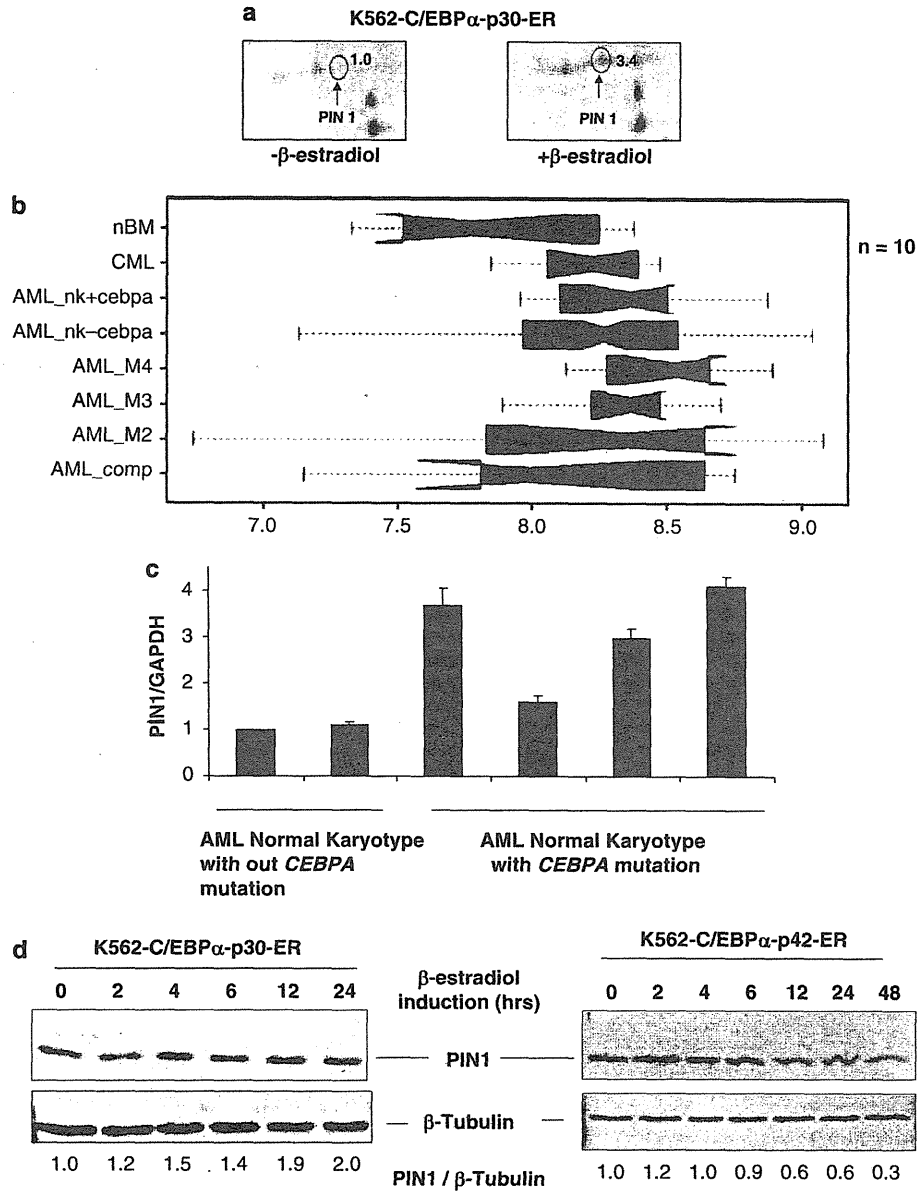

Figure 1 C/EBPα-p30 induces PIN1 mRNA and protein expression. (**a**) K562-C/EBPα-p30-ER cells were induced with β-estradiol for 6 h followed by 2D gel electrophoresis and protein identification by mass spectrometry. The values represented are mean normalized spot volume from the Proteom Weaver software (Definiens, Munich, Germany). (**b**) Affymetrix analysis was carried out with AML blast cells using total RNA isolated from leukemic patient samples, processed and analyzed on the Affymetrix HG-U133A and HG-U133B chips. Expression signal intensities are expressed in logarithmic scale. A total of 10 samples were used in each subtype of AML. The dark bars represent median and boxes represent 25–75% quantile range. If the notches of the box plots do not overlap, there is a strong indication that the medians are different. nBM, normal bone marrow; CML, chronic myeloid leukemia; AML_NK+cebpa, normal karyotype with C/EBPα mutation; AML_NK–cebpa, normal karyotype without C/EBPα mutation; AML_Comp, complex karyotype; AML_M4, AML with the CBF/MYH11 fusion gene; AML_M3, AML with PML/RARA fusion gene; AML_M2, AML with AML1/ETO fusion gene. (**c**) Total RNA was isolated from AML blast cells and analyzed for PIN1 mRNA expression by quantitative real-time PCR. (**d**) K562-C/EBPα-p30-ER cells (left) and K562-C/EBPα-p42-ER cells (right) were induced with β-estradiol (5 μM) for respective time points and cells were lysed and subjected to western blot analysis using PIN1 antibody. Values below the gel image indicate the upregulation (fold) of PIN1 protein level normalized to β-tubulin.

PIN1 inhibition by PiB can overcome the differentiation block observed in human myeloid cells

To further understand how silencing of the PIN1 activity is biologically significant in the context of C/EBPα mutation, we carried out myeloid differentiation experiments in AML blast cells with C/EBPα mutation and in Kasumi-6 cells. Kasumi-6 is a myeloid leukemia cell line established from the bone marrow cells of an individual with AML, subtype M2 having C/EBPα mutation and endogenously expressing both the p42 and the p30 isoforms of the C/EBPα protein.[22] For silencing PIN1, we used the PIN1 inhibitor, PiB.[28] PiB has been shown to inhibit the PIN1 activity by binding to the peptidyl-prolyl *cis/trans*

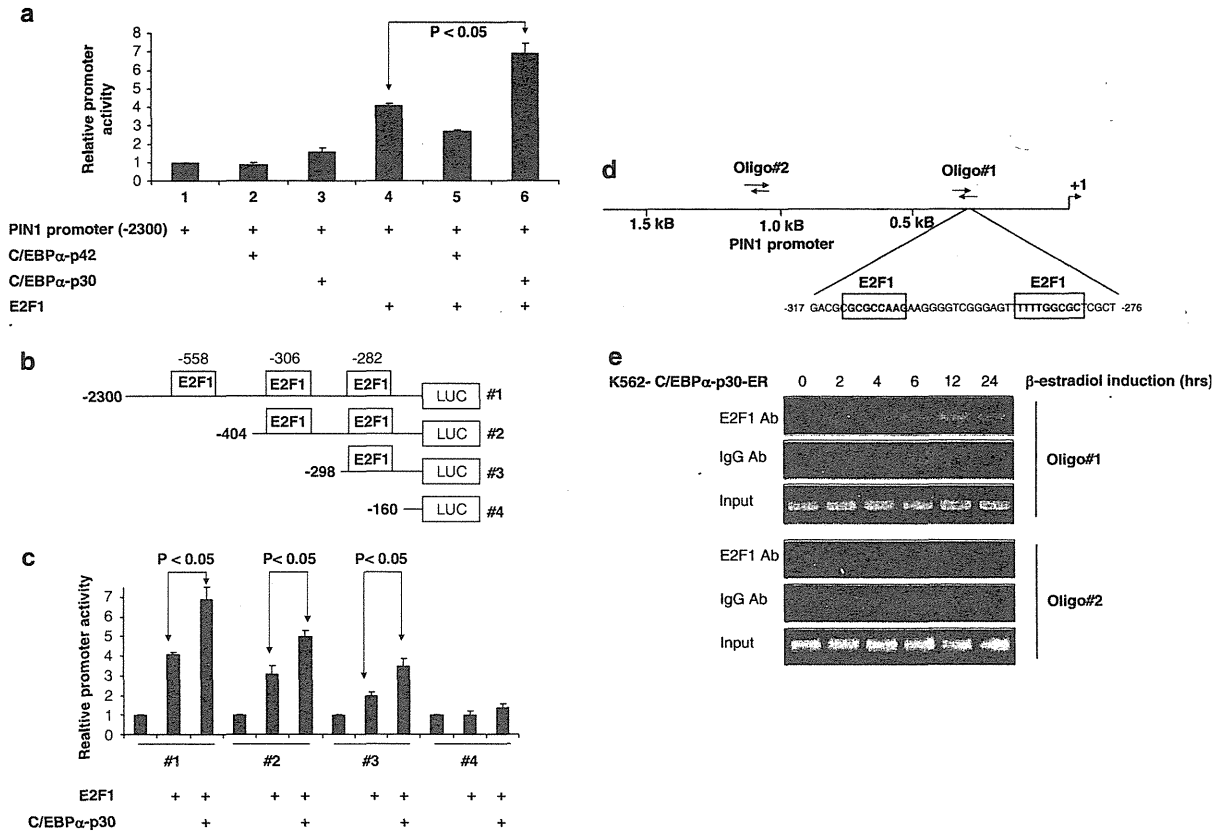

Figure 2 C/EBPα-p30 recruits E2F1 to the PIN1 promoter. (**a**) Transient transfection in 293T cells with a reporter construct of 2.3 kb of the human PIN1 promoter cloned in pGL3 basic vector and expression plasmids for C/EBPα-p42, C/EBPα-p30 and E2F1. Luciferase activities were measured 24 h after transfection and values were normalized using *Renilla* luciferase PRL0. The results are the means from three independent experiments, and error bars represent the standard deviation. (**b**) Schematic presentation of various PIN1 promoter 5′ deletion constructs used for promoter assays. (**c**) 293T cells were transfected with different PIN1 promoter constructs in combination with C/EBPα-p30 and E2F1. (**d**) Human PIN1 promoter sequence with putative E2F1-binding sites. (**e**) K562-C/EBPα-p30-ER cells were induced with β-estradiol for respective time points and chromatin was immunoprecipitated with anti-E2F1 or IgG antibodies, and the recovered DNA was PCR amplified with primers specific for E2F1-binding sites in the PIN1 promoter as shown in Figure 2d.

isomerase domain of PIN1. The myeloid cell differentiation was assessed by CD11b and CD15 expression by flow cytometry analysis, as well as by the level of granulocyte colony-stimulating factor receptor (G-CSFR) expression by real-time reverse transcription-PCR. Treatment of AML blast cells and the Kasumi-6 cells with PiB induced myeloid differentiation (Figure 3). We did not observe any significant change in granulocyte morphology during PIN1 inhibition by PiB (data not shown). Hence, we assume that PIN1 inhibition by PiB is not sufficient to induce terminal stages of differentiation. We investigated the role of PIN1 silencing in NB4 cells, which do not have any C/EBPα mutation. We observed that PIN1 inhibition in NB4 cells leads to granulocytic differentiation (Supplementary Figure 9). However, compared with Kasumi-6, the differentiation effects were modest during PIN1 silencing in NB4 cells. We also analyzed the effect of PIN1 silencing in normal bone marrow cells. We did not observe any change in granulocytic differentiation (data not shown). These data suggest that C/EBPα mutation status (presence of C/EBPα-p30) is significant in granulocytic differentiation during PIN1 silencing. The granulocytic differentiation during PIN1 inhibition by PiB was found to be independent of apoptosis (Supplementary Figure 10) and cell proliferation (data not shown).

PIN1 blocks granulocytic differentiation

The C/EBPα-p30 has been shown to block granulocytic differentiation mediated by C/EBPα,[3] as well as to be able to induce AML.[5] On the basis of our data that C/EBPα-p30 induces PIN1 in AML (Figure 1) and silencing PIN1 leads to granulocytic differentiation (Figure 3), we assessed the ability of PIN1 to block granulocytic differentiation. We overexpressed PIN1 in myeloid U937 cells and treated the cells with retinoic acid to induce granulocytic differentiation. Overexpression of PIN1 blocked granulocytic differentiation, as shown by around 50% reduction in CD15 expression as well as reduction in G-CSFR expression (Figure 4). Taken together, these data show that PIN1 could have significant role in granulocytic differentiation block observed in AML.

PIN1 controls the stability of the c-Jun protein

We have previously reported that c-Jun expression is high in AML patient samples with C/EBPα mutation.[17] On c-Jun N-terminal kinase activation, PIN1 binds to c-Jun, which is phosphorylated on Ser63/73-Pro motifs.[10] Owing to the fact that C/EBPα-p30 could induce PIN1 and that PIN1 regulates the protein stability of many targets with which it interacts,[8] we

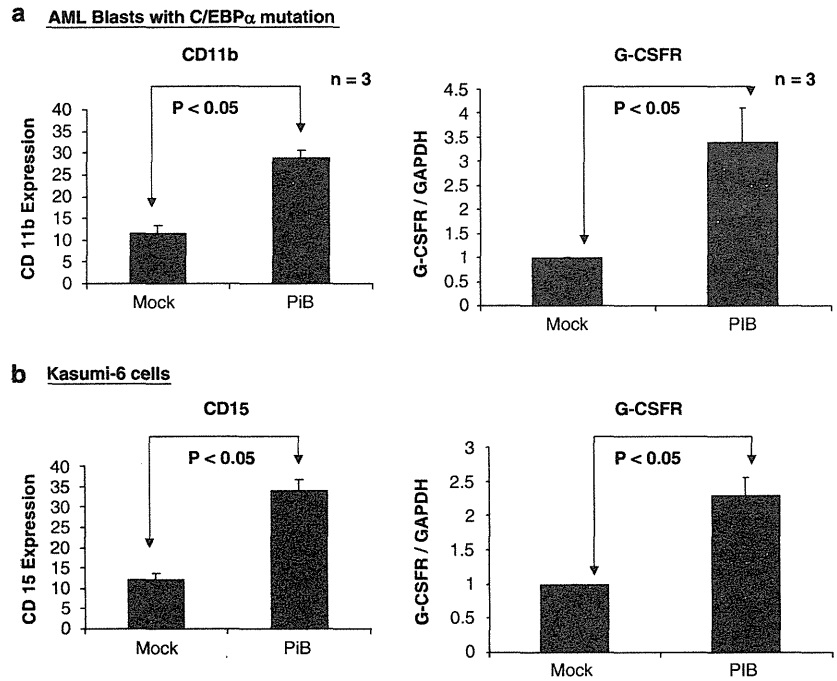

Figure 3 PIN1 inhibition using PiB can overcome the differentiation block observed in human myeloid cells. (**a**,**b**) AML blast cells with C/EBPα mutation (**a**) and Kasumi-6 cells (**b**) were treated with PiB (5 μM) for 6 days and myeloid cell differentiation was assessed by FACS analysis using CD11b and CD15 expression, as well as G-CSFR expression by real-time RT-PCR analysis. The results are the means from three independent experiments, and error bars represent the standard deviation.

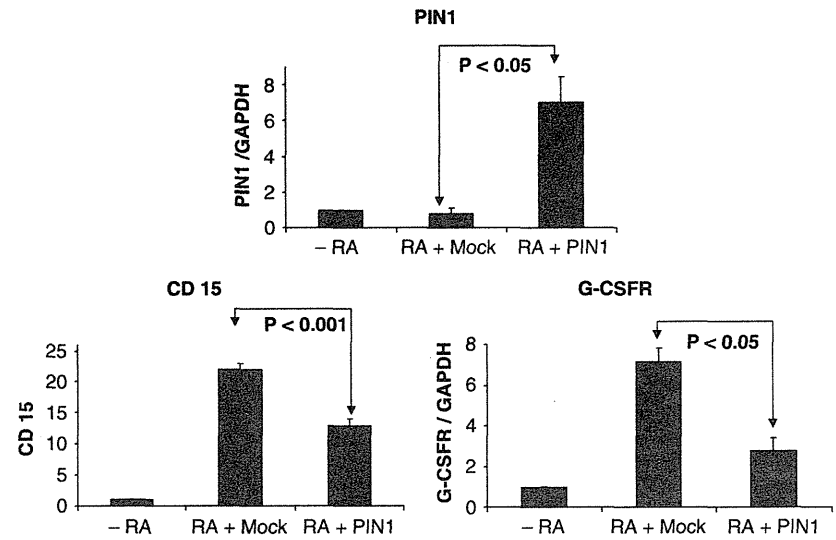

Figure 4 PIN1 inhibits granulocytic differentiation. U937 cells were transfected with mock vector or PIN1 vector followed by treatment with retinoic acid (1 μM). At 3 days after transfection, CD15 expression was analyzed with FACS analysis, and PIN1 and G-CSFR expressions were analyzed with real-time RT-PCR. The results are the means from three independent experiments, and error bars represent the standard deviation.

hypothesized that PIN1 could have a significant role in regulating c-Jun protein stability. To test this hypothesis, PIN1 expression was silenced in 293T cells by RNA interference and c-Jun protein stability was then assessed by blocking protein synthesis with cyclohexamide and by measuring the c-Jun protein remaining at various time points. Our data show that the stability of c-Jun was reduced when PIN1 expression was knocked down by a specific siRNA (Figure 5a and supplementary Figure 11). These results indicate that PIN1 is an important factor in regulating the stability of c-Jun protein.

PIN1 protects c-Jun from ubiquitination-mediated protein degradation

c-Jun has been shown to be subjected to ubiquitin-mediated degradation in a c-Jun N-terminal kinase-dependent manner.[29,30]

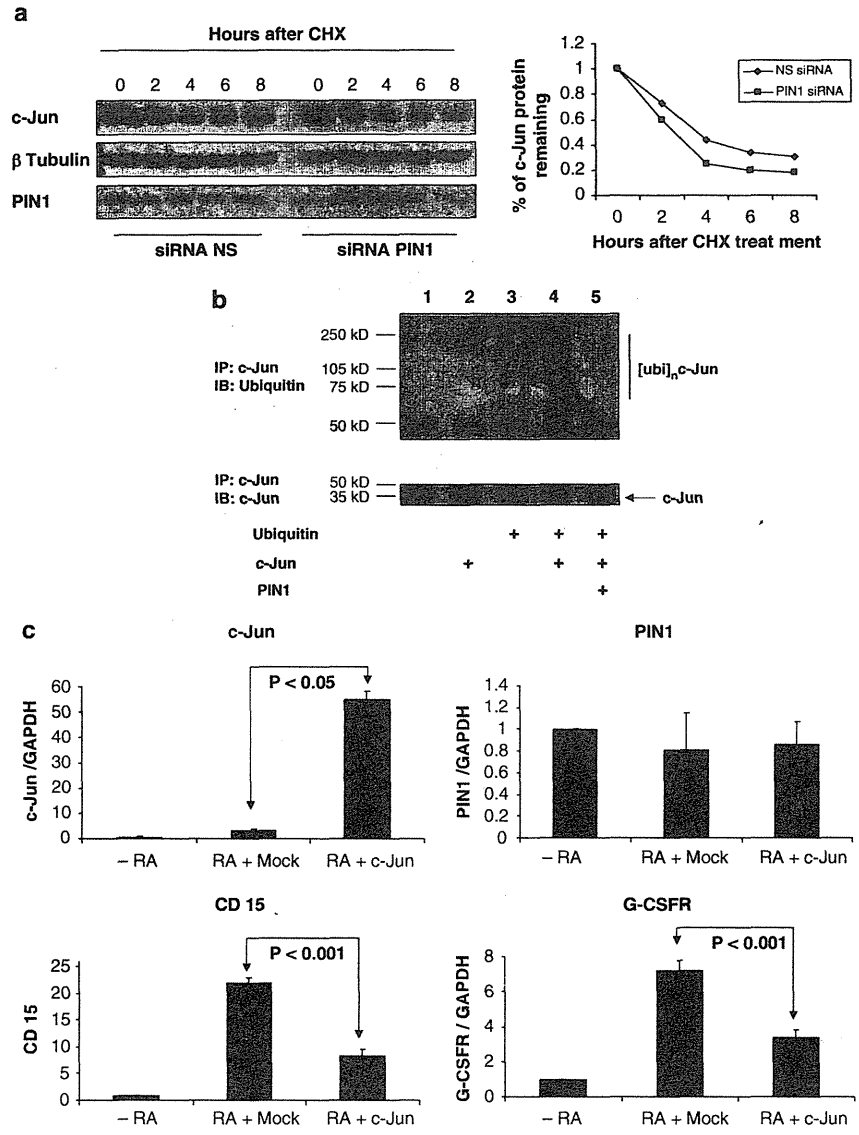

Figure 5 PIN1 stabilizes c-Jun protein by inhibiting c-Jun ubiquitination and c-Jun blocks the granulocytic differentiation. (**a**) 293T cells were transfected with PIN1 and control siRNA. After 24 h, protein synthesis was blocked with cyclohexamide (CHX) and cells were harvested at different time points. The amount of c-Jun protein remaining was analyzed by western blot followed by densitometric scanning. (**b**) Ubiquitination assay was performed by transfecting 293T cells with the expression plasmids for HA-ubiquitin, c-Jun and PIN1 as indicated; at 24 h post-transfection, cells were lysed and c-Jun was immunoprecipitated and immunoblotting against HA antibody (upper panel) was carried out. Membrane was stripped and and reprobed for c-Jun (lower panel) as control for c-Jun immunoprecipitation (**c**) U937 cells were transfected with mock vector or c-Jun vector followed by treatment with retinoic acid (1 μM). At 3 days after transfection, CD15 expression was analyzed with FACS analysis, and c-Jun, PIN1 and G-CSFR expressions were analyzed with real-time RT-PCR. The results are the means from three independent experiments, and error bars represent the standard deviation.

On the basis of our finding that PIN1 regulated c-Jun protein stability (Figure 5a), we tested the role of PIN1 in the degradation of c-Jun. We performed ubiquitination assays in 293T cells by overexpressing c-Jun, PIN1 and hemagglutinin-tagged ubiquitin. When c-Jun was cotransfected with hemagglutinin-tagged ubiquitin, higher molecular weight bands were detected in the immunoprecipitation with the hemagglutinin antibody, which reflects c-Jun ubiquitination (Figure 5b, lane 4). Interestingly, when PIN1 was cotransfected, the ubiquitination of c-Jun was prevented, suggesting a role for PIN1 in the regulation of c-Jun degradation (Figure 5b, lane 5).

Our data suggest that PIN1 prevents the degradation of c-Jun protein (Figure 5a, b). The Jun protooncogene is positively autoregulated by its product, Jun/AP-1.[31] On the basis of these findings, we hypothesized that PIN1 could have a major role in regulating c-Jun mRNA levels. We overexpressed PIN1 in Kasumi-6 cells and analyzed c-Jun mRNA levels by quantitative real-time reverse transcription-PCR. We observed that overexpression of PIN1 leads to an increase in c-Jun mRNA levels (Supplementary Figure 12a). We next investigated the effect of silencing PIN1 on the c-Jun mRNA levels. We found that silencing PIN1 with the inhibitor PiB downregulated the c-Jun

mRNA level (Supplementary Figure 12b). These data show that PIN1 stabilizes c-Jun protein, which in turn induces c-Jun mRNA by autoregulation.

c-Jun blocks granulocytic differentiation

We have previously shown that downregulation of the proto-oncogene c-Jun by C/EBPα is important for granulocytic lineage commitment.[16] We have also reported that c-Jun expression is high in different subtypes of AML including AML samples with C/EBPα mutation and that c-Jun is able to block the DNA binding of C/EBPα.[17] On the basis of these findings, we hypothesized that c-Jun is able to block the granulocytic differentiation induced by C/EBPα. To assess this, we over-expressed c-Jun in human myeloid U937 cells and treated the cells with retinoic acid. The granulocyte differentiation was assessed by FACS (fluorescence activated cell sorting) analysis for CD15 expression and by quantitative real-time reverse transcription-PCR for G-CSFR. The expression of CD15 during granulocytic differentiation was downregulated to around threefold in the presence of c-Jun (Figure 5c). The myeloid markers such as G-CSFR were also found to be downregulated during overexpression of c-Jun (Figure 5c). These data suggest that c-Jun is able to block granulocytic differentiation induced by C/EBPα.

Discussion

Initially, it was suggested that the dominant-negative effect of C/EBPα-p30 is the result of heterodimerization of the mutant form with the C/EBPα-p42. A recent report shows that a C/EBPα-p30 with modification in the leucine zipper, which cannot form heterodimers with C/EBPα-p42, still exhibits dominant-negative properties over C/EBPα-p42.[32] This suggests that rather than heterodimerization, regulatory networks activated by C/EBPα-p30 could have a critical role in its dominant-negative function. One recent study showed that C/EBPα-p30 has quite distinct functional properties compared with the C/EBPα-p42.[33] This study showed that C/EBPα-p30 is able to bind to a unique set of target genes with higher affinity than did C/EBPα-p42. Mice with targeted disruption of C/EBPα-p42, which express C/EBPα-p30, develop leukemia.[5] This finding is of particular interest as C/EBPα knockout mice did not develop leukemia even though they exhibited a block of granulocytic differentiation.[34] Taken together, these findings show that the disruption of C/EBPα-p42 alone is not sufficient to initiate leukemogenesis, but that pathways modulated by C/EBPα-p30 might be critical in the development of AML.

By using a proteomic screen, we have recently shown that C/EBPα-p30 is able to upregulate the expression of a number of proteins including PIN1.[6] Our finding that PIN1 is upregulated in leukemic patient samples including in those with C/EBPα mutations (Figure 1b,c) is intriguing as PIN1 overexpression was shown to amplify multiple signaling pathways important in oncogenesis.[35] We have shown that C/EBPα-p42 is able to downregulate PIN1 protein levels (Figure 1d, right). One possible explanation for the upregulation of PIN1 in different leukemic samples could be that C/EBPα has been shown to be downregulated by diverse mechanisms in different subtypes of leukemia.[36] This downregulation of C/EBPα function might then lead to PIN1 overexpression in leukemia (Figure 1b). However, we did not find any significant upregulation of PIN1 during C/EBPα silencing in cell lines (Supplementary Figure 4). It has been observed that BRCA1 tumor suppressor (breast cancer-associated gene 1) represses PIN1.[37] Women with germline heterozygous mutations in BRCA1 are at increased risk of developing breast cancers[38] and PIN1 is overexpressed in breast cancer.[10] These findings suggest the possibility that other tumor suppressors could also be regulating PIN1 expression in granulopoiesis. Further studies are needed to show how C/EBPα as well as other tumor suppressors regulate PIN1 and how PIN1 is overexpressed in AML without C/EBPα mutation.

A recent study proposes that C/EBPα could be a PIN1 target.[39] This study points out that several Ser/Thr-Pro motifs in C/EBPα could be regulated by PIN1-mediated isomerization. Most of the Ser/Thr-Pro motifs in the C/EBPα-p42 are also present in C/EBPα-p30. Even though C/EBPα has been shown to be phosphorylated, there is no report about PIN1-mediated post-phosphorylation mechanisms regulating the C/EBPα function. Further studies are needed to explain how PIN1 regulates C/EBPα-mediated transactivation and whether CEBPA might be a target of PIN1-mediated proline isomerization.

In granulopoiesis, the inhibition of the E2F1 activity has been proven to be the unique mechanism for the antimitotic activity of C/EBPα.[19,40] E2F1 activates the transcription of the *MYC* oncogene, which had been shown to block granulopoiesis. C/EBPα inhibition of E2F1 has been shown to result in the downregulation of c-Myc, leading to granulopoiesis.[41] This raises the possibility that other E2F1 targets could also be regulated by C/EBPα in a similar mechanism, as observed for c-Myc. In this context, it should be noted that PIN1 has been shown to be an E2F1 target gene,[27] and lack of E2F inhibition by C/EBPα has been shown to be a key event in AML.[5,42] Our finding that C/EBPα interferes with the E2F1 transactivation of PIN1 promoter (Figure 2a) suggests that the downregulation of PIN1 by C/EBPα could be an important event in granulopoiesis. Our observation points out the intrinsic differences between C/EBPα-p42 and C/EBPα-p30 in regulating PIN1 expression. C/EBPα-p30 cooperates with E2F1 in regulating PIN1 promoter activity (Figures 2a, c), and C/EBPα-p30 is capable of recruiting E2F1 to the PIN1 promoter (Figure 2e). This suggests that regulatory networks coordinated by C/EBPα-p30 and E2F1 could have an important role in AML with C/EBPα mutations.

A recent study shows that C/EBPα-p30 not only blocks granulopoiesis but also has the potential to induce AML.[5] This study also indicates that loss of C/EBPα function alone is not sufficient for the development of AML. Rather, a restricted modulation of C/EBPα-p42 function by C/EBPα-p30 is a prerequirement for AML to develop. Our findings that C/EBPα-p30 induces PIN1 (Figure 1), inhibition of PIN1 leads to granulocytic differentiation (Figure 3) and that PIN1 blocks granulopoiesis (Figure 4), point out the fact that PIN1 might have a major role in contributing to the differentiation block observed during C/EBPα mutation.

PIN1 is overexpressed in breast cancer and increases the transcriptional activity of c-Jun.[10] PIN1 regulates the protein degradation of many targets with which it interacts, including p53, p73, nuclear factor-κB and cyclin D1.[8] In addition, PIN1 has been shown to bind to c-Jun[10] and has been proposed to increase the protein stability of c-Jun.[8] Our data show that PIN1 increases the stability of c-Jun protein by inhibition of its ubiquitination (Figure 5a,b). As observed for other PIN1 targets, we assume that stabilization of c-Jun by PIN1 is a general phenomenon in cancer. c-Jun is an effector of many signal cascades and has crucial functions in diverse mechanisms of oncogenesis.[43] An oncogenic role for c-Jun in AML was suggested by the finding that c-Jun is overexpressed in different subtypes of AML.[17,44] One explanation for the upregulation of

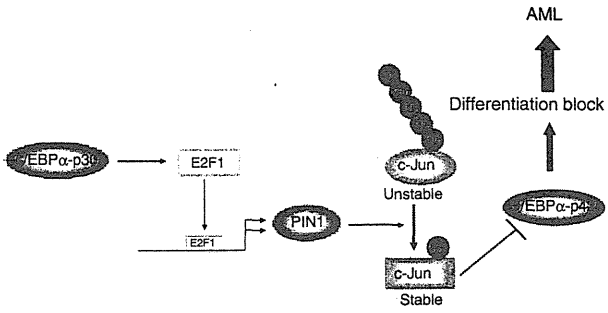

Figure 6 Model depicting the PIN1-mediated dominant-negative role of C/EBPα-p30 in AML with C/EBPα mutation. C/EBPα-p30 cooperates with E2F1 and increases the PIN1 mRNA and protein levels. PIN1 stabilizes the c-Jun protein, which in turn blocks C/EBPα functions and leads to differentiation block and AML.

c-Jun expression in AML might be the downregulation of C/EBPα by different mechanisms.[36] Furthermore, leukemic fusion proteins such as BCR-ABL[45] and AML1-ETO[44] have been shown to induce c-Jun expression through the c-Jun N-terminal kinase signaling pathway. We cannot rule out the possibility that loss of activity of other factors that negatively regulate PIN1 could also upregulate c-Jun in AML. Our laboratory had shown that c-Jun inactivates C/EBPα through direct protein–protein interaction through their leucine zipper domains.[17] One recent study suggested that C/EBPα–c-Jun interaction has the potential to induce monocytic differentiation,[46] pointing out how protein–protein interactions of C/EBPα are critical in lineage commitment decisions. Another report showed that c-Jun has the ability to promote monocytic differentiation at the expense of granulocytic differentiation.[47] Our data also indicate that overexpression of c-Jun induces monocytic differentiation (Supplementary Figure 13). Our findings that PIN1 can stabilize c-Jun (Figure 5a) and prevent c-Jun ubiquitination (Figure 5b) suggest that PIN1 might have an important role in regulating the c-Jun protein turn over. The stabilized c-Jun might bind to its own promoter and increase its expression in a positive feedback loop.[31] The ability of c-Jun to block DNA binding of C/EBPα,[17] as well as our finding that c-Jun is able to inhibit granulocytic differentiation (Figure 5d), suggests a model in which c-Jun promotes proliferation and leads to differentiation block by inactivating C/EBPα. In other words, the relative protein concentrations of C/EBPα and c-Jun regulate granulopoiesis—higher C/EBPα levels favor differentiation, whereas higher c-Jun levels favor proliferation.

In summary, our study identifies PIN1 as an important player that might contribute to myeloid leukemia development through the inhibition of the C/EBPα function. Here, we show that PIN1 is upregulated by C/EBPα-p30 and that silencing of PIN1 is able to overcome the differentiation block mediated by C/EBPα-p30. Our observations suggest a model in which C/EBPα-p30 induces PIN1 expression and increases the stability of c-Jun, which in turn inhibits the function of C/EBPα-p42 (Figure 6). Inhibiting PIN1 function could provide a novel strategy in the treatment of myeloid leukemic patients.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Molecular and enzymatic characterization of XMRV protease by a cell-free proteolytic analysis

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ABSTRACT

Xenotropic murine leukemia virus-related virus (XMRV) is a virus generated under artificial conditions by the recombination of 2 murine leukemia virus (MLV) proviruses, PreXMRV-1 and PreXMRV-2, during the in vivo passage of human prostate cancer cells in athymic nude mice. The molecular etiology of XMRV infection has not been characterized and its implication in human prostate cancer progression remains equivocal. As a step toward resolving this issue we developed an in vitro enzymatic assay system to characterize XMRV protease (PR)-mediated cleavage of host-cell proteins. Enzymatically-active XMRV PR protein was synthesized using a wheat-germ cell-free system. By monitoring cleavage activity of XMRV PR by AlphaScreen and 2-color immunoblot analyses, we revealed that the catalytic activity of XMRV PR is selectively blocked by the HIV PR inhibitor, Amprenavir, and identified several human tumor suppressor proteins, including PTEN and BAX, to be substrates of XMRV PR. This system may provide an attractive means for analyzing the function of retrovirus proteases and provide a technology platform for drug screening.

1. Introduction

Xenotropic murine leukemia virus-related virus (XMRV) was originally isolated from a human prostate cancer (PC) in 2006 [1]. This virus is highly homologous to several endogenous Murine leukemia viruses (MLVs) found in mice [2]. Although previous reports suggested the involvement of XMRV in PC as well as chronic fatigue syndrome (CFS), as an etiological agent, no evidence of this etiological link between XMRV and human disease has been shown to date [3–5].

The nucleotide sequence of XMRV isolated from humans indicates that the virus is nearly identical with the XMRV isolated from the human prostate tumor cell line 22Rv1 [6]. This cell line was generated by serial passage of human prostate tumor tissue in nude mice. Sequence analysis revealed that the genomes of these mouse strains contain two different proviral

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DNAs related to XMRV (Pre-XMRV-1 and 2). It appears that these proviral genomes recombined to produce the XMRV isolated from 22Rv1 cells. It is plausible that the reported association of XMRV with human disease is due to contamination of human samples with virus originating from this recombination event in mice prior to the analysis.

While XMRV arose from an unusual recombination, several lines of study have indicated that XMRV can indeed infect, and proliferate in, several human prostate cancer cell lines including LNCaP and PC3 [7]. It is of significance that dihydrotestosterone (DHT) was shown to stimulate transcription and replication of XMRV through the transactivation of the XMRV-LTR via the hormone response element (HRE) [8]. Mutations in the HRE of XMRV impaired basal transcription and androgen responsiveness, suggesting a relationship between virus productivity and prostatic hypertrophy and neoplasia.

If XMRV is indeed an etiological agent in PCs, detection and elimination of XMRV infection could provide an effective strategy for early diagnosis and treatment of this tumor. To date, however, conflicting epidemiological data has precluded investigations into whether the virus is truly pathogenic or not and, moreover, whether this virus is oncogenic and associated with human PCs.

Retroviral protease (PR) is essential for virus replication and has been the major target for anti-retroviral therapy. Concomitant with particle release, the virally-encoded PR cleaves Gag into its four mature protein domains; MA, CA, NC and p12, in case of XMRV. Gag-Pol is also cleaved by PR, creating the viral enzymes RT, IN and PR itself. The maturation step coupled with PR activation is essential to confer viral infectivity. Therefore, for the effective inhibition of XMRV infection, should this virus be found to be pathogenic, development of anti-retroviral drugs targeting the XMRV protease would seem logical.

Several recent studies have indicated that viral-protease cleavage of host proteins that promotes viral replication and cytopathic effects [9–11]. It remains elusive whether this is the case for XMRV PR. One of the major bottlenecks in PR research has been the difficulty in producing recombinant protein with enzymatic activity in conventional cell-associated protein expression systems (e.g. *E. coli* or insect cell) due to host cell toxicity.

The wheat germ protein production system is a robust in vitro cell-free protein synthesis system comprising a crude wheat germ extract containing ions (buffer) and all the macromolecular components required for protein translation (e.g. ribosomes, tRNAs, aminoacyl-tRNA synthetases, initiation, elongation and termination factors) [12,13]. The extract is further supplemented with amino acids, energy sources such as ATP, energy generating systems and cofactors for efficient and abundant protein production. This system is a powerful tool for the preparation of multiple proteins at one time, and large amounts of specific proteins for both biochemical and biomedical applications. It also enables synthesis of specific proteins that are difficult to express and/or purify in *E. coli* or other cell-based systems. The wheat germ system can produce a wide variety of proteins, including viral proteins, in sufficient amounts for functional assays. Furthermore, this system can yield enzymatically-active proteins in their naturally folded state owing to the constituent eukaryotic translation and folding machinery.

In our current study, we utilized the wheat germ cell-free system to synthesize catalytically active XMRV PR for the identification of potential inhibitors and substrates. By this approach we delineated a molecular link between XMRV PR and human tumor suppressor proteins pointing to a potential role in oncogenesis.

2. Materials and methods

2.1. *Construction of DNA template for transcription and the protein expression*

DNA templates were made using the Gateway and split-primer polymerase chain reaction (PCR) systems [12,14,15]. The tumor suppressor genes were amplified from MGC, a human cDNA resource purchased from Danaform (Tokyo, Japan).

The XMRV protease fragment was amplified by PCR from XMRV VP62 clone [1,16] using the following forward and reverse primers, respectively: (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGAAGGACTGCCCAAAGAAGCC-3′) and (5′-GGGGACCACTTTGTACAAGAA AGCTGGGTCTTATAGAGGAACATCTGGCTC-3′). For HIV-1 protease, cDNA fragment was amplified by PCR from HIV-1 NL4-3 clone by using the following forward and reverse primers, respectively: (5′-CCACCCACCACCACCAATGTTTTTTAGGGAAGATC-3′) and (5′-TCCAGCACTAGCTCCAGATTAGCCATCCATTCCTGGC-3′). Subsequently, attB-flanked fragment were amplified by PCR using attB1-S1 primer (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCCACCCACCACCACCAATG-3′) and attB2-T1 primer (5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCTCCAGCACTAGCTCCAGA-3′). The single Capsid (CA) and Nucleocapsid (NC) fragment of XMRV Gag (control substrate), and the 24 tumor suppressor gene fragments (test substrates) were amplified by two-step PCR (without stop codon). The first round of PCR was performed on using 10 nM of S1 (forward) primer (5′- CCACCCACCACCACCAatg(n)19-3′) and T1 (reverse) primer (5′-TCCAGCACTAGCTCCAGA(n)19-3′). The second round of PCR was carried out using the first PCR product as template, with 100nM of attB1-FLAG-S1 (forward) primer (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGACTACAAGGATGACGATGACAAGCTCCACCCACCACCACCAATG-3′) and T1-biotin ligase site (bls)-stop-attB2-anti (reverse) primer (5′-GGGGACCACTTTGTACAAGAAAGCTGGGTTTATTCGTGCCACTCGATCTTCTGGGCCTCGAAGATGTCGTTCAGGCCGCTTCCAGCACTAGCTCCAGA-3′) [17].

The amplified attB-flanked fragments were each inserted into the pDONR221 vector using the Gateway BP Clonase II enzyme mix (Invitrogen, Carlsbad, CA, USA) to give pDONR-XMRV PR vector and pDONR-FLAG-gene-bls vectors, respectively.

pDONR-XMRV PR and pDONR-FLAG-CA/NC-bls vectors were subcloned into pEU-E01-GW [18] to generate the pEU-based-plasmids by LR reaction. BP and LR reactions were performed according to the manufacturer's instructions (Invitrogen). PCR reactions were performed using PrimeStar enzyme according to the manufacturer's instructions (Takara Bio, Otsu, Japan).

For HIV-1 PR substrate, the p2–p7 fragment in HIV-1 Gag precursor was amplified by PCR from HIV-1 NL4-3 clone by using the following forward and reverse primers, respectively: (5′-GAGACTCGAGGCCGAGGCCATGAGCCAGG-3′) and

(5′-GAGCGGTACCTTATTCGTGCCACTCGATCTTCTGGGCCTC-GAAGATGTCGTTCAGGCCATTAGCCTGCCTCTCGGTGCA-3′). The p2–p7-bls fragment was inserted into the pEU-E01-GST-MCS vector (Cellfree Sciences, Yokohama, Japan). The transcription template from pEU vector was amplified using the following forward and reverse primers, respectively: SPu primer (5′-GCGTAGCATTTAGGTGACACT-3′) and AODA2303 (5′-GTCA-GACCCCGTAGAAAAGA-3′). PCR was carried out using the TaKaRa Ex Taq (Takara Bio Inc, Shiga, Japan) according to the manufacturer's instructions.

DNA templates of human genes for transcription were constructed using split-primer PCR in two steps as described previously [18]. For the first step, S1 primers and pDONR221-1st_4080 (5′-ATCTTTTCTACGGGGTCTGA-3′) or AODA2306 (5′- AGCGTCAGACCCCGTAGAAA -3′) were used. For the second step, the primers SPu and pDONR221-2nd_4035 (5′-ACGTTAAGG-GATTTTGGTCA-3′) or AODA2303 were used to generate the final DNA template for transcription.

2.2. *Cell-free protein synthesis*

In vitro transcription and cell-free protein synthesis was performed as described previously [19]. Transcripts were made from each of the DNA templates mentioned above using SP6 RNA polymerase. The synthetic mRNAs were then precipitated with ethanol, collected by centrifugation and washed. Each mRNA (typically 30–35 μg) was added to the translation mixture and the translation reaction was performed in the bilayer mode [20] with slight modifications. The translation mixture that formed the bottom layer consisted of 60 A260 units of wheat germ extract (CellFree Sciences) and 2 μg creatine kinase (Roche Diagnostics K. K., Tokyo, Japan) in 25 μl SUB-AMIX solution (CellFree Sciences). SUB-AMIX contained (final concentrations) 30 mM Hepes/KOH at pH 8.0, 1.2 mM ATP, 0.25 mM GTP, 16 mM creatine phosphate, 4 mM DTT, 0.4 mM spermidine, 0.3 mM each of the 20 amino acids, 2.7 mM magnesium acetate, and 100 mM potassium acetate. SUB-AMIX (125 μl) was placed on the top of the translation mixture, forming the upper layer. After incubation at 16 °C for 16 h, protein synthesis was confirmed by SDS-PAGE. For biotin labeling, 1 μl (50 ng) of crude biotin ligase (BirA) produced by the wheat germ cell-free expression system was added to the bottom layer, and 0.5 μM (final concentration) of D-biotin (Nacalai Tesque, Inc., Kyoto, Japan) was added to both upper and bottom layers, as described previously [21].

2.3. *Detection of cleavage activity of XMRV protease by luminometry*

In vitro cleavage activity assays of XMRV protease were carried out in a total volume of 15 μl consisting of 100 mM Tris–HCl pH 8.0, 0.01% Tween-20, 1 mg/ml BSA, 1 μl crude recombinant protease (~0.75 μM) and 0.5 μl crude recombinant FLAG-biotin-tagged CA/NC (~0.037 μM) at 37 °C for 1 h in a 384-well Optiplate (PerkinElmer, Boston, MA, USA). To assay the effects of HIV protease inhibitors on XMRV protease, after 3 μl recombinant viral protease and HIV protease inhibitor was incubated at 37 °C for 10 min, FLAG-biotin-tagged CA/NC or GST-biotin-tagged p2–p7 was added and the reaction further incubated at 37 °C for 1 h in a 384-well Optiplate. In accordance with the AlphaScreen IgG (Protein A) detection kit (PerkinElmer) instruction manual, 10 μl of detection mixture containing 100 mM Tris–HCl pH 8.0, 0.01% Tween-20, 1 mg/ml BSA, 5 μg/ml Anti-FLAG antibody (Sigma-Aldrich, St. Louis, MO, USA) or Anti-GST antibody (GE Healthcare, Buckinghamshire, UK), 0.1 μl streptavidin-coated donor beads and 0.1 μl anti-IgG (Protein A) acceptor beads were added to each well followed by incubation at 26 °C for 1 h. Luminescence was analyzed by the AlphaScreen detection program. Each assay was performed in triplicate, and the data represent the means and standard deviations of three independent experiments.

2.4. *Detection of cleavage activity by immunoblotting*

3 μl crude recombinant viral protease (~0.75 μM) and 7 μl crude FLAG-biotin-tagged recombinant proteins were incubated at 37 °C for 2 h. To assay the effect of HIV protease inhibitors, 3 μl crude recombinant XMRV protease and 1 μl of 10 μM HIV protease inhibitor were incubated at 37 °C for 10 min followed by addition of 6 μl crude FLAG-biotin-tagged recombinant proteins, and incubated at 37 °C for 120 min. Proteins were separated by SDS-PAGE and transferred to a PVDF membrane (Millipore Bedford, MA, USA) according to standard procedures. Immunoblot analysis was carried out with anti-FLAG (M2) antibodies (Sigma-Aldrich) or Streptavidin-HRP conjugate (GE Healthcare) according to the procedure described above. For fluorescent imaging, immunoblotted proteins were detected by Alexa592-anti-mouse antibodies (N-cleaved fragments), and Alexa488-streptavidin (C-cleaved fragments). The labeled proteins were visualized using a Typhoon Imager (GE Healthcare).

2.5. *Homology modeling of XMRV PR in complex with APV*

To predict interactions between XMRV PR and APV, we performed homology modeling [22] of the complex structure formed between XMRV PR and APV using the Molecular Operating Environment (MOE) software ver. 2008.10. (Chemical Computing Group, Canada). Firstly, the homologues of XMRV PR were searched for with the MOE-search PDB module from the MOE homology databank. Secondly, to minimize misalignments of the target sequence, multiple alignments were made using sequences of the homologues and those of HIV-1 PR (PDB code: 1HPV) and HTLV PR (PDB code: 3LIN) with the MOE-Align module. The aligned sequences showed that amino acids at the active site of HIV-1, HTLV PRs and those likely to be at the active site of XMRV PR were comparatively conserved, suggesting a structure of HIV-1 PR with APV (PDB code: 1HPV) would be appropriate for a template structure to predict interactions between XMRV PR and APV. Thirdly, homology modeling was performed with MOE-Homology, using the structure of HIV-1 PR in complex with APV (PDB code: 1HPV) as a template structure. During the modeling, the MMFF94x force field and the GB/VI implicit solvent function [23] were applied for energy calculation. In this study, we predicted ten structures of the complex, and selected the structure with the lowest energy as the model for the XMRV PR-APV complex.

3. Results

3.1. *Synthesis of an enzymatically active XMRV PR using the wheat germ cell-free system*

To synthesize enzymatically-active XMRV PR, we generated a transcription template of this enzyme derived from the XMRV VP62 clone. The template cDNA encodes the open reading frame of XMRV PR flanked by N-terminal 20 amino acid and a C-terminal 20 amino acid regions, as shown in Fig. 1A (PR; 20aa-PR-20aa). This PR differs from the synthesized inactive native PR template by an introduced substitution of the termination codon at the 3′-terminus of the Gag coding sequence for Glu-coding codon (CAG) to avoid translational termination at the end of Gag protein. As a catalytic-incompetent PR, we also designed a PR mutant harboring the catalytic active site substitution D32N (PR_D32N) (Fig. 1A). All 3 cDNA templates were subjected to cell-free transcription-translation. The protein yield of XMRV PR produced by this system was approximately 0.75 μM and the solubility was ~90%, as calculated by semi-quantitative CBB staining following SDS-PAGE (Fig. 1B). By immunoblotting (IB), two specific protein bands appeared at 14 kDa and 17 kDa, corresponding to the expected mobility of the full-length PR and the truncated form of PR by auto-cleavage of the flanking 20 a.a. at both ends (Fig. 1B). The auto-cleavage site of the XMRV PR was also confirmed by amino acid sequencing of the truncated protein band (Fig. 1C).

We next examined the enzymatic activity of the XMRV PR by monitoring its cleavage activity upon a native FLAG-pr55^{Gag} protein substrate. Wheat germ-synthesized XMRV PR was incubated with FLAG-pr55^{Gag} protein followed by immunoblotting analysis. p55^{Gag} was efficiently digested into the expected cleavage products (FLAG-MA-p12 and FLAG-MA) predicted from the known cleavage sites (Fig. 1D).

3.2. *Evaluation of protease activity using AlphaScreen*

For the quantitative and high-throughput measurement of XMRV PR activity using AlphaScreen technology, we designed a reporter substrate comprising a partial capsid (CA)-nucleo capsid (NC) junction peptide flanked by N-terminal FLAG and C-terminal biotin binding sequence (FLAG-CA/NC-biotin), as described in Materials and methods [14,15]. Fig. 2A shows a schematic representation of our assay system. Briefly, cell-free synthesized active XMRV PR, its D32N mutant or dihydrofolate reductase (DHFR) as a negative control, were incubated with the reporter substrate at 37 °C for 1 h, followed by the addition of AlphaScreen streptavidin donor and protein A acceptor beads as depicted in Fig. 2A. The cleavage of the reporter substrate was measured by level of luminescence (Fig. 2A). Wild-type XMRV PR, but not D32N_PR diminished the Alphascreen luminescent signal indicating proteolytic cleavage of the reporter polypeptide (Fig. 2B). The cleavage activity of PR was normalized relative to the luminescent activity of DHFR (Fig. 2C). Parallel immunoblot analysis with an anti-FLAG antibody demonstrated that the substrate protein was selectively cleaved by PR alone (Fig. 2D).

3.3. *Screening of XMRV PR inhibitors by AlphaScreen*

We next tested whether our assay system is applicable for drug screening targeting XMVR PR. As an initial approach, we examined the susceptibility of XMRV PR to six HIV-1 PIs: SQV (saquinavir), APV (amprenavir), IDV (indinavir), NFV (nelfinavir), DRV (darunavir) and LPV (lopinavir). Although all HIV-1 PIs tested showed marked inhibitory effects on HIV-1 PR, only two of them, APV and DRV, were found to block the activity of XMRV PR at the 1 μM concentration (Fig. 3A). This was also confirmed by IB of the blockade of cleavage of the reporter polypeptide containing the CA-NC junction (Fig. 3B). We next determined the IC_{50} value by titration of PIs (Fig. 3C). For XMRV PR, the IC_{50} values for APV, DRV, IDV and LPV were 0.2 μM, 1.0 μM, 60 μM and 17 μM, respectively. We next delineate the sensitivity of XMRV PR to APV in comparison with HIV-1 PR. Parallel experiment using recombinant HIV-1 PR and XMRV PR proteins revealed that IC_{50} values for APV was 34.7 nM in HIV PR and 200 nM in XMRV PR, respectively (Fig. 3D). These results indicate that this assay system can provide a tool to screen for selective PR inhibitors.

Retroviruses often exhibit drug resistant properties against anti-retrovirals due to their highly frequent genomic mutation. We next asked whether our assay system is useful for investigating drug-resistant properties of XMRV PR. To predict the sites of interaction between APV and XMRV PR, we modeled the three-dimensional (3D) complex of XMRV PR bound to APV. A recently published report on the crystal structure of XMRV PR shows that XMRV PR has a structural topology similar to that of HIV-1 PR [24]. Thus, we constructed our 3D structural model of the XMRV PR-APV complex by homology modeling, using the X-ray crystal structure of the HIV-1 PR-APV complex as a starting template (Fig. 4A). The constructed model indicates that APV interacts with aspartate Asp32 of the catalytic domain of XMRV PR, and also contacts the residues Val39, Lys61, Tyr90, and Leu92. Moreover, a water molecule would intermediate interactions between APV and Ala57 of the PR. A sequence alignment of PRs between XMRV and HIV-1 shows that the Val39, Ala57, Lys61, Tyr90, and Leu92 in XMRV PR are corresponding to the Val32, Ile50, Ile54, Val82, and Ile84 in HIV-1 PR, respectively (Fig.4B). These residues in HIV-1 PR are reported to be important for interactions between HIV-1 PR and APV and associated with viral resistance against APV [25].

We then created selected site-directed mutants (V39I, K61L, A57V, V39I/A57V, Y90A/L92V) and investigated the catalytic activity and drug-resistant properties of these mutants to APV (Fig. 4C). As shown in Fig. 4C, V39I and A57V substitutions resulted in significantly ($P<0.01$) (Fig. 4C) higher drug resistance as compared with wild-type PR. This effect resulted in the 2.8-fold drug-resistance based on IC_{50} value (Fig.4D). These results indicate that our current assay system can predict drug-susceptibility of mutated proteases and may be useful for drug development targeting XMRV PR.

3.4. *Identification of human proteins cleaved by XMRV PR*

As it is known that viral proteases can cleave cellular proteins [26–28], we hypothesized that XMRV PR might be capable of digesting human proteins. As a representative demonstration

we selected twenty-four tumor suppressor proteins and synthesized them with N-terminal FLAG and C-terminal biotin tags by wheat cell-free system. These tester proteins were then incubated with XMRV PR followed by 2-color immunoblot analysis (Fig.5B). The result revealed that XMRV PR, but not DHFR as a negative control, can digest 4/24 tumor suppressor proteins examined: BAX, PTEN, DKK3 and ARL11 (Fig. 5C). Cleavage of the tumor suppressor proteins by XMRV PR was clearly inhibited by APV (Fig. 5D). For BAX and PTEN, the cleavage sites by XMRV PR were determined by peptide sequencing of the C-terminal cleavage products (Fig. 5E). The cleavage sites were found to be located in functional domains of both proteins, suggesting that proteolytic digestion by XMRV PR may diminish the native function of these tumor suppressor proteins by proteolytic digestion.

Since XMRV PR and HIV-1 PR have some similarity and can both be inhibited by APV, it is highly possible that XMRV PR can cleave the same substrates as HIV-1 PR. To this end, we tested whether XMRV PR could cleave two reported HIV-1 PR substrates, caspase-8 and NDR2 [28,29]. Interestingly, XMRV PR was found to digest caspase-8 although the cleavage site was distinct from that of HIV-1 PR. In contrast, XMRV PR was not able digest NDR2. Conversely, HIV-1 PR did not cleave Bax whereas XMRV PR can cleave it. Furthermore, both proteases could not cleave p53. These results indicate that there is certain substrate specificity of retroviral proteases toward host proteins (Fig. 6).

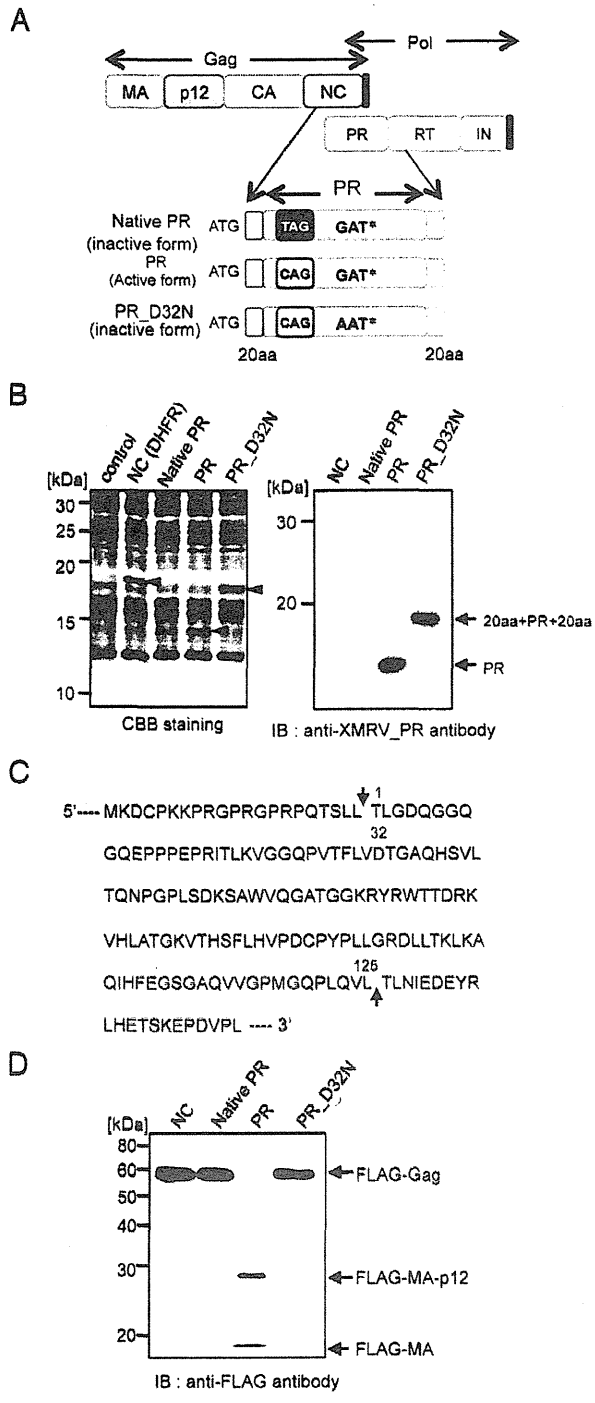


Fig. 1 – Synthesis of enzymatically active XMRV protease (PR) by wheat cell-free protein production system. A. Construction of expression vector of XMRV PR for wheat cell-free synthesis. TAG (stop) codon between Gag and PR was substituted for CAG (Q) codon. Non-active form of PR was generated by the substitution of AAT (N) for catalytic center GAT (D). B. XMRV PRs (DHFR as a negative control) were separated by SDS-PAGE followed by CBB-stained (left panel) and immunoblotting using anti-XMRV PR antibody (right). The arrows depict protein products. C. Amino acid sequence of XMRV PR. The arrows indicate self-cleavage site in XMRV PR. D. Cleavage of XMRV Gag by XMRV PR produced by wheat cell-free system. XMRV PR was incubated with cell-free synthesized FLAG-tagged XMRV Cag (arrow), and the cleaved Gag was detected by immunoblot analysis with anti-FLAG antibody.

4. Discussion

In the current study, we developed a cell free protease assay with XMRV PR which can evaluate the cleavage activity via AlphaScreen or immunoblot analysis. We demonstrate the advantage of utilizing wheat cell-free system that was able to systematically produce catalytically active viral protease with a large amount for biochemical assays. Furthermore, our in vitro enzymatic assay revealed that APV is a potent inhibitor of XMRV PR. We have also delineated the physical interaction between APV and XMRV PR and identified the amino acid residues involved in the binding. Finally, we demonstrated the substrate specificity for XMRV PR as compared with HIV-1 PR. These results might reveal that our current assay system is a powerful tool to characterize viral proteases and to screen their specific inhibitors.

XMRV is a virus that was generated as the result of a unique recombination event between two endogenous MLV-like viruses in a nude mouse carrying the CWR22 prostate cancer xenograft [6]. Although XMRV is an unusual virus, XMRV has been associated with prostate cancer [2]. In fact, the human cell line 22Rv1, which was established from a human prostate tumor (CWR22), produces infectious XMRV particles [30]. While the absence of XMRV in non-prostatic tumors

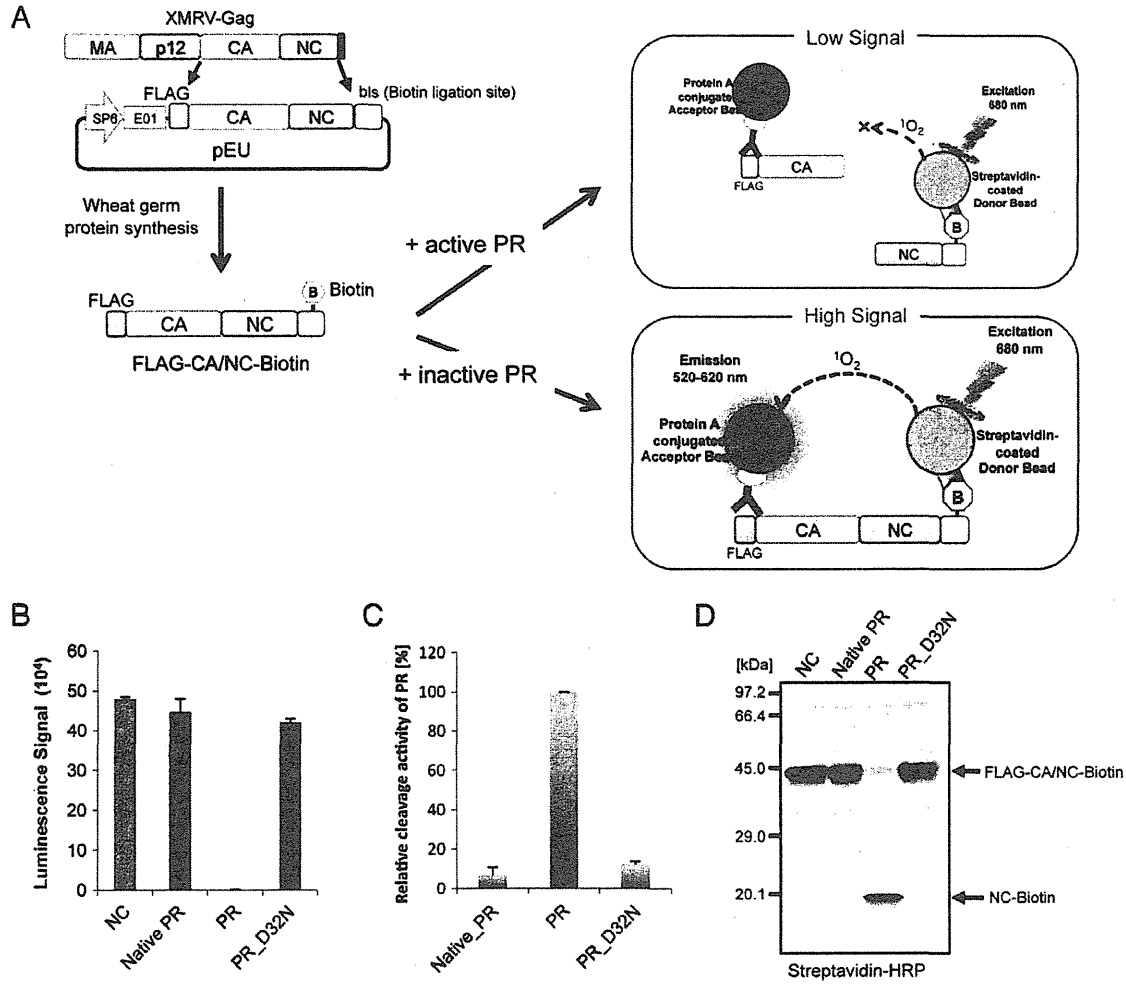


Fig. 2 – Development of a cleavage activity assay for XMRV PR using the luminescent assay AlphaScreen. A. Schematic diagram of the substrate construction of XMRV PR and detection system for the cleavage activity of XMRV PR by luminescent analysis. Substrate was designed as XMRV Gag capside (CA) and nucleocapside (NC) flanked by N-terminal FLAG and C-terminal biotin (FLAG_CA-NC_biotin). PR was incubated with the substrate, for 1 h at 37 °C. Subsequently, protein A-conjugated acceptor beads with anti-FLAG antibody and streptavidin coated donor beads were added and bound to the tagged substrate. Upon laser excitation, Donor beads convert ambient oxygen to a singlet oxygen. In the case of non-activity PR, singlet oxygen transfers across to activate Acceptor beads and subsequently emit light at 520–620 nm. In the case of active PR, no light is produced because the singlet oxygen can not transfer from Donor beads to Acceptor beads due to the distance (>200 nm). B,C,D. Cleavage activity of XMRV PR was quantitated by the luminescent assay (Fig.2B). Actual cleavage of XMRV Gag substrate was also confirmed by immunoblotting with streptavidin-HRP (Fig.2D). The arrow indicates the band for the non-cleaved substrates (FLAG_CA/NC_biotin).

remains controversial [31], XMRV can however proliferate in other human prostate cancer cells such as LNCaP or PC3 without severe cytopathic effects [32]. Such conditions of persistent infection without cell death could conceivably lead to prolonged exposure of host cell proteins to XMRV PR, increasing their susceptibility to cleavage with oncogenic consequences. The important question remains, however, as to whether this virus has indeed tumorigenic capability. Previous reports have indicated that XMRV integration is characterized by a strong preference for transcriptional start sites, CpG islands, and DNase-hypersensitive regions, all features that are frequently associated with structurally-open transcription regulatory regions of the chromosome in prostate cancer cells [33]. Integration of XMRV occurs preferentially in actively-transcribed genes and gene-dense regions within the chromosome [33]. Oncogenic properties of XMRV have been investigated in cell culture models. Although XMRV has been reported to lack direct transforming activity, the virus is able to induce low rates of transformation in cultured fibroblast cells [34]. Therefore, the molecular link between XMRV infection and cell transformation merits further investigation.

Our current data demonstrates that APV is a potent antagonist of XMRV PR. During the preparation of this manuscript, Li et al. reported the crystal structure of complexes

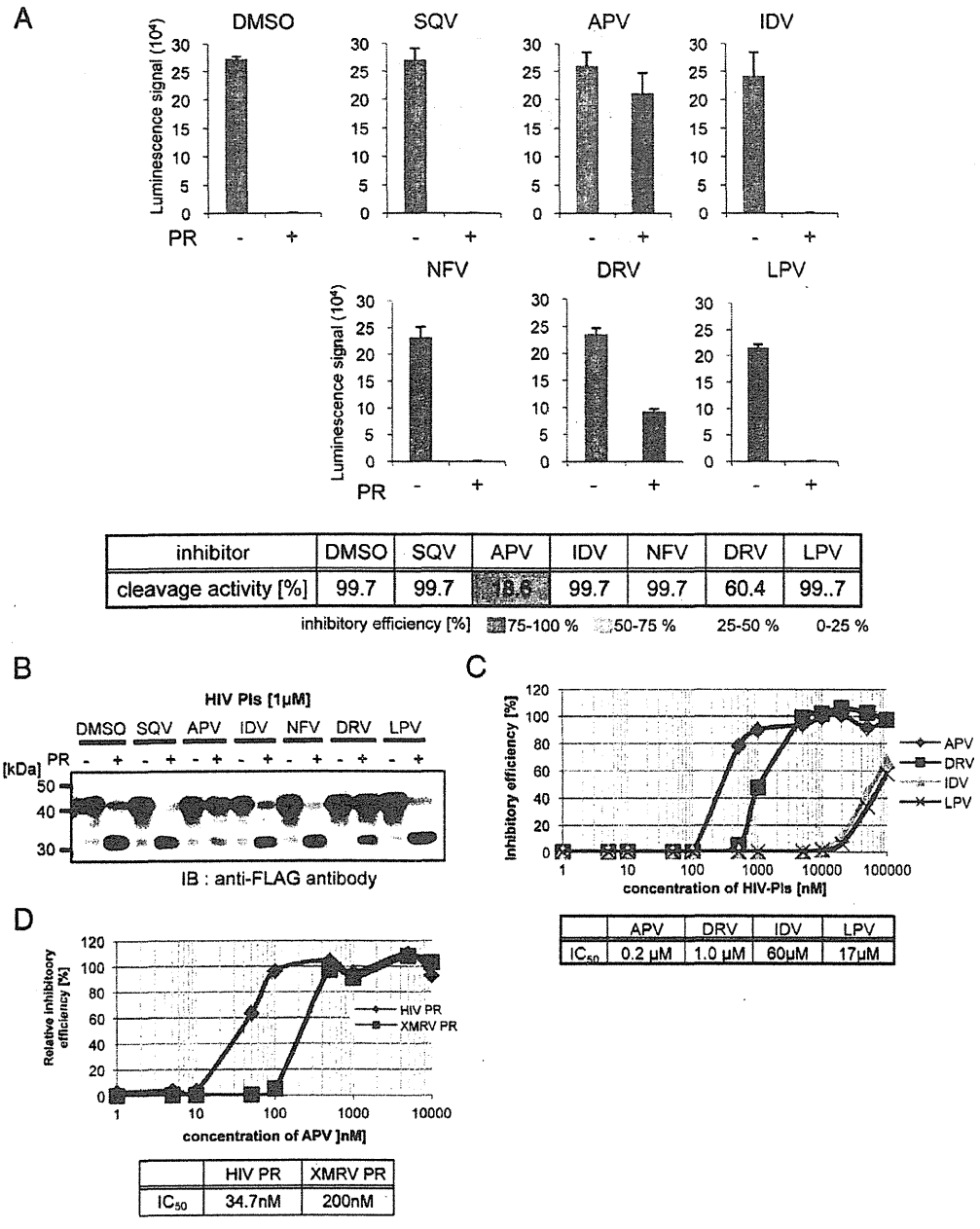
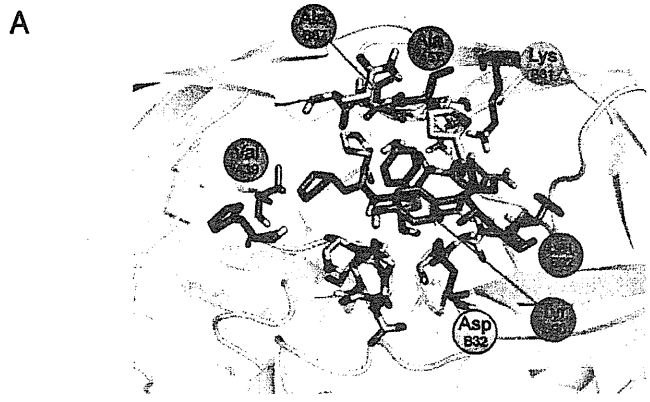


Fig. 3 – Drug screening for XMRV PR based on the cleavage activity. A,B. XMRV protease (+) or DHFR (–) was pre-incubated with indicated HIV PIs (SQV, saquinavir; APV, amprenavir; IDV, indinavir; NFV, nelfinavir ; DRV, darunavir; LPV, lopinavir; 1 μM each) and then subjected to AlphaScreen. Luminiscent AlphaScreen signal (upper panel) and relative enzymatic activity (lower panel) were listed. C. Conformation of the cleavage of the tester polypeptide by immunoblot analysis with anti-FLAG antibody. D. Dose–response curve of XMRV PR with HIV PIs using AlphaScreen (upper panel). IC_{50} values were calculated for each inhibitor (lower panel). E. Dose–response curve of XMRV PR and HIV-1 PR with APV using AlphaScreen (upper panel). IC_{50} values were calculated for each protease (lower panel).

formed between XRMV PR and several protease inhibitors, including APV [24,35]. In the current study we moved a step closer to clarifying the molecular interactions between XMRV PR and APV during drug-resistance, by developing an effective cell-free in vitro protease assay for XMRV PR. This assay revealed that an Ala57Val substitution induced significant drug-resistance to APV regardless of the integrity of the protease activity. The data indicates that this cell-free assay is useful for analyzing the drug-resistance properties of retroviral proteases.

Proteases often modify the activities of their target substrates [36]. Identification of the specific substrates cleaved by viral PR is of great significance for understanding the molecular etiology of virus infection. Proteomic studies with mass spectrometry could, theoretically, exhaustively identify the cellular proteins cleaved



B

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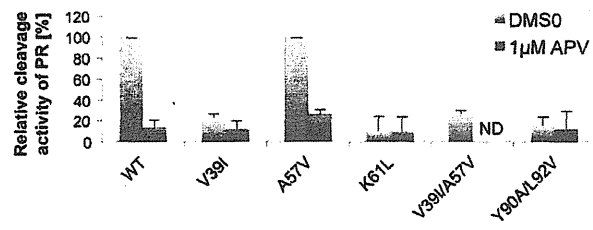
                10          20          30          40          50
XMRV  TLGDQGGQGQ EPPPEPRITL KVGGQPVTFL VDTGAQHSVL TQNPGPLSDK
HIV-1 -----PQITL W--QRPLVTI KIGGQLKEAL LDTGADDTVL EEMSLPGRWK
                5          13          23          33          43
               60          70          80          90         100
XMRV  SAWVQGATGG KRYRWTTDRK VHLATGKVTH SFLHVPDCPY PLLGRDLLTK
HIV-1 PKMIGGIGGF IKVRQYDQIL IEICGHKAIG TVL-VGPTPV NIIGRNLLTQ
               53          63          73          82          92
              110         120    125
XMRV  LKAQIHFEGS GAQVVGPMGQ PLQVL
HIV-1 IGCTLNF--- ---------- -----
           99
```

C

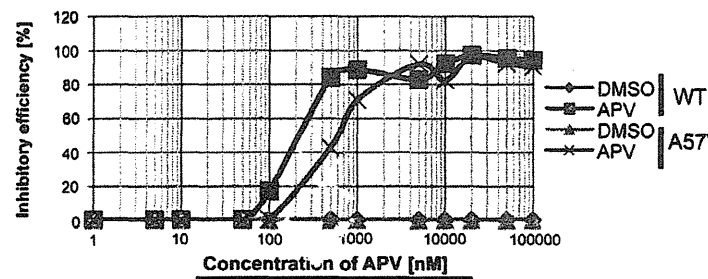


	WT	V39I	A57V	K61L	V39I/A57V	Y90A/L92V
cleavage activity [%]	99.7	23.5	99.7	10.3	27.0	15.6
inhibitory efficiency [%]	13.9	53.0	26.6		0.0	

cleavage activity [%] 75-100 % 50-75 % 25-50 % 0-25 %

inhibitory efficiency [%] 75-100 % 50-75 % 25-50 % 0-25 %

D



	WT	A57V
IC_{50}	200 nM	596 nM

Fig. 4 – Prediction of the amino acid residues of XMRV PR interacting with APV. A. The predicted 3D-structure for the interaction between XMRV PR and APV. This homology modeling was based on the HIV-1 PR and APV complex as a template. B. Sequence alignment of XMRV PR and HIV-1 PR. The amino acids related to interaction of APV with HIV-1 PR and the corresponding amino acids in XMRV PR are highlighted with red letters. C. Cleavage activity of XMRV PR-WT and its mutants in the presence of 1 μM APV or equivalent amount of DMSO (control). Lower panel is cleavage activity and inhibitory efficiency (APV value/DMSO value) for each XMRV PR. D. Dose–response curve of the inhibitory rate of PR-WT or PR-A57V by APV.

A

No.	Symbol	Gene bank accession No.	aa	Da	Cleavage by XMRV PR
1	ANXA1	BC001275	346	38714	No
2	GJB2	BC017048	226	26215	No
3	ARL11	BC013150	196	21391	Yes
4	RND3	BC012513	244	27368	No
5	FHIT	BC032336	147	16858	No
6	KCNRG	BC032343	272	31048	No
7	SFRP1	BC036503	314	35386	No
8	WNT5A	BC064694	380	42339	No
9	SMAD3	BC050743	425	48081	No
10	p21	BC000312	164	18119	No
11	NME1	BC000293	152	17149	No
12	MAL	BC000458	153	16714	No
13	SPINT2	BC001668	252	28228	No
14	SLC39A1	BC003152	324	34250	No
15	CHEK2	BC004207	543	60915	No
16	DKK3	BC007660	350	38291	Yes
17	TIMP3	BC014277	211	24145	No
18	BAX	BC014175	192	21184	Yes
19	ALPL	BC021289	524	57305	No
20	CST6	BC031334	149	16510	No
21	KLF5	BC042131	457	50792	No
22	CAV1	BC082246	178	20472	No
23	PTEN	BC005821	403	47166	Yes
24	TP53	BC003596	393	43653	No

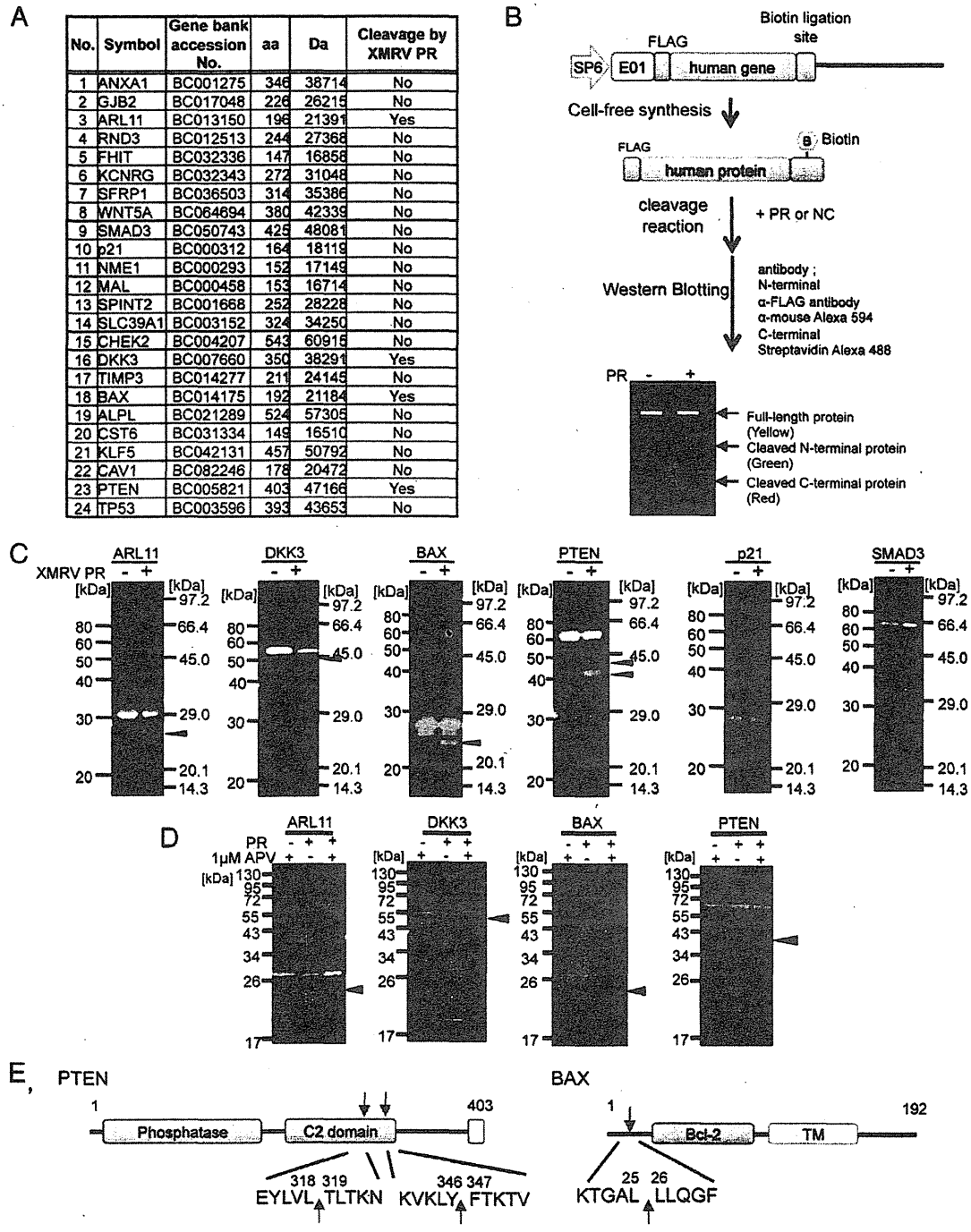


Fig. 5 – Screening of host proteins cleaved by XMRV PR in vitro. A. The list of human tumor suppressor proteins tested in this study. B. Scheme of the tester proteins construction and the cleavage assay system by immunoblotting. The genes were amplified by PCR with primer sets containing either FLAG or biotin ligation site (bls) in the flanking sequence, respectively. The recombinant host proteins flanking FLAG and biotin (FLAG-X-biotin) were incubated with XRMV PR at 37 °C for 2 h followed by SDS-PAGE. The proteins were detected using anti-FLAG-Alexa592 antibody (green) and Alexa488-conjugated streptavidin (red). Full-length protein is seen as a yellow band. C. Tester proteins were treated with XMRV PR or carrier. 2-color immunoblot analysis was performed as in Materials and methods. D. Tester proteins were treated with XMRV PR in the absence or presence of amprenavir. Immunoblot analysis was performed as in C. E. Identification of the cleavage site in the XMRV PR amino acid sequence.

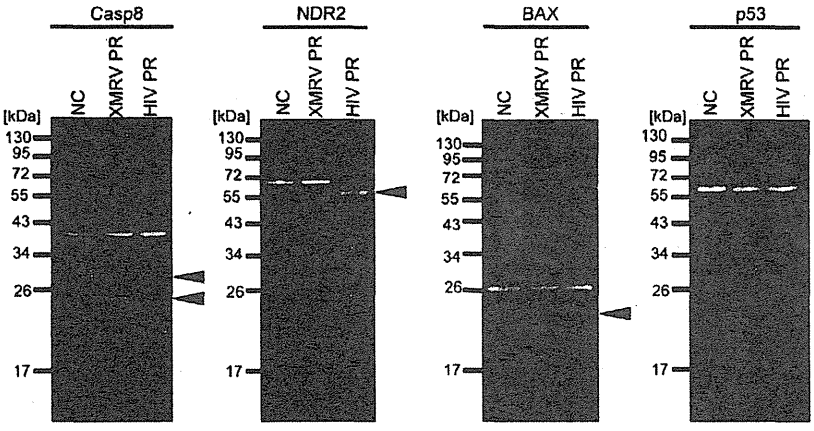


Fig. 6 – Comparative analysis of host proteins cleaved by XMRV PR and HIV-1 PR. The recombinant host proteins flanking FLAG and biotin (FLAG-X-biotin) were incubated with either XRMV PR or HIV-1 PR at 37 °C for 2 h followed by SDS-PAGE. The proteins were detected using anti-FLAG-Alexa592 antibody (green) and Alexa488-conjugated streptavidin (red). Full-length protein is seen as a yellow band. Arrows depict the cleavage products.

by retroviral proteases in infected cells. However, this cell-based will run into difficulty identifying individual substrates if several host proteases act simultaneously on the substrate. To circumvent this potential problem we developed the cell-free in vitro method for the identification of substrates cleavable by XMRV PR. Wheat extracts purified rarely include endogenous proteases that can interfere with the proteolytic reaction, making them suitable for the cell-free protease assay.

Tumor suppressor proteins play a major role in preventing tumor initiation. Our current results demonstrate that XMRV PR can cleave PTEN and BAX tumor suppressors as well as the intrinsic substrate XMRV Gag. It has been reported that the C-terminal region of PTEN is important for the protein's stability, and the C-terminal deletion mutant is degraded rapidly in cells [37]. Since XMRV cleaves within the C-terminal region, the native function and stability of PTEN might be abrogated by XMRV infection. The N-terminal region of BAX has been demonstrated to mediate its activity in apoptosis [38]. We demonstrated in the present study that XMRV PR can cleave the N-terminal region of BAX, suggesting that XMRV infection might affect the activity of BAX protein.

A biochemical approach to the evaluation of PR-inhibitor susceptibility has been attempted previously using several related methods [39,40]. The essence of each of these procedures is the synthesis of catalytically-active PR and substrate peptide and inhibitor in vitro, and measurement of the amount of substrate cleavage. The advantage of this approach is that it can directly detect the catalytic activity of PR. However, it is often difficult to produce sufficient quantities of enzymatically active viral PR in conventional cell-based protein expression systems such as E. coli or insect cells. In our current study, we successfully created catalytically-active XMRV PR in a cell-free system that, when mixed with a reporter substrate flanked with N- and C-terminal fluorophores, substrate cleavage could be assayed by AlphaScreen or 2-color IB. This approach directly evaluates the cleavage activity of the PR and, in addition, cleavage sites can be estimated by the size of cleavage products. The current availability of full-length cDNA libraries, derived from higher eukaryotes, will facilitate the in vitro synthesis of full-length proteins, making this cell-free system approach could further be applicable to the assay of a broad range of, not only viral, but also host proteases.

5. Conclusion

We have delineated the molecular and enzymatic characteristics of XMRV PR by utilizing wheat-germ cell-free protein synthesis and AlphaScreen. Furthermore, we have developed an in vitro cleavage assay for drug screening based on the enzymatic activity. Our results suggest that XMRV-protease cleavage of certain host proteins and inhibited by APV. Further in vivo studies with XMRV-infected cells will be necessary to confirm a molecular link between XMRV and human diseases.

Acknowledgments

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Pinning down viral proteins: a new prototype for virus–host cell interaction

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Pin1 is an enzyme that specifically catalyzes the *cis–trans* isomerization of phosphorylated serine/threonine-proline (pSer/Thr-Pro) motif in its substrate proteins. Recent studies demonstrate that stability of several viral proteins is regulated by phosphorylation-dependent prolyl-isomerization by a host factor Pin1. Pin1 is now positioned as an important modulator of the molecular crosstalk between virus and host cells and could be a unique target for anti-virus therapy. This new type of post-translational modification by Pin1 might be involved in the regulation of other viral proteins.

Keywords: phosphorylation, prolyl-isomerization, protein stability, Pin1

Post-translational modifications of proteins are major regulatory events in many cellular processes such as cell proliferation, differentiation, and cell death. In particular, protein phosphorylation is a major mode of post-translational modifications and an important regulatory event for many cellular processes by modulating intracellular signaling pathways (Hunter, 1995). It has been reported that phosphorylated proteins are subsequently subjected to a new type of "post-phosphorylation" regulation by a peptidylprolyl isomerase Pin1. Pin1 is a peptidylprolyl *cis–trans* isomerase and only binds to serine or threonine residue immediately preceding a proline residue (Ser/Thr-Pro). After binding to the motif, Pin1 dynamically changes the conformation of target proteins via *cis–trans* isomerization of the peptide bonds. Such conformational changes have profound effects on the function of substrate proteins by modulating their catalytic activity, protein–protein interaction, sub-cellular localization, and protein stability (Ryo et al., 2003; Lu et al., 2007). With the diverse physiological roles of Pin1, it has been shown that Pin1 is linked to the etiology of several diseases that include cancers, Alzheimer's disease and immune diseases (Lu and Zhou, 2007). In addition to this, recent studies demonstrate that the stability and function of several viral proteins are also regulated by phosphorylation-dependent Pin1-mediated prolyl-isomerization (**Figure 1**).

Pang et al. (2007) firstly identified Pin1 as a novel binding partner for the hepatitis B virus X protein (HBx), a viral encoding oncoprotein. The interaction appears to have significant effects on the stability and pro-tumorigenic activity of the viral protein (Pang et al., 2007). Pin1 overexpression was found to be related to HBx expression in HBV-related tumors. Pang et al. (2007) confirmed that Pin1 binds HBx at the specific phosphorylated the Ser41-Pro motif. This interaction was shown to be inhibited by the mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK) inhibitor, suggesting the possible role of mitogen-activated kinase (MAPK) family in the phosphorylation of the Ser41-Pro motif. Pin1 overexpression was shown to increase the protein stability of HBx as well as HBx-mediated transactivation. Concomitant expression of Pin1 and HBx in the non-tumorigenic human hepatocyte cell line MIHA led to a synergistic increase in tumor growth. Moreover, in hepatocellular carcinoma Hep3B cells with suppressed Pin1 expression, HBx-mediated tumor growth in nude mice was abrogated. These results together indicate that Pin1 enhance hepatocarcinogenesis in HBV-infected hepatocytes by activating both stability and function of HBx.

The second target for Pin1–viral protein interaction is HTLV-1 Tax. Two groups have reported the functional interaction between HTLV-1 Tax oncoprotein and Pin1 (Jeong et al., 2009; Peloponese Jr. et al., 2009). Pin1 is highly expressed in adult T cell leukemia (ATL) cells expressing Tax protein and forced expression of Pin1 in turn increases the Tax protein expression. Pin1 prolonged the protein stability of Tax by suppressing the ubiquitination and subsequent lysosomal degradation of Tax. Pin1 interacts with phosphorylated Tax on its Ser160-Pro motif. On the other hand, a Pin1 inhibitor Juglone suppressed cell proliferation of the Tax-expressing T cell line. Thus, Pin1 plays a supporting role in Tax-mediated cell transformation in the post-translational regulation of Tax. The targeting of Pin1 may offer a new insight into the pathogenesis of HTLV-1 related diseases such as ATL.

A recent study has demonstrated that HIV integrase (IN) is a new target for Pin1. Indeed, the protein stability HIV IN was found to be regulated by phosphorylation-dependent Pin1-catalyzed prolyl-isomerization (Manganaro et al., 2010). Exogenously transfected HIV IN can associate with Pin1. Furthermore, such interaction is dependent on phosphorylation of HIV IN, specifically on the Ser57-Pro motif, which can be phosphorylated by host kinase c-Jun N-terminal kinase (JNK). Importantly, this interaction has indeed profound functional significance. HIV IN is a protein with a short half-life, but its steady-state levels were shown to significantly

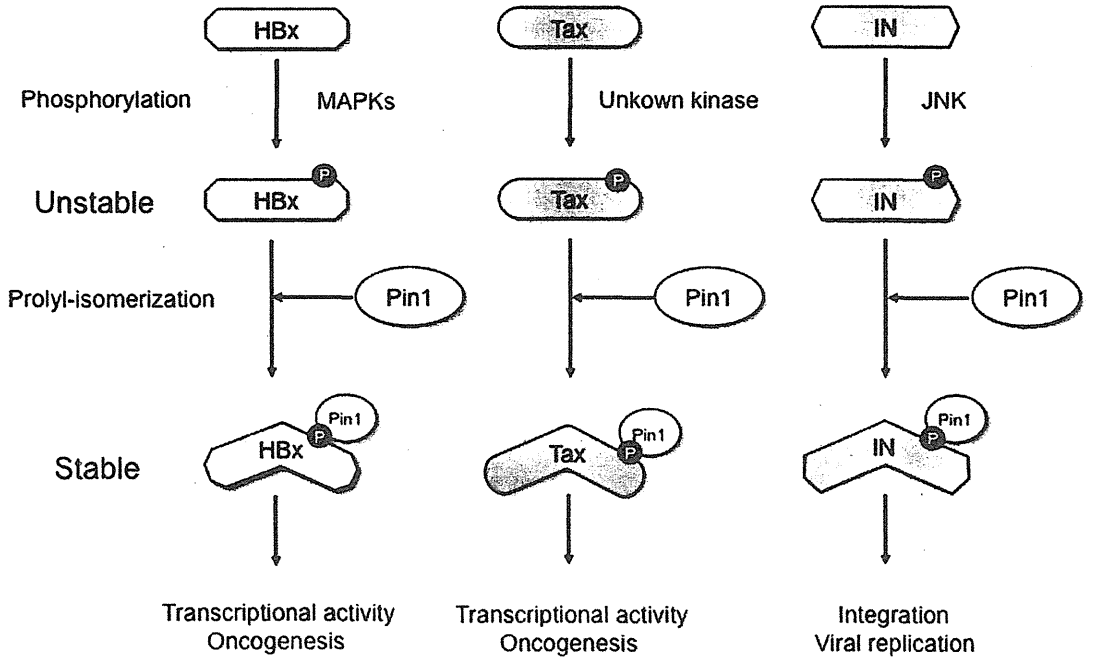

FIGURE 1 | A novel post-phosphorylation regulatory mechanism for viral protein stability. The phosphorylation of viral proteins (HBV HBx, HTLV-1 Tax, and HIV IN) by the proline-directed kinases (JNK, MAPK etc.) generates binding modules for Pin1. Subsequent prolyl-isomerization by Pin1 induces conformational changes and thereby enhances the viral protein stability.

increase during Pin1 interaction. In addition to regulating protein stability, Pin1 can concomitantly enhance the activity for HIV IN thereby facilitating the HIV-1 proviral integration into the host cell genome. These concerted activities of Pin1-dependent prolyl-isomerization can in turn lead to efficient HIV-1 replication. Since the lack of these modifications restricts viral infection, Pin1 could be an intriguing target for anti-HIV therapy.

In conclusion, these observations are of special relevance to Pin1 for the natural course of viral replication via modulating the stability and function of viral proteins. Because it is quite likely that other viral proteins in different viral species could also be Pin1 targets, future studies for Pin1–viral protein interaction would shed new light on molecular etiology of virus–host cell interaction. Furthermore, these studies may also have therapeutic implications of Pin1 inhibition in viral infection. With specific Pin1 inhibitors being developed, the application of such molecules in combination with current antiviral therapies could change the course of viral replication and prevent the development of viral pathogenicities.

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