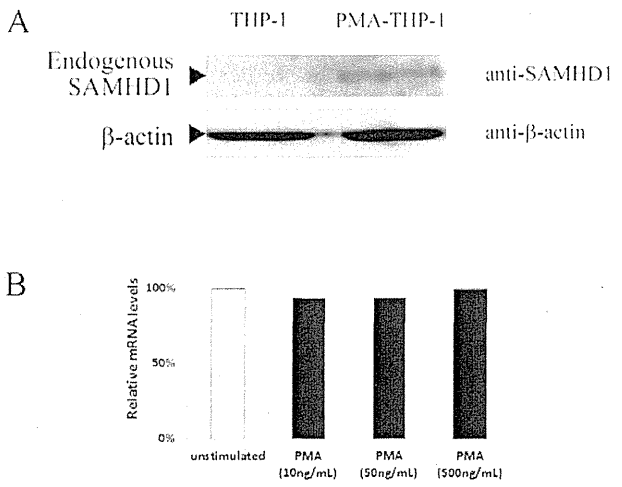

Figure 1. The SAMHD1 protein is post-translationally-regulated. (A) The endogenous SAMHD1 protein expression was analyzed in undifferentiated THP-1 and PMA-differentiated THP-1 cells. THP-1 cells were stimulated with 500ng/mL PMA for overnight. A WB analysis with a SAMHD1-specific antibody was performed. β-actin was detected as a loading control. (B) Real-time PCR for quantitation of SAMHD1 mRNA with specific primers were performed on THP-1 cells stimulated with different quantity of PMA. Relative quantities of SAMHD1 mRNA to GAPDH mRNA are indicated. Data are representative of similar results from two independent experiments.

We next wanted to identify which domain is responsible for the post-translational regulation of SAMHD1. We generated the C-terminally HA-tagged full-length SAMHD1 (1-626 aa), the N-terminal SAM domain-containing aa 1-119 region (SAMHD1n), and the C-terminal HD domain-containing aa 120-626 region (SAMHD1c) expression plasmids (Fig. 2A). Each plasmid was transfected into U937 cells and the cells were PMA-differentiated. As in undifferentiated THP-1 cells, none of the proteins were detected in undifferentiated U937 cells (Fig. 2B lane 1, 3, and 5). In contrary, all the proteins were expressed upon the PMA-induced differentiation, and the expression levels of the full-length SAMHD1 and SAMHD1n proteins were much higher than that of the SAMHD1c protein in PMA-differentiated U937 cells (Fig.2B, lane 2, 4, and 6), leading us to speculate that the SAMHD1n stabilizes the SAMHD1 protein in differentiated U937 cells. However, it may simply reflect the PMA stimulation-induced protein expression from CMV promoter [24]. We had previously searched for various cell lines in which SAMHD1 restricts HIV-1 infection without the PMA stimulation and, as a result, we found TE671 rhabdomyosarcoma cells moderately restricted the infection by transiently expressed SAMHD1 (manuscript in press). Of note, transiently expressed SAMHD1-mediated HIV-1 infection restriction was not observed in HeLa, H292, C33A, and NP2 (manuscript in press). Thus, in this study, we transfected each plasmid into TE671 cells and HeLa cells, the cells in which SAMHD1 does and does not restrict the HIV-1 vector infection, respectively, and the cells were treated with the proteasome inhibitor, MG132. As in PMA-differentiated U937 macrophage cells, the SAMHD1c protein was barely detected in TE671 cells and in HeLa cells, but the MG132 treatment significantly enhanced the protein abundances (Fig. 2C, lane 5 versus lane 6, and Fig. 3, lane 5 versus lane 6). These results indicated that the SAMHD1c protein is degraded by the proteasome. On the other hand, the amounts of the full-length SAMHD1 and SAMHD1n proteins were much higher than that of the SAMHD1c protein in TE671 cells (Fig.2C lane 1, 3, and 5), indicating that the SAMHD1n protects the full-length SAMHD1 protein from proteasomal degradation induced by SAMHD1c in TE671 cells. These former two protein expression levels are not significantly affected by the MG132 treatment (Fig. 2C lane 1 versus 2, and lane 3 versus 4), In contrary to that observed in TE671 cells, the amount of full-length protein was much lower than SAMHD1n protein in HeLa cells (Fig. 3 lane 1 versus 3), indicating that the SAMHD1n-mediated full-length SAMHD1 protein stabilization occurs more efficiently in TE671 cells than in HeLa cells. Moreover, the expression of full-length SAMHD1 was much higher than the SAMHD1n protein in U937 macrophage cells compare to that observed in TE671 cells (Fig. 2B lane 2 versus 4 and Fig.2C lane 1 versus 3). Thus, we speculate that the full-length SAMHD1

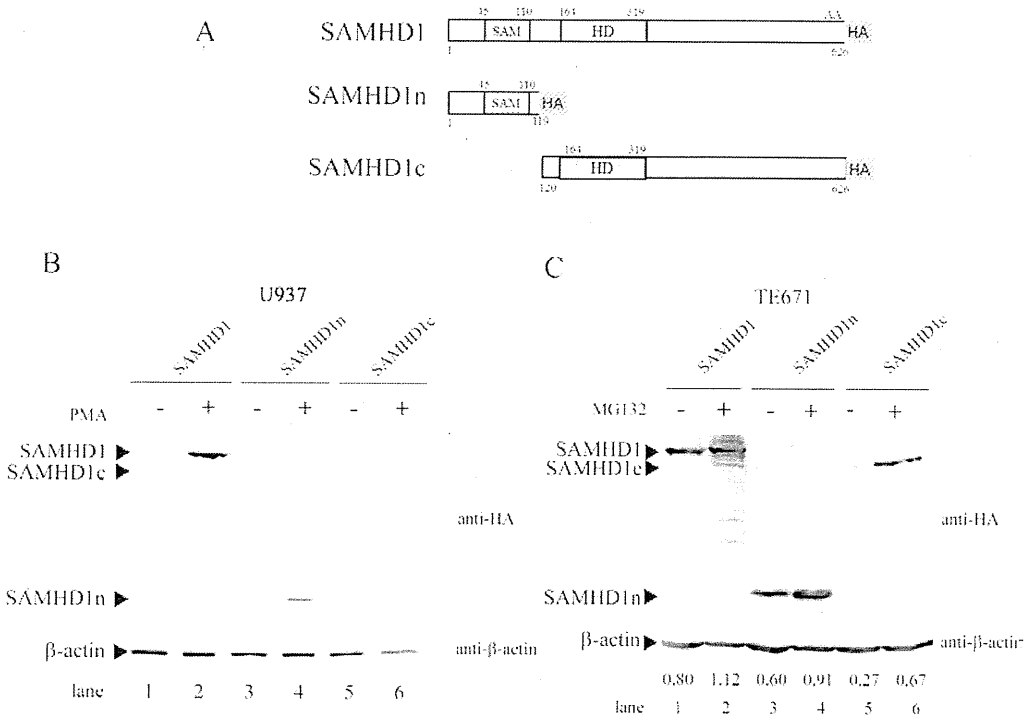

Figure 2. The SAMHD1c protein is degraded in the proteasome, and the SAMHD1n inhibits the degradation. (A) Schematic representations of SAMHD1 and its truncated mutants are indicated. SAMHD1 consists of the sterile-α motif (SAM) domain (aa 45-110) and the hydrolysis (HD) domain (aa 164-319). The SAMHD1n deletion mutant contains amino acid residues 1-119. The SAMHD1c mutant contains amino acid residues 120-626. Each plasmid encodes a C-terminally HA-tagged protein. Vectors encoding SAMHD1(1-626 aa), SAMHD1n (1-119 aa), and SAMHD1c (120-626 aa) were transfected into (B) U937 cells, followed by PMA untreatment or treatment for overnight, and into (C) TE671 cells, followed by MG132 untreatement or treatement for overnight. The samples were analyzed by WB, using anti-HA and anti-β-actin antibodies, in which β-actin was used as a loading control (B and C). Relative levels of each protein compared to β-actin are shown above the lane numbers (C). Data are representative of similar results from two independent experiments.

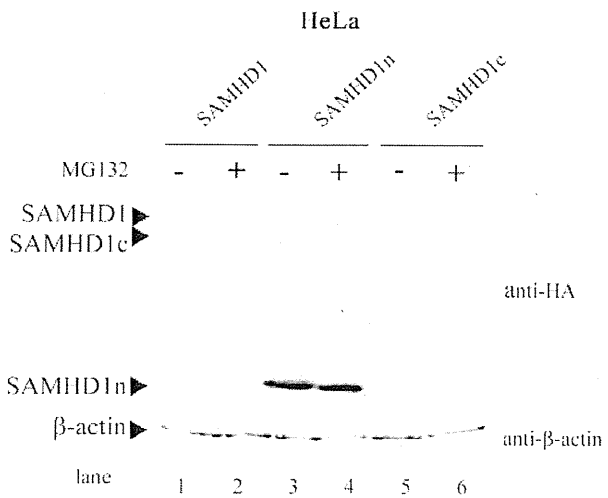

Figure 3. HeLa cells were untreated or treated with MG132 for overnight, followed by SDS-PAGE and WB. Each sample was analyzed with anti-HA and anti-β-actin antibodies. Data is a representative of similar results from two independent experiments.

protein stability may correlate with SAMHD1-mediated HIV-1 vector infection restriction. Overall, these results suggested that the SAMHD1n protects the SAMHD1 protein from the SAMHD1c -mediated proteasome degradation.

The SAMHD1 C-terminal region leads to the proteasome-dependent degradation of a heterologous protein. To assess the involvement of the SAMHD1c (120-626 aa) in the protein degradation, we constructed an expression plasmid encoding the C-terminally SAMHD1c-fused EGFP protein (GFP-HD) (Fig. 4A), and compared its expression with that of the control EGFP protein (GFP) in TE671 cells. As shown in Fig. 4B, the amount of the GFP-HD protein was increased by MG132 treatment, while the MG132 treatment did not significantly affect the GFP protein abundance. Our results clearly demonstrated that the SAMHD1c region has the ability to lead not only SAMHD1 but also a heterologous protein into proteasome-dependent degradation.

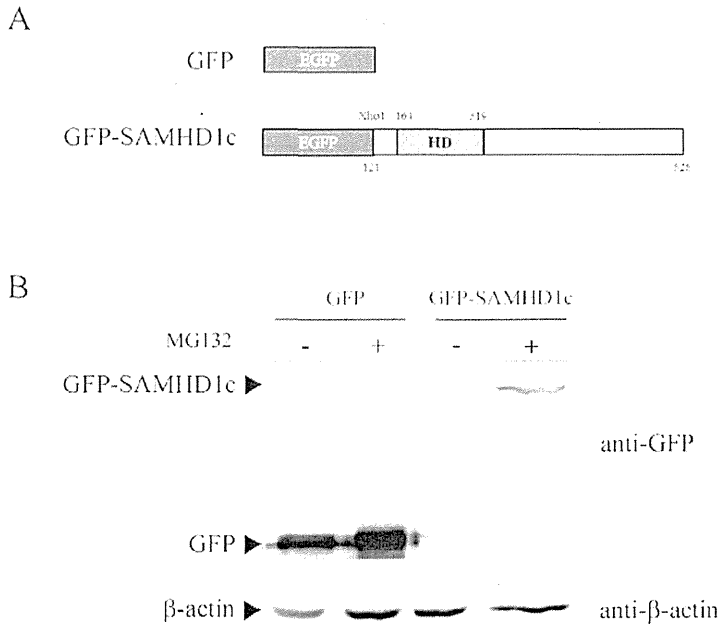

Figure 4. The SAMHD1c leads to proteasome-dependent degradation of a heterologous protein. (A) Schematic representations of GFP and C-terminally SAMHD1c fused GFP proteins are indicated. GFP and SAMHD1c were fused at the *Xho*I site. (B) GFP and GFP-SAMHD1c were transfected into TE671 cells. After 24 hrs of culture, the transfected cells were treated with MG132, and then further cultured for 24 hrs. Whole cell lysates were collected, fractionated by SDS-PAGE and analyzed by WB. The expression of the GFP and GFP-SAMHD1c proteins was analyzed with an anti-GFP antibody. *β*-actin was used as a loading control. Data are representative of similar results from two independent experiments.

The SAMHD1 N-terminal region protects full-length SAMHD1 from protesome-dependent degradation. To assess the role of the SAMHD1n in protein stabilization, we constructed an expression plasmid encoding an N-terminally SAMHD1n-fused, C-terminally Myc-tagged APOBEC3G protein (SA3G) (Fig. 5A). Human APOBEC3G (A3G) is degraded in the proteasome by the SIV or HIV-1 accessory protein, Vif [25,26]. The SA3G and A3G expression plasmids were co-transfected together with the Vif or control pcDNA3.1 expression plasmid into TE671 cells. As shown in Fig. 5B, similar levels of the SA3G and A3G proteins were detected without Vif. The Vif co-expression decreased the A3G protein expression (28-fold), while, the SA3G protein was slightly but steadily resistant to the Vif-mediated protesomal degradation (8-fold), indicating that the SAMHD1n can protect the heterologous A3G protein from proteasomal degradation in TE671 cells.

The SAMHD1 N-terminal region is critical for SAMHD1 stabilization in differentiated U937 cells. To know if the SAMHD1n-mediated protein stabilization is correlated to cell-type specific HIV-1 infection restriction ability of SAMHD1, the same experiment was performed on U937 cells, followed by PMA treatment. As in TE671 cells, the addition of Vif dramatically reduced the amount of the A3G protein (70-fold), while that of the SA3G protein was hardly affected (3-fold), indicating that SA3G was highly resistant to Vif-mediated proteasomal degradation in U937 cells (Fig. 5C). Although the SAMHD1n protected the A3G protein from Vif-mediated proteasomal degradation in TE671 cells, Vif still reduced the amount of the SA3G protein significantly in this cell line. Thus, these results suggested that the ability of the SAMHD1n to confer resistance to proteasome-dependent degradation was more prominent in U937 cells than in TE671 cells.

Trans-expression of SAM mutant protein does not affect SAMHD1 protein stability.

In addition, we tested if the *trans*-expressed SAMHD1n can rescue protein from the degradation. SAMHD1n expression plasmid was transfected into TE671 cells, together with the full-length SAMHD1 or SAMHD1c expression plasmid. As a result, neither the SAMHD1 nor SAMHD1c protein abundance was elevated by the trans-expression of

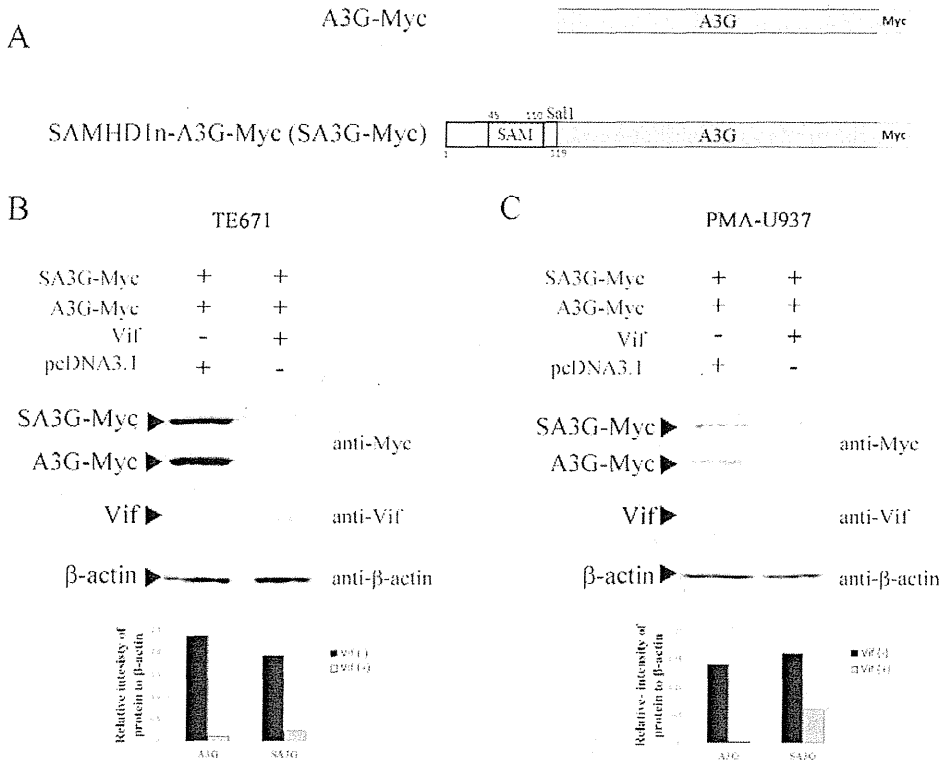

Figure 5. The SAMHD1n can protect a heterologous protein from proteasome-dependent degradation in TE671 and PMA-differentiated U937 macrophage cells. (A) Schematic representations of the A3G and N-terminally SAMHD1n-fused A3G (SA3G) proteins are indicated. SAMHD1n and A3G were fused at the *SalI* site. Each plasmid encodes a C-terminally Myc-tagged protein. (B) TE671 cells and (C) U937 cells were co-transfected with A3G and SA3G, together with pcDNA3.1 or Vif. U937 cells were treated with PMA after the transfection. The expression of the A3G and SA3G proteins was analyzed by WB, using the anti-Myc antibody. *β*-actin was used as a loading control and relative levels of each protein compared to *β*-actin are shown. Data are representative of similar results from two independent experiments.

SAMHD1n protein (Fig. 6), indicating that only the *cis*-linked SAMHD1n can protect the full-length SAMHD1 from degradation.

Discussion

Our study is the first report showing that the HD domain-containing C-terminal region of SAMHD1 is responsible for the proteasome-dependent degradation of SAMHD1 in the absence of Vpx, whereas the SAM domain-containing N-terminal region is critical for the stability of the full-length SAMHD1 protein. SAMHD1 can restrict HIV-1 vector infection in PMA-differentiated U937 macrophage and TE671 rhabdomyosarcoma cells, but not in HeLa cells. The SAMHD1n-mediated stabilization of the SAMHD1 protein occurs more efficiently in differentiated U937 cells than in TE671 cells, correlated to the degree of restriction of these cells. Interestingly, while we were preparing this manuscript, White *et al.* and Cribier *et al.* showed that the phos-

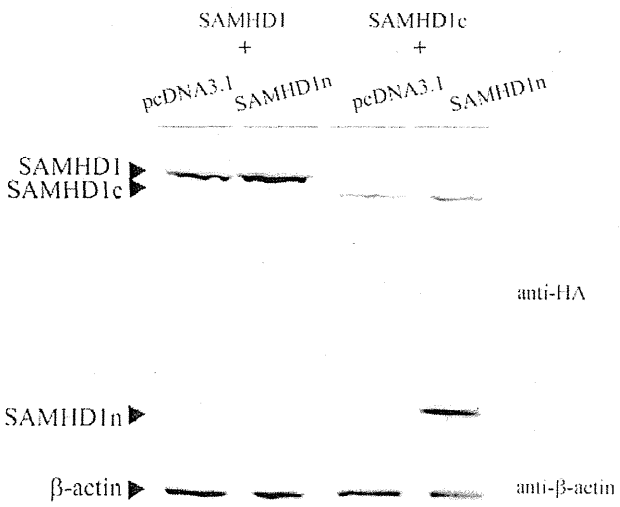

Figure 6. Trans expression of SAMHD1n mutant protein does not affect SAMHD1 protein stability. TE671 cells were transfected with a vector encoding the full-length SAMHD1 (left panel) or the SAMHD1c mutant (right panel) protein, together with pcDNA3.1 or the SAMHD1n mutant. Cell lysates from the transfected cells were analyzed by WB. Data are representative of similar results from two independent experiments.

phorylation of SAMHD1 determines the cell-type specific restriction of HIV-1 infection by SAMHD1 [27,28]. They proposed that the phosphorylation of SAMHD1 C-terminal at T592 aa regulates the retroviral restriction ability of SAMHD1. The close relationship between phosphorylation and ubiquitination has been well understood [29]. It is highly speculated that the ubiquitination of the SAMHD1c is also regulated by the phosphorylation of this region. Nevertheless, our study suggests that the stabilization of SAMHD1 by the SAMHD1n contributes to its restriction of HIV-1 infection.

Previous report has shown that N-terminal region-deleted SAMHD1 protein (112-626 aa) could still restrict HIV-1 vector infection in PMA-differentiated U937 cells [15], suggesting that the SAM domain containing N-terminal region is dispensable for the HIV-1 restrictive function of SAMHD1. However, it is still possible that the SAM domain participates in the infection restriction, since a SAM domain (45-110)-deleted SAMHD1 mutant protein reportedly fails to restrict HIV-1 infection in PMA-differentiated U937 cells, and its abundance is lower than that of the wild type SAMHD1 [15]. Several reports have shown that the amounts of N-terminally truncated SAMHD1 proteins are lower than that of the full-length protein [9,10,11,15,23]. In addition, recent report revealed that the SAMHD1 N-terminal region (1-118) is necessary to maximize the dGTP triphosphatase and nuclease activities, and indispensable for nucleic acid and viral genome binding activities of SAMHD1 [14]. Together, these reports suggested that the SAM domain is necessary for the SAMHD1-mediated restriction of HIV-1 infection and protein stability.

Notably, the SA3G protein was remarkably resistant to the Vif-induced degradation in differentiated U937 cells. The tertiary structure of SA3G is considered to have minor or no effect to the A3G interaction with Vif by the SAMHD1n fusion, because the amount of the SA3G protein was significantly reduced by Vif in TE671 cells. To further support the involvement of the SAM domain in the protein protection from proteasome-dependent degradation, Park *et al.* [30] reported an interesting feature about the SAM domain of the tyrosine kinase-type A2 receptor (EPHA2) protein: the group showed that the SAM domain of the EPHA2 protein plays a critical role in its stability, by modulating the proteasome-dependent process. The SAMHD1 SAM domain shares 40% similarity and 16% identity with that of EPHA2 (Phyre2 program). The SAM domain is responsible for protein-protein or protein-RNA interactions [31]. Therefore, an interaction between the SAMHD1n and an unknown cellular factor may stabilize the SAMHD1 protein.

Vpx at its N-terminal region forms a complex with SAMHD1 C-terminal region (606-626 aa) and loads it to the $CRL4^{DCAF1}$ ubiquitin E3 ligase, followed by proteasomal degradation[11]. However, we have shown that the SAMHD1c contains a motif that leads SAMHD1 into proteasome-dependent degradation, in the absence of Vpx. The SAMHD1 protein may be stabilized by an intramolecular interaction between the SAMHD1n and SAMHD1c regions. Vpx may compete with the SAMHD1n, and induce the degradation of the full-length SAMHD1 protein. Identifying the relationship between these core domain containing regions and the Vpx is our next target of interest in parallel with disclosing the contribution of SAMHD1c phosphorylation to the full-length SAMHD1 stability.

In summary, the HD domain-containing C-terminal region leads the SAMHD1 protein to proteasomal degradation. The SAM domain-containing N-terminal region stabilizes the SAMHD1 protein efficiently in differentiated U937 cells and in TE671 cells, in which SAMHD1-mediated HIV-1 infection restrictions were observed, suggesting that the SAM-mediated stabilization of the SAMHD1 protein is important for its anti-virus activity. Further studies are required to understand the mechanism by which the SAMHD1n stabilizes the SAMHD1 protein.

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Androgen-independent proliferation of LNCaP prostate cancer cells infected by xenotropic murine leukemia virus-related virus

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ABSTRACT

Xenotropic murine leukemia virus-related virus (XMRV) is a novel gammaretrovirus that was originally isolated from human prostate cancer. It is now believed that XMRV is not the etiologic agent of prostate cancer. An analysis of murine leukemia virus (MLV) infection in various human cell lines revealed that prostate cancer cell lines are preferentially infected by XMRV, and this suggested that XMRV infection may confer some sort of growth advantage to prostate cancer cell lines. To examine this hypothesis, androgen-dependent LNCaP cells were infected with XMRV and tested for changes in certain cell growth properties. We found that XMRV-infected LNCaP cells can proliferate in the absence of the androgen dihydrotestosterone. Moreover, androgen receptor expression is significantly reduced in XMRV-infected LNCaP cells. Such alterations were not observed in uninfected and amphotropic MLV-infected LNCaP cells. This finding explains why prostate cancer cell lines are preferentially infected with XMRV.
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1. Introduction

Xenotropic murine leukemia virus-related retrovirus (XMRV) is a novel human gammaretrovirus that was originally isolated from human prostate cancer tissues [1]. Although it is widely believed at present that XMRV is not the etiologic agent of prostate cancer, human prostate cell lines are frequently infected with XMRV [2].

It is known that some retroviruses play a critical role in leukemogenesis in various mammalian species including human [3,4]. The xenotropic MLV infection receptor (XPR1), which is also recognized by XMRV [5,6], varies among wild mice species as a mechanism of resistance to xenotropic virus infection [7,8]. The latter observation suggests that xenotropic viruses may be pathogenic in some species and implies that XMRV may affect growth of certain cell lineages.

Prostate cancer cell lines exhibit a propensity for infection by XMRV when compared to other types of human cancer cell lines [2,9]. It has been reported that amyloidogenic fragments originating from prostatic acid phosphatase greatly increase XMRV infections of primary prostatic epithelial and stromal cells [10]. In vivo infection of macaques with XMRV has confirmed that prostate tissue has a high affinity for XMRV, and the prostate tissues remain continuously infected even after 5 months, when XMRV was undetectable in blood [11]. Dihydrotestosterone (DHT) stimulates XMRV expression in cells expressing a functional androgen receptor (AR) [12,13]. These results suggest that XMRV infection specifically confers an advantage to prostate cancer cells.

In this study, we aimed to determine whether XMRV infection affects androgen-dependent growth of the LNCaP human prostate cancer cell line. Our results indicate that XMRV infection may provide an androgen-independent growth advantage to prostate cancer cells.

2. Materials and methods

2.1. Cells

PC-3 and LNCaP cells were obtained from ATCC. PC-3 cells were cultured in RPMI 1640 medium (Wako) supplemented with 8% (v/v) fetal bovine serum (FBS) (Biofuies), L-glutamine and penicillin–streptomycin (both from Sigma–Aldrich). LNCaP cells [14] were maintained in the same medium but additionally supplemented with 10 nM dihydrotestosterone (DHT) (Sigma–

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Aldrich). Rat F10, human HeLa, and human 293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 8% FBS and penicillin–streptomycin. All cell lines were grown in a tissue culture incubator at 37 °C with a 5% CO_2 atmosphere.

2.2. Retrovirus infection

The XMRV plasmid DNA was obtained from Dr. R.H. Silverman and Dr. B. Dong [1] through the AIDS Research and Reference Reagent Program (NIAID, NIH, USA) and was used for transfection of rat F10 cells. Culture supernatants of transfected F10 cells were used to inoculate target cells in presence of polybrene (4 μg/ml) (Sigma). Inocula containing MLV were from culture supernatants of amphotropic MLV-producing cells, obtained from Dr. Y. Iwatani. Infected LNCaP cells were maintained in presence of DHT. In tests of androgen responses, target cells were cultured in various combinations of DHT (10 nM) and bicalutamide (10 μM).

2.3. Cell counts and viability

The cells to be counted were collected and stained with trypan blue. Numbers of unstained (viable) cells were counted using a counting chamber under a microscope to estimate cell viability.

2.4. Western blot analysis

Cell lysates were subjected to electrophoretic separation in SDS-containing polyacrylamide gels (BioRad), after which proteins were transferred onto a PVDF membrane. The membrane was first treated with the primary antibodies: mouse anti-β-actin (Santa Cruz Biotechnology), goat anti-dynamin (Santa Cruz Biotechnology), rabbit anti-human AR (Santa Cruz Biotechnology), goat anti-MLV p30 Gag (ViroMed), or goat anti-MLV SU (ViroMed) antibody. Following these procedures, the membrane was treated with secondary horse radish peroxidase (HRP)-conjugated anti-mouse IgG antibody, or HRP-conjugated protein G (Bio-Rad). Secondary antibody- or protein G-bound polypeptides were detected by ECL Western Blotting Detection Reagents (GE healthcare).

2.5. Semi-quantitative RT-PCR

Total RNA and genomic DNA samples were isolated by standard protocols. Fist-strand cDNA was synthesized using reverse transcriptase (TaKaRa) from the total RNA (500 ng). Semiquantitative PCR was performed to detect XMRV env, AR, and GAPDH sequences. Nucleotide sequences of the PCR primers for the XMRV *env* sequences were 5′-GACTTGTGTGATTTAGTTGGAGAC-3′ and 5′-CCCCGGTGTGGCACC-3′; for AR, 5′-AGCCCCACTGAGACAACC-3′ and 5′-ATCAGGGGCGAAGTAGAGCAT-3′; and for GAPDH, 5′-AGG TXGGAGTXAAXGGATTTGGT-3′ and 5′-GTGGGCCATGAGGATCCAC-CAC-3′. These primers were synthesized by Genenet Inc.

2.6. Statistical analysis

Differences between two sets of data were determined by Student's *t*-test, and these differences were considered significant when $P < 0.05$.

3. Results

3.1. XMRV infection converts LNCaP cells to an androgen-independent phenotype

To analyze the effect of XMRV infection on androgen-dependent growth of LNCaP cells, the proliferation of XMRV-infected and -uninfected LNCaP cells was compared. As it has been reported that XMRV can replicate in rat cells but not in human 293T cells [12,13,], virus was first rescued by transfection of an XMRV expression plasmid [1] in rat F10 cells. Undiluted culture supernatant from these cells was then added to LNCaP cells with polybrene and cultured for 24 h. The XMRV-infected and -uninfected cells were maintained in the presence of 10 nM DHT for more than 3 months. Uninfected LNCaP cells did not grow in the absence of DHT (Fig. 1A) but did in the presence of DHT (Fig. 1B), indicating a strong androgen-dependent growth requirement, as reported [14]. As a control for specific AR effects, DHT-induced growth of uninfected LNCaP cells was shown to be abrogated by the antagonist bicalutamide, an androgen blocker (Fig. 1C). Bicalutamide (10 μM) alone had no effect on the growth of either infected or uninfected LNCaP cells (Fig. 1D). LNCaP cells chronically infected with XMRV grew even in the absence of DHT (Fig. 1A), and bicalutamide did not suppress growth of XMRV-infected LNCaP cells (Fig. 1C and D). In the presence of DHT, the number of XMRV-infected LNCaP cells was greater than control uninfected cells after 3 days in culture (Fig. 1B). Three independent XMRV-infected LNCaP cell pools were constructed, and all of them could grow in the absence of DHT. When uninfected LNCaP cells were maintained in the presence of DHT, the cells did not gain androgen-independent growth property during this study. These results indicate that XMRV infection converts LNCaP cell growth from androgen dependence to independence.

On the other hand, LNCaP cells chronically infected with amphotropic MLV did not efficiently proliferate even in the presence of DHT (Fig. 1A–D), suggesting that the amphotropic MLV infection is cytotoxic for LNCaP cells.

To determine the time course of the conversion of XMRV-infected LNCaP cells to androgen independence, growth kinetics were analyzed after XMRV infection (from 1 to 2 months, 2 to 3 months, and >3 months). Cultures initially contained 5×10^3 cells and were counted again after 6 days, because differences between the uninfected and XMRV-infected LNCaP cells in androgen dependence were apparent 6 days after the culture was started (Fig. 1A–D). DHT dependence of LNCaP cell growth was reduced by XMRV infection, but DHT still activated cell proliferation 1–2 months after XMRV infection (Fig. 1E). Cell numbers of the infected LNCaP cells in the absence of DHT were comparable to those in its presence 2–3 months after infection. These results suggest that the complete conversion of LNCaP cells to androgen independence takes more than 2 months. The XMRV infection did not increase cell numbers in the absence of DHT 1–3 months after the XMRV inoculation, but cell increases were observed longer than 3 months after inoculation, showing that the activation of LNCaP cell growth by the XMRV infection requires at least 3 months.

Uninfected PC-3 cells, whose growth is androgen-independent [15], grew as efficiently as XMRV-infected PC-3 cells in the absence or presence of DHT (data not shown). These results indicate that XMRV infection did not affect growth of androgen-independent PC-3 cells.

3.2. XMRV infection inhibits androgen receptor expression in LNCaP cells

Because androgen agonistic (DHT) and antagonistic (bicalutamide) effects are mediated through androgen receptor (AR), we analyzed the effects of XMRV infection on its expression in LNCaP cells. As demonstrated by western blot analysis, the expression of AR protein gradually decreased after XMRV infection of LNCaP cells (Fig. 2A). Expression was significantly decreased but still detectable 2–3 months after infection, but by >3 months no expression was observed. Therefore, the reduction of AR

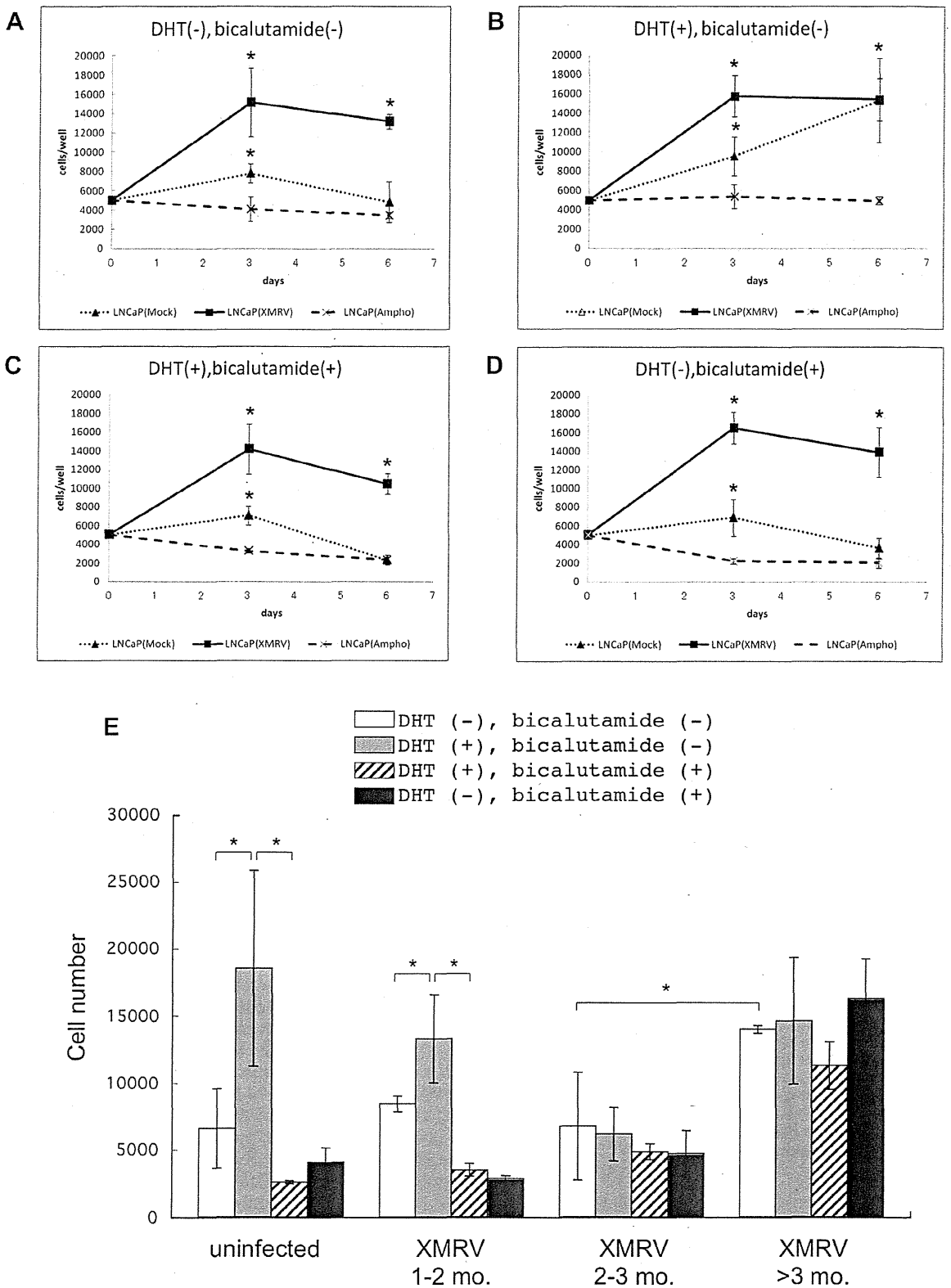

Fig. 1. XMRV infection converts the phenotype of LNCaP cells from androgen-dependent to androgen-independent. (A–D) Growth of uninfected, chronically XMRV-infected, and amphotropic MLV-infected LNCaP cells cultured in media supplemented with or without the androgen dihydrotestosterone (DHT) and/or the androgen receptor antagonist bicalutamide. (E) Effects of DHT and bicalutamide on growth of uninfected and XMRV-infected LNCaP cells were analyzed. Cells were counted after 6 days in culture. In all the panels, the data presented are average values ± SD from two independent experiments performed in triplicate. The concentrations of DHT used in the experiments were (+) 10 nM, or (−) 0 nM; and of bicalutamide, (+) 10 μM, or (−) 0 μM.

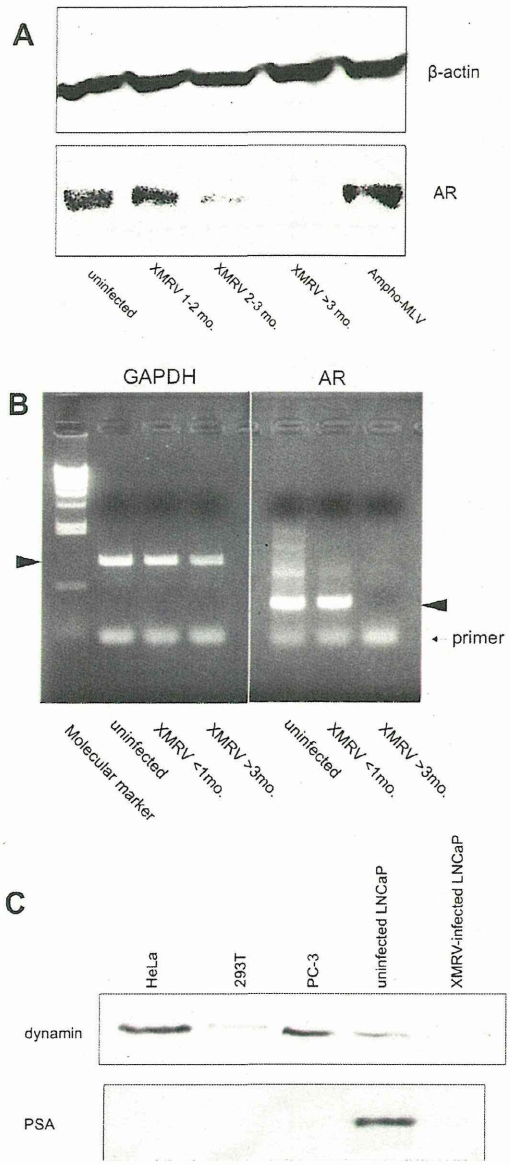

Fig. 2. XMRV infection results in reduced androgen receptor (AR) protein and mRNA expression. (A) Results of western blot analysis of cell lysates from uninfected, XMRV-infected (at three time points after infection, indicated below the chart), and amphotropic MLV-infected LNCaP cells. Levels of AR (lower panel) and β-actin protein expression (internal positive control, in upper panel) are shown. (B) AR and GAPDH mRNA expression levels from uninfected and XMRV-infected LNCaP cells analyzed by semiquantitative RT-PCR. Arrow heads indicate predicted sizes of the PCR products. (C) PSA protein expression was analyzed in HeLa, 293T, PC-3, uninfected LNCaP, and XMRV-infected LNCaP cells by western blot. As control, dynamin expression was also examined.

expression occurred in parallel with the conversion to androgen-independent proliferation. In LNCaP cells chronically infected with amphotropic MLV, AR expression was not affected (last lane of Fig. 2A).

To determine whether the reduction of AR protein expression by XMRV infection was associated with decreased AR transcript levels, we examined mRNA expression by semiquantitative RT-PCR. As the result, we found that the AR mRNA level in LNCaP cells is counteracted by the XMRV infection (Fig. 2B). These findings demonstrated that the XMRV infection induces the androgen-independent growth and attenuates the AR gene transcription in LNCaP cells.

LNCaP cells have been shown to exhibit androgen-dependent expression of the prostate-specific antigen (PSA) [14]. We therefore analyzed PSA expression in LNCaP cells converted to androgen independence by XMRV. PSA expression was not detected in HeLa, 293T, or androgen-independent PC-3 prostate cancer cells (Fig. 2C). Uninfected LNCaP cells expressed PSA, but chronically XMRV-infected LNCaP cells did not, even though the cells were cultured in the presence of DHT.

3.3. The expression of XMRV proteins depends on androgen stimulation

Because it has been reported that XMRV expression is dependent on androgen and AR [12,13], we analyzed XMRV Gag and Env protein expression in infected LNCaP cells. Our results show that expression of XMRV Gag gradually decreased after infection, correlating with the time course of conversion to androgen-independent growth (Fig. 3A). Three months after XMRV infection, both the Gag precursor and mature protein levels were much lower than after 1–2 month. The XMRV Env protein was expressed for as long as 3 months after infection, but at periods longer than 3 months expression was not detected (Fig. 3B).

Amount of XMRV sequence integrated into genomes of chronically infected LNCaP cells were comparable to that at shorter than 1 month (Fig. 4A), indicating that XMRV-infected cells were maintained during the culture. XMRV RNA level at periods longer than 3 months after XMRV infection was lower than that at shorter than 1 month (Fig. 4B). These results indicate that XMRV expression is reduced during LNCaP cell phenotypic conversion and support the conclusion that XMRV expression is dependent on androgen [12,13].

The XMRV Gag proteins were not detected by western analysis of XMRV-infected PC-3 cells (Fig. 3A), but the XMRV env sequence-containing RNA was detected by RT-PCR (Fig. 4C), showing that XMRV genome was integrated and transcribed at low level in PC-3 cells. Because PC-3 cells are androgen-independent and lack AR expression [16], the androgen-dependent XMRV Gag protein level was presumably below detectable limits (Fig. 3A). In contrast, Gag protein was detected by western analysis in LNCaP cells chronically infected with amphotropic MLV (Fig. 3A), showing that the amphotropic MLV expression was independent of androgen.

4. Discussion

In this study, we found that XMRV infection converts the androgen-dependent phenotype of LNCaP cells to androgen independence, and it reduces AR expression. This effect seems to be relatively specific to XMRV, as it was not observed with amphotropic MLV infection of the same cells. Consistently, it has been reported that XMRV activates tumor growth and invasiveness of LNCaP cells [17–19], but androgen dependence of LNCaP cell proliferation was not analyzed in these studies. Many human cancer cells have been transplanted into nude mice, but prostate cancer cells are preferentially infected with xenotropic MLVs [2]. The XMRV-mediated androgen-independent growth of prostate cancer cells may explain the propensity for XMRV infection observed in prostate cancer cells.

It has been reported that androgen-independent LNCaP cells spontaneously appear during culture in the absence of androgen [14,20]. However, XMRV-infected LNCaP cells became androgen-independent even in the presence of androgen. Because the XMRV infection suppressed AR protein expression, DHT cannot induce growth activation. Indeed, less than 3 months after the XMRV infection, growth of the infected LNCaP cells was not activated even in the presence of DHT. Then, spontaneous alterations inducing androgen-independent growth might be selected in the

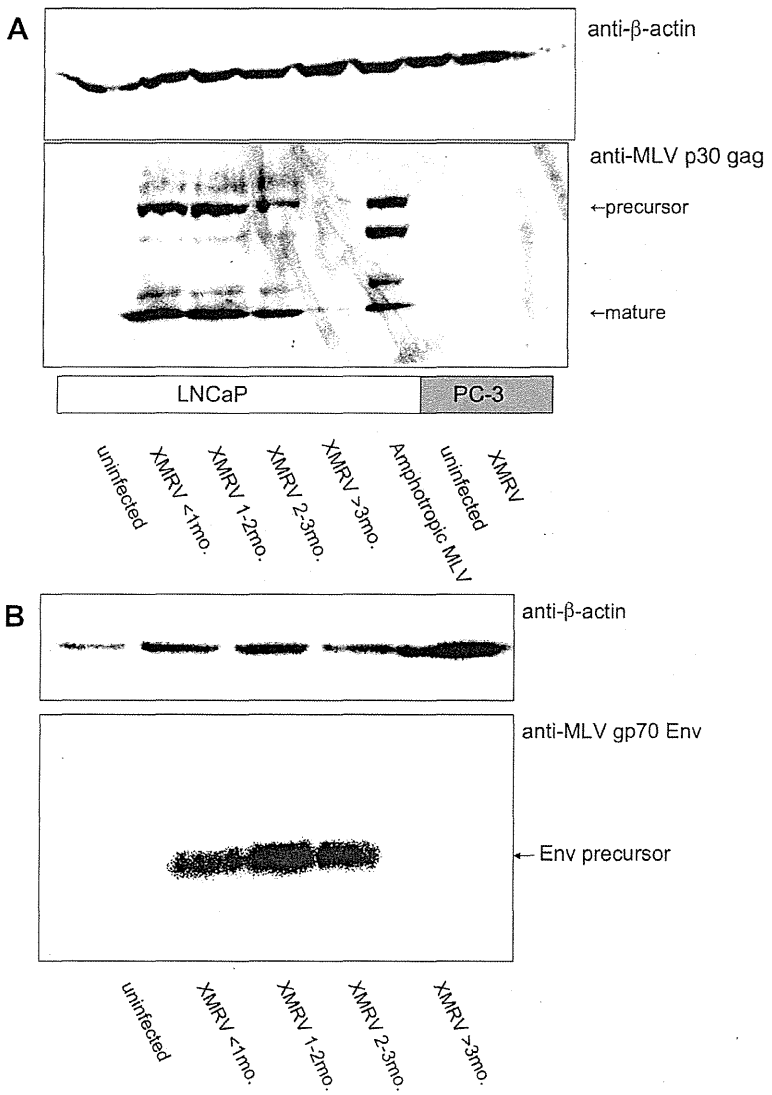

Fig. 3. XMRV expression was decreased in parallel with XMRV-induced conversion of LNCaP cells from androgen-dependent to -independent growth. Western blot analysis of MLV Gag (panel A) and Env (panel B) protein levels were evaluated in uninfected, amphotropic MLV-infected, and XMRV-infected LNCaP, and PC-3 cells. As a positive internal control, β-actin protein expression levels were also analyzed.

XMRV-infected LNCaP cell culture. Because the expression of XMRV proteins was suppressed in chronically XMRV-infected LNCaP cells, the viral proteins are not necessary for maintenance of the androgen-independent state. This observation supports the above speculation. If so, the XMRV infection did not directly induce the androgen-independent growth. However, the abrogation of AR expression by the XMRV infection triggered the conversion to androgen-independent growth.

How does XMRV reduce AR expression? Amphotropic MLV infection inhibited growth of LNCaP cells. Thus, though the XMRV infection did not clearly suppress the cell growth, XMRV proteins may be slightly toxic to LNCaP cells. Because XMRV expression is androgen-dependent, lower level of AR expression induces lower amount of XMRV proteins. Due to this mechanism, LNCaP cells expressing AR at lower level might be selected during the culture. Further study is required to understand the mechanism by which XMRV infection abrogates AR protein expression.

The expression of XMRV was reduced in the androgen-independent XMRV-infected LNCaP cells. However, xenotropic MLVs are constitutively expressed in the androgen-independent CWR22Rv1 prostate cancer cells [9] and in several human cancer cell lines other than prostate cancer [21–23]. These results indicate that expression of these xenotropic MLVs is independent of androgen.

As a clinical problem, most prostate cancer patients treated with combined androgen blockage (CAB) therapy develop castration resistant prostate cancer (CRPC) [24]. Growth of the prostate cancer cells is androgen-dependent in the first stage, and thereafter androgen-independent cancer cells are selected during CAB therapy. The androgen refractory mechanisms are explained by the following hypotheses [24]: (i) mutations in or enhanced expression of the AR gene; (ii) mutated AR is activated by other steroids; (iii) mutated AR is activated by other signals, e.g., peptide growth factors or cytokines; or (iv) an AR bypassing pathway is activated. Mechanisms of the fourth case are not completely understood, and it is to this category that XMRV-induced conversion belongs. This is because AR expression is significantly reduced in XMRV-infected LNCaP cells. The mechanism of AR-deficient CRPC development in human patients may be similar to that of the XMRV-induced LNCaP androgen independence. Elucidation of the mechanism by which XMRV induces

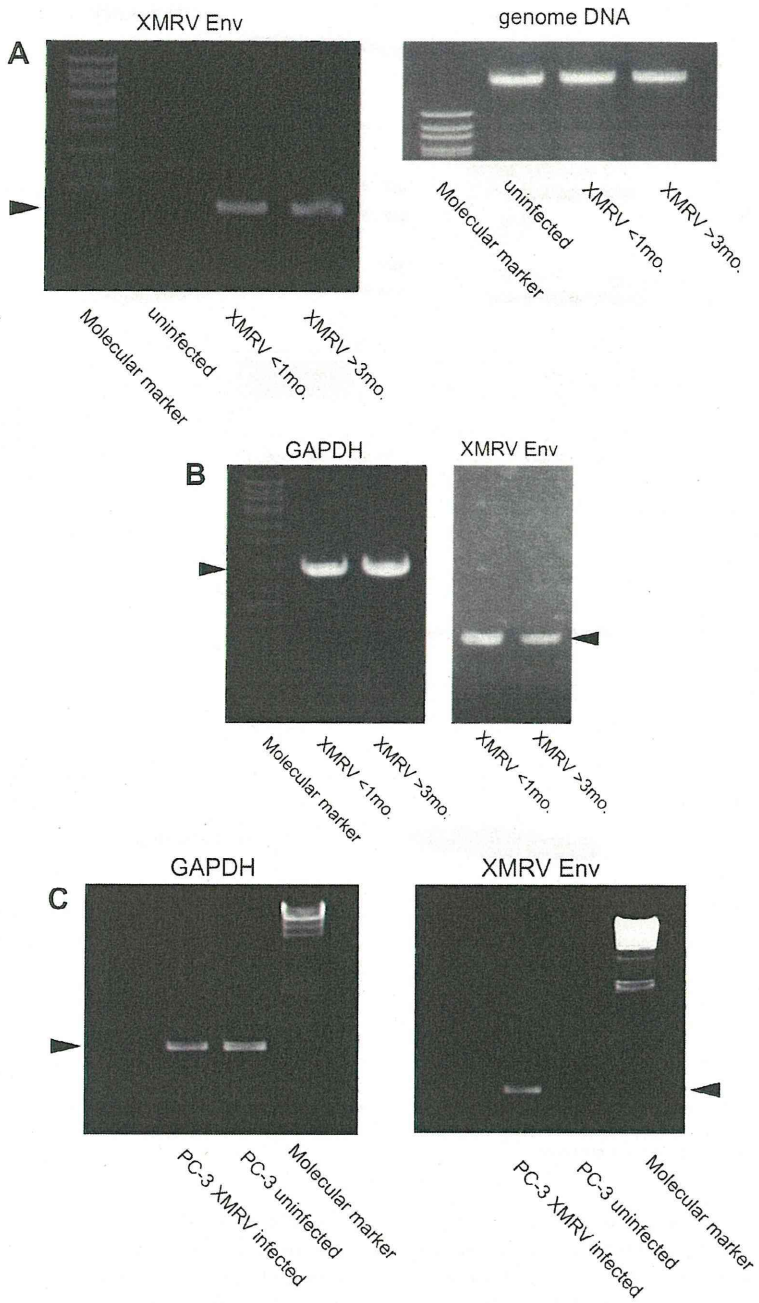

Fig. 4. XMRV transcription was decreased in XMRV-infected LNCaP cells. (A) XMRV sequences integrated into LNCaP cell genomes were detected by PCR (left panel). Equal amounts of genomic DNAs (500 ng) were analyzed (right panel). (B) Levels of XMRV env mRNA was quantified by RT-PCR in XMRV-infected LNCaP cells. (C) XMRV env mRNA levels was measured in uninfected and XMRV-infected PC-3 cells (right panel). As control, GAPDH mRNA was analyzed in the same samples (left panel). Arrow heads indicate predicted sizes of the PCR products.

androgen-independent growth of LNCaP cells would contribute to a more complete understanding of CRPC development and novel therapies for human prostate cancer patients.

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