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Moloney murine leukemia virus integrase and reverse transcriptase interact with PML proteins

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Pull-down assay and co-immunoprecipitation of cell extracts in which the integrase or reverse transcriptase of Moloney murine leukemia virus was transiently expressed showed that both enzymes interacted with PML proteins. In infected cells, interaction between the integrase and PML was also observed. Transient expression of PIASy and SUMO proteins facilitated SUMOylation of the integrase but had no apparent effects on the interaction with PML. A FLAG-tagged integrase co-localized with PML protein possibly in the PML body. Knockdown of PML by small interfering RNA resulted in reduced viral cDNA levels and integration efficiency. This suggested that PML proteins activated reverse transcription.

Keywords: integrase/PML/retrovirus/reverse transcriptase.

Abbreviations: IN, integrase; MoMLV, Moloney murine leukemia virus; PIAS, protein inhibitor of activated STAT; PIC, pre-integration complex; PML, promyelocytic leukemia; RT, reverse transcriptase; RTC, reverse transcription complex; SUMO, small ubiquitin-related modifier.

In the early stage of the retroviral life cycle, a reverse transcription complex (RTC) is assembled after the entry of retroviral particles into cells, and viral complementary DNA (cDNA) is synthesized. The RTC contains several viral proteins, such as integrase (IN), reverse transcriptase (RT) and capsid in Moloney murine leukemia virus (MoMLV) (*1*). Newly synthesized viral cDNA, IN and various viral and cellular proteins then form preintegration complex (PIC) and the complex is imported into the nucleus and viral double-stranded cDNA is integrated into the host chromosome (*1*). The precise mechanism by which the RTC transforms into PIC remains to be elucidated (*2*), although detailed analyses of the mechanism of integration have been conducted (*3*, *4*). PIC associates with various cellular proteins depending on virus type. For instance, PIC of human immunodeficiency virus type 1 (HIV-1) contains lens epithelium-derived growth factor (LEDGF/p75) (*5*, *6*) and promyelocytic leukemia (PML) protein (*2*, *7*). MoMLV PIC contains lamina-associated polypeptide 2α (Lap2α) (*8*) and possibly the transcription factor Ying Yang 1 (YY1) (*9*). It is well established that IN itself physically interacts with various proteins (*10*).

The PML body is a distinct structure mainly observed in the nucleus, and PML proteins are key organizers of the body proposed to regulate a variety of cellular processes, including the sequestration, activation or degradation of partner proteins (*11–13*). The PML body recruits an ever-increasing number of proteins, and many of the proteins found in the PML body are modified by small ubiquitin-related modifier (SUMO) (*14*). It has been supposed that non-covalent interaction between the SUMO moiety and SUMO-interacting motif (SIM) is crucial to the formation of the PML body and partly contributes to the recruitment of partner proteins to the body (*11–13*). Recently, SUMOylation of HIV-1 IN has been reported but the physiological importance of the modification has not been clear (*15*). In the present article, we also found the SUMOylation of MoMLV IN and that IN and RT interacted with PML proteins. We describe the physiological importance of the interaction between these viral proteins and PML proteins.

Materials and Methods

Cell culture

In this study, we used five cell lines including NIH3T3, HeLa, clone no. 4 (*16*), 293FT and GP293. The former three cell lines were cultured at 37°C in atmosphere containing 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS). The latter two cell lines were cultured in DMEM supplemented with 10% FCS, 1 mM sodium pyruvate and Non-Essential Amino Acids (Invitrogen).

Vector construction

phCMV2 (Genlantis) was used as an expression vector for FLAG-tagged (phCMV2/FLAG-MLV IN) or HA-tagged (phCMV2/HA-MLV IN) MoMLV IN. As a template for PCR, pFLAG/MLV IN was used (*9*). For the construction of FLAG-tagged RT, the RT sequence was amplified by PCR with the pGP plasmid in a retrovirus packaging kit (Takara) as the template, and 5′-AAAGAATTCATGACCCTAAATATAGAAGATGAGCATC GGCTA-3′ and 5′-AAAAAGCTTGAGGAGGGTAGAGGTGTC TGGAGT-3′ as primers. The amplified DNA was cloned in pETBlue-2 (Novagen), and then re-cloned into pcDNA4/HisC derivative containing the FLAG tag (Invitrogen). SUMO-1, SUMO-2

and SUMO-3 sequences were amplified by PCR using cDNA from 293FT cells as the template and the following primers, SUMO-1: 5′-CATGGATCCTCTGACCAGGAGGCAAAACC-3′, 5′-CATCTCGAGCTAAACTGTTGAATGACCCC-3′; SUMO-2: 5′-CATGGATCCGCCGACGAAAAGCCCAAGGA-3′, 5′-CATCTCGAGCTGGAGTAAAGAAGCAGGTTC-3′; SUMO-3: 5′-CATGGATCCTCCGAGGGGAGAAGCCCAAGGA-3′, 5′-CATCTCGAGCTAGAAACTGTGCCCTGCC-3′, (underlines show a *Bam*HI or *Xho*I site). The amplified DNA was ligated to the HA-tag sequence and cloned to pcDNA4/His.

For the construction of the PML VI-expression vector, PML VI cDNA was amplified from cDNA of NIH3T3 cells using 5′-CATGGATCCGAAACTGAACCAGTTTCCGTG-3′ and 5′-CATGCGGCCGCCTGTTTCCCCTGTGTCACT-3′ (*Bam*HI and *Not*I sites are underlined). The amplified DNA was introduced into pcDNA4/His C and pGEX-6P-2 (GE Healthcare). The FLAG-tagged (pFLAG/mPIASy) and non-tagged (pcDNA4/mPIASy) PIASy-expression plasmids were constructed from pSPORT6/mPIASy (*17*).

Knockdown of PML

siRNA: 5′-AAUUCCUCCUGUAUGGCUUGCUCUG-3′ was used. NIH3T3 cells (4.0×10^4 per 35-mm dish) were cultured for 24 h, then siRNA was introduced at a final concentration of 80 nM by Lipofectamine 2000^{TM} (Invitrogen). After incubation for another 24 h, siRNA was transfected again. Cells were then cultured for 24 h and infected with a MoMLV-based viral vector pQEGFP (*9*). DNA was isolated by using a QIAamp DNA mini kit (Qiagen), and viral cDNA and two-LTR circle viral cDNA were quantified by real-time PCR, and integrated cDNA was determined by nested PCR as reported (*9*). Rescue experiments were performed by simultaneously introducing the PML VI-expression vector and siRNA.

Immunoprecipitation

To investigate the interaction of endogenous PML with MoMLV IN and RT, the expression plasmid for FLAG-tagged MoMLV IN or FLAG-tagged MoMLV RT was transfected into 293FT cells using Lipofectamine 2000^{TM}. Cells were then harvested between 36 and 48 h post-transfection, and lysed in IP lysis buffer [50 mM Tris–HCl (pH 7.4), 1 mM EDTA, 150 mM NaCl, 0.5% sodium deoxycholate and 1% NP-40]. After the cell lysates had been incubated with ANTI-FLAG M2 affinity gel (Sigma) for 4 h, the gel was washed twice with the lysis buffer. The gel was treated twice with 100 U/ml DNase I, 10 μg/ml RNase A and 200 U/ml Benzonase (a broad spectrum endonuclease from *Serratia marcescens*, Novagen) in 20 mM Tris–HCl (pH 7.3), 50 mM KCl and 40 mM $MgCl_2$ at 37°C for 10 min and washed twice with the lysis buffer. The samples were analysed by western blotting using anti-PML antibody (H-238). Anti-FLAG and anti-PML antibodies were purchased from Wako and Santa Cruz, respectively. For confirmation of the SUMOylation of IN, the FLAG-tagged IN expression plasmid was introduced into 293FT cells together with expression plasmids for HA-tagged SUMO-1, −2 or −3 and PIASy. Cells were lysed in 20 mM Tris–HCl (pH 7.4), 1 mM EDTA, 500 mM NaCl, 0.5% sodium deoxycholate, 0.5% SDS and 0.5% NP-40 (*18*), and the lysates were heated at 100°C for 5 min, then IN was immunoprecipitated with M2 affinity beads. SUMOylation of IN was estimated by western blotting using anti-HA (3F10) (Rosch).

To analyse whether IN and PML interact each other during virus infection, NIH3T3 cells and MoMLV-producer clone no. 4 (*16*) were cocultured in DMEM containing 10% FCS. NIH3T3 and the producer cells were inoculated at a ratio of 3:1. The co-cultured cells were lysed with the lysis buffer and supplied for immunoprecipitation using anti-MoMLV IN antiserum (*9*).

In vitro binding assay

To study whether IN or RT physically interacts with the PML protein, purified proteins were prepared. His-tagged IN and GST-tagged full-length, N-terminal, core catalytic and C-terminal portions of IN were produced in *E. coli* BL21(DE3) or BL21, and purified as described previously (*9*). *Escherichia coli* BL21 containing the GST-tagged PML VI-expression vector was grown in LB medium to an optical density at 600 nm of 0.5. Then 1 mM isopropyl β-D-thiogalactoside was added, and *E. coli* was cultured at 37°C for 3 h. The cells were suspended in phosphate buffered saline (PBS) containing 300 mM NaCl and disrupted by sonication. For the purification of the GST-tagged PML VI, glutathione-Sepharose 4B (GE healthcare) was used. After the purification, samples were dialysed against PBS containing 300 mM NaCl. FLAG-tagged RT and His-tagged PML VI were produced in 293FT cells. For the purification of these proteins, M2 affinity beads or TALON metal affinity resin (Clontech) was used under denaturing conditions [20 mM Tris–HCl (pH 7.4), 50 mM NaCl, 0.5% sodium deoxycholate, 0.5% NP-40 and 0.5% SDS] to remove associated proteins. After the purification, samples were dialysed against 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.5% sodium deoxycholate and 1% NP-40. GST-tagged MoMLV IN and its truncated forms (125–250 ng) were incubated with glutathione-Sepharose 4B in the lysis buffer. Cell lysates were then mixed with the immobilized GST-IN fusion proteins on the beads, and the mixture was allowed to stand for 4 h at 4°C. The beads were washed three times with the lysis buffer, treated with a mixture of Benzonase, DNase I and RNase A, and then mixed with SDS sample buffer [62.5 mM Tris–HCl (pH 6.8), 5% 2-mercaptoethanol, 2% SDS, 5% sucrose and 0.002% bromophenol blue]. The bound proteins underwent SDS–polyacrylamide gel electrophoresis (PAGE) followed by western blotting using anti-PML antibody. Pull-down assay using GST-tagged PML VI and His-tagged IN, and *in vitro* immunoprecipitation assays using His-tagged PML VI and FLAG-tagged RT were also performed.

Immunocytochemical analyses

Hela cells were transfected with the FLAG-tagged IN expression vector with or without HA-tagged SUMO-1 and PIASy expression vectors and cultured on coverslips. The cells were rinsed with cold PBS three times, fixed with freshly prepared 4% paraformaldehyde for 30 min at room temperature, washed three more times in PBS, and permeabilized with PBS containing 0.5% Triton X-100 for 30 min at room temperature. After another three washes with PBS, the cells were incubated with PBS containing Block Ace^{TM} powder (DS Pharmabiomedical) for 1 h at room temperature. The coverslips were rinsed with PBS three times and dipped in primary antibodies in PBS for 1 h at room temperature. The primary antibodies used were rabbit anti-PML and mouse anti-FLAG antibodies. After being rinsed with cold PBS three times, cells were incubated with goat anti-mouse IgG coupled to fluorescein isothiocyanate and goat anti-rabbit IgG coupled to tetramethylrhodamin-isothiocyanate (ZyMax^{TM}, Zymed Laboratories) for 30 min at room temperature. After three more washes with PBS, the coverslips were mounted onto glass slides with 90% glycerol and 10% PBS. Samples were analysed using a laser scanning microscope (Olympus FV300 and FV1000).

Results

Interaction between MoMLV IN and PML

To clarify the host–viral interaction with MoMLV, we screened cellular proteins that interact with IN as reported previously (*9*) and found that the PML protein interacted with MoMLV IN in transient expression and co-immunoprecipitation experiments. As shown in Fig. 1A, immunoprecipitation was performed with cells in which FLAG-tagged IN was transiently expressed. For PML, it is well known that a variety of carboxy-terminal domains generated by alternative splicing yield isoforms (*11*). Several endogenous PML isoforms such as PML I/II, III/IV, V, VI and VII were detected in the immunocomplex and PML VI gave the most intense band among them. These bands were estimated to be each isoform based on their molecular weight and a recent report describing human PML (*19*). However, several proteins detected in the immunocomplex could not be identified (marked by asterisks in Fig. 1A). Since SUMO-conjugation of the PML protein is crucial for the PML-body's formation, the blot was re-probed with anti-SUMO antibody. However, SUMOylated PML was not detected in the immunocomplex. Under the condition, SUMOylated IN was not detected either.

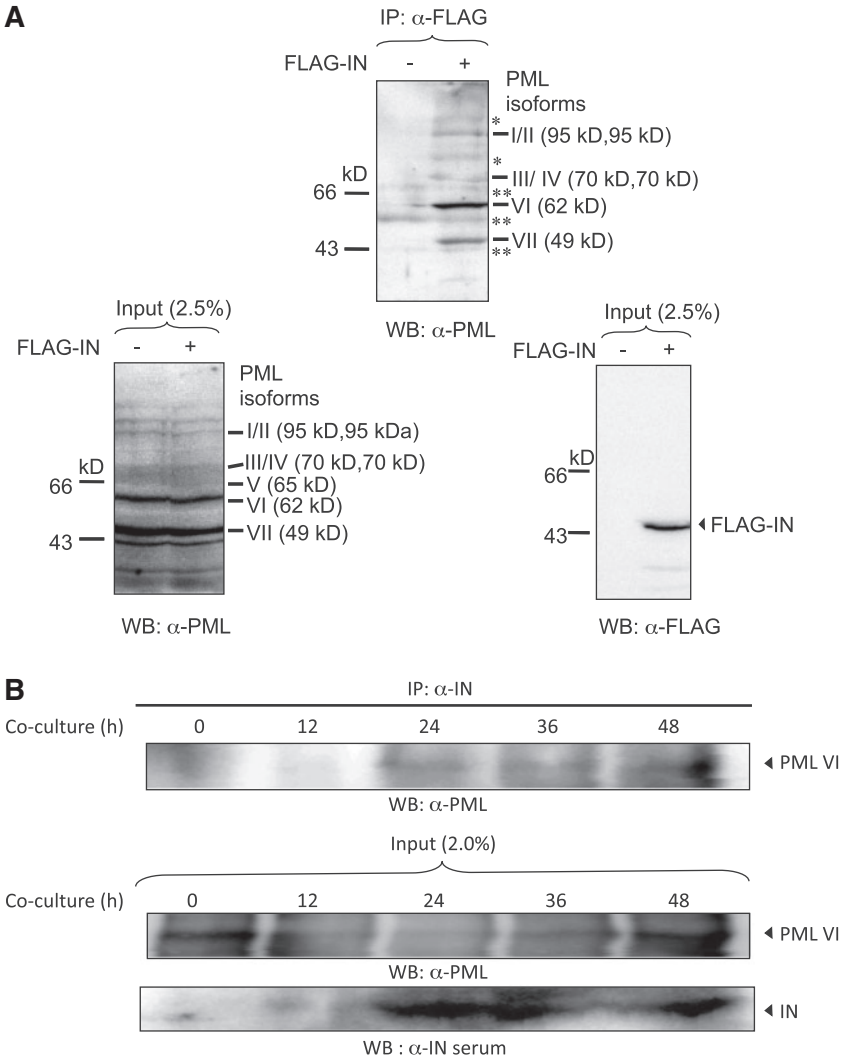

Fig. 1 PML interacts with MoMLV IN. (A) Results of co-immunoprecipitation of endogenous PML and FLAG-MoMLV IN expressed in 293FT cells (upper panel). A control without IN was included to estimate nonspecific interaction between PML and anti-FLAG antibody since the interaction between IN and some PML isoforms seemed to be weak. PML was detected with anti-PML antibody in PBS with 2% skim milk. This antibody was also used for detection of input PML isoforms (2.5%, lower left panel). FLAG-IN was detected with anti-FLAG antibody (lower right panel). PML isoforms were identified from their molecular mass and several unidentified bands (*) were detected. The non-specific bands possibly caused by the anti-FLAG antibody were also detected (**). A result representative of several experiments is presented. (B) Results of co-immunoprecipitation of endogenous PML and IN in MoMLV infected cells. Cell extracts were prepared from cocultures of NIH3T3 and clone no. 4 cells at 0, 12, 24, 36 and 48 h. IN complex was precipitated with anti-MoMLV IN antiserum and analysed with anti-PML antibody (upper panel). Each sample (2.0%) was used to confirm the viral IN and endogenous PML (lower panels). Immunoreaction enhancer solution (Can Get Signal, Toyobo) was used in the western analyses.

We then checked whether IN interacts with PML protein in the viral context. For this, MoMLV-producing cells were co-cultured with normal NIH3T3 cells (*9*) and the interaction was studied by co-immunoprecipitation. As shown in Fig. 1B, PML protein, possibly isoform VI judging from their molecular mass could be detected in the immunocomplex precipitated by anti-MoMLV IN antibody. We also could detect PML I/II isoforms in this experiment (data not shown). This result is reasonable since PIC contains PML (*2*, *7*).

SUMOylation of MoMLV IN

Generally, it has been reported that the binding between SUMO and SIM of PML protein is crucial for the PML-body's formation (*20*), and PML-body-associated proteins are recruited to the body through SUMO–SIM interaction or SUMO-independent interaction between PML and the partner proteins (*11–13*). Furthermore, it has recently been reported that HIV-1 IN is potentially SUMOylated (*15*). Therefore, we studied whether MoMLV IN is SUMOylated, although we did not detect a SUMO-conjugated form of IN in the transient expression and immunoprecipitation experiments shown in Fig. 1A. As shown in Fig. 2A, one of the SUMO-E3 ligases PIASy interacted with MoMLV IN and the IN protein was SUMOylated in the presence of exogenous SUMOs and PIASy (Fig. 2B). In the Fig. 2B, we performed immunoprecipitation under the denaturing condition. Bands marked by asterisks in lanes 9–11 of the middle left panel seemed to be consistent with

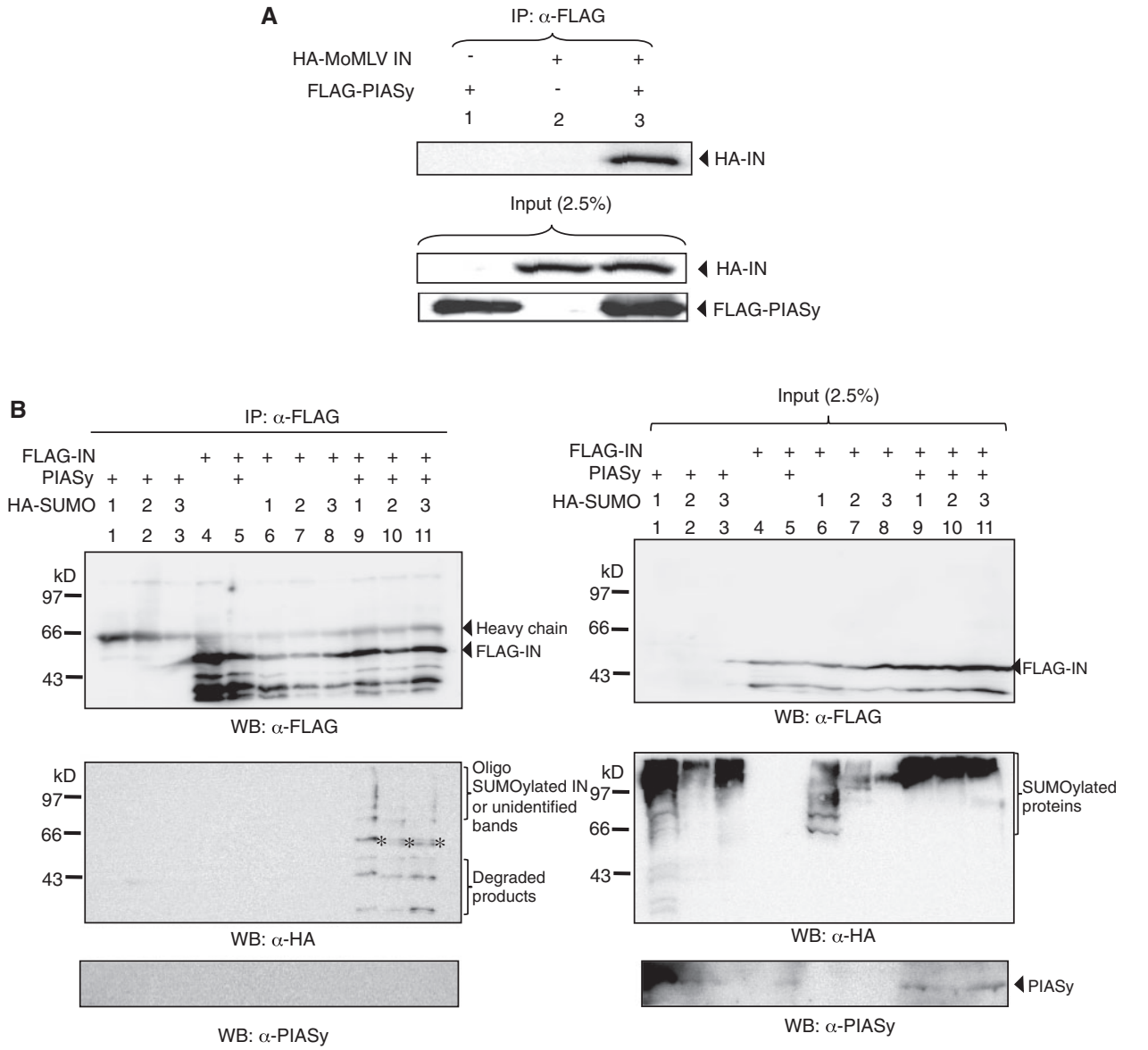

Fig. 2 PIASy is a SUMO E3 ligase of MoMLV IN. (A) Results of co-immunoprecipitation of FLAG-PIASy and HA-MoMLV IN expressed in 293FT cells (upper panel). HA-IN precipitated with anti-FLAG antibody was detected with anti-HA antibody after extensive washing with the lysis buffer. Each sample (2.5%) was used to confirm the expression of FLAG-PIASy or HA-IN (input, lower panels). A result representative of several experiments is presented. Can Get Signal was used in the western analyses. (B) PIASy stimulates SUMO conjugation of MoMLV IN in the cells. 293FT cells were co-transfected with plasmid encoding FLAG-IN and PIASy, and HA-SUMO-1, SUMO-2, or SUMO-3. Cells were lysed in the denaturing buffer and heated at 100°C for 5 min 48 h post-transfection followed by immunoprecipitation with anti-FLAG antibody. The SUMOylated IN proteins were analysed with anti-FLAG antibody and anti-HA antibody (left panels). Low molecular weight bands detected by anti-HA antibody may be due to degradation of SUMOylated IN. Each sample (2.5%) was used to confirm the expression of FLAG-IN, HA-SUMO-1, SUMO-2, or SUMO-3 and PIASy (input, right panels). FLAG-IN was detected with anti-FLAG antibody. HA-SUMO paralogues were detected with anti-HA antibody. PIASy was detected with anti-PIASy antibody. Can Get Signal was used in the western analyses.

mono-SUMOylated INs judging from their molecular weight (~57 kDa), although the bands could not be detected by anti-FLAG because of the presence of heavy chain (upper left). Putative oligo-SUMOylated bands were also detected by anti-HA antibody. Sometimes, SUMO-E3 ligase tightly bound to its substrate. However, under the condition, PIASy was not precipitated in the immunocomplex. Therefore, bands detected by anti-HA were not SUMOylated PIASy. Either SUMO-1, −2 or −3 seemed to be conjugated with IN. We also studied another E3 ligase, Pc2 (*21*), but could not detect any SUMO-conjugated IN (data not shown). Although we could not rule out the possibility that other E3 ligase catalyses the SUMOylation of IN, PIASy is a candidate for IN-E3 ligase.

IN physically interacts with PML

In Fig. 3A, GST pull-down was performed with *E. coli*-produced full-length, N-terminal, catalytic core and C-terminal portions of IN (*9*) and the PML protein produced by cultured cells. The results of the pull down indicated that PML VI mainly bound to IN, although the degradation of GST-IN was evident. This result did not rule out the possibility that other

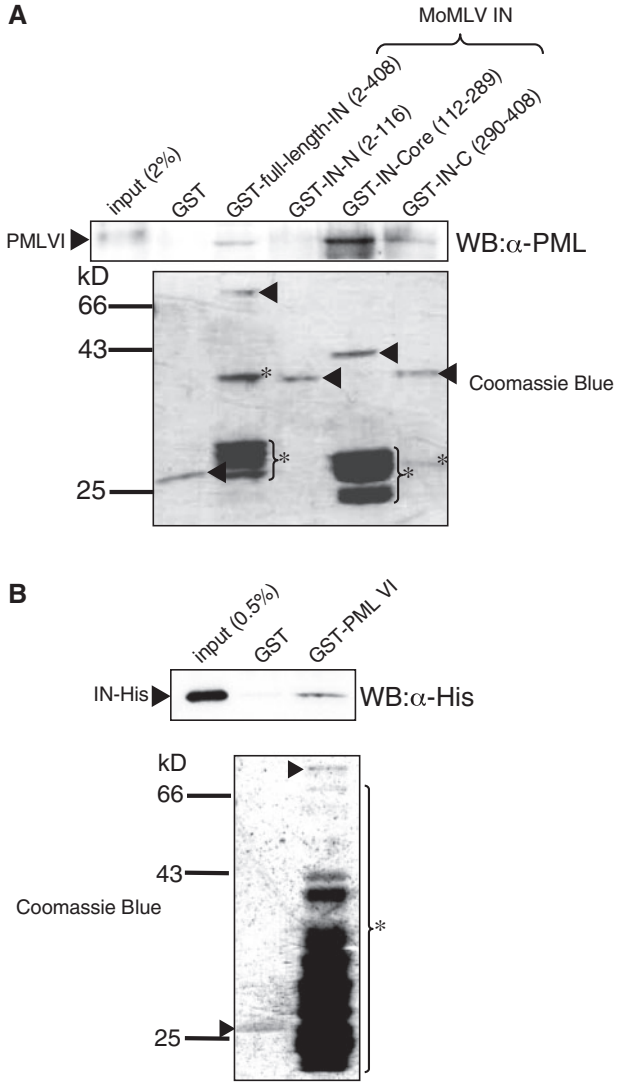

Fig. 3 *In vitro* binding assay for detection of the interaction between IN and PML. (A) Results of pull-down assay of GST-tagged full-length and portions of IN with extracts of 293FT cells. PML protein was detected by western blotting using the anti-PML antibody (upper panel). Can Get Signal was used in the western analysis. GST, GST-tagged full-length IN and GST-tagged portions of IN were subjected to SDS–PAGE, and the gel was stained with Coomassie brilliant blue (lower panel). Arrowheads show GST-INs and GST. Asterisks indicate probable degradation products of GST-IN. A result representative of several experiments was presented. (B) Physical interaction between MoMLV IN and purified PML. GST-PML VI and purified IN-His were mixed and pull-down was performed. GST-PML VI was confirmed with Coomassie brilliant blue. Arrowheads show GST-PML VI and GST. Asterisk indicates probable degradation products of GST-PML VI. Pull-down product was detected with anti-His antibody in PBS containing 2% skim milk. A result representative of several experiments is presented.

isoforms of PML bound to IN because of the low detection level of protein under the conditions. In fact, with a longer exposure, we could detect other PML isoform, possibly PML I/II. Then, we re-probed the blot with anti-SUMO-1 antibody but could not detect SUMO modification. We also tried pull-down assays using cell extracts from SUMO-1 knockdown cells and cells that transiently expressed SUMO-1 and PIASy. However, obvious difference of binding between IN and PML was not observed (data not shown). We could not clarify whether the interaction between IN and PML was based on SUMO–SIM interaction or SUMO-independent interaction from these experiments, since detection of SUMO-conjugated PML protein itself was difficult under our experimental condition due to low sensitivity of anti-SUMO-1 antibody we used, or low abundance of SUMOylated PML species. Furthermore, the result of pull-down assay using truncated INs showed that catalytic core and C-terminal portions of IN played an important role in the interaction (Fig. 3A). We then performed the pull-down experiment with purified PML VI protein (Fig. 3B). In the experiment, GST-fused PML VI expression vector was introduced into *E. coli* BL21, and PML protein was purified. PML VI interacted with His-tagged IN, indicating again that IN directly bound to PML VI protein.

Localization of IN and PML

We then studied the subcellular localization of transiently expressed IN by using laser scanning microscopy. As shown in Fig. 4A, IN was detected as speckle-like structures with the FITC-labelled antibody and the PML protein was also detected as speckles with the TRITC-conjugated antibody. Merged colour (yellow) indicated co-localization of IN and PML proteins, suggesting that IN may be included in the PML body. We also confirmed that the speckle-like structures localized within nuclei by DAPI staining (Fig. 4B). When PIASy alone or PIASy plus HA-SUMO-1 were expressed together with FLAG-IN, IN was also detected as speckles, and the size and numbers of the speckle structure seemed not to be significantly affected (Fig. 4A).

Function of PML in MoMLV infection

To know the physiological function of the PML protein in the viral life cycle, PML was knocked down by siRNA in NIH3T3 cells, and then, a VSV-G-pseudotyped retroviral vector (pQEGFP) was introduced with a multiplicity of infection of 0.5–1.0. We analysed viral cDNA synthesis and integration efficiency as well as two-LTR circle viral cDNA formation that roughly reflects the nuclear entry of PICs (*9*). As shown in Fig. 5A, the amount of PML protein was reduced by ~20% of untreated cells after two cycles of siRNA transfection. The amounts of total viral cDNA and two-LTR circle viral cDNA were determined after the infection. In PML-knockdown cells, the amounts of viral cDNA decreased <50% of the control after 10 h post-infection (Fig. 5B), and two-LTR circle cDNA was ~60% of the control (Fig. 5C). The amount of integrated viral cDNA was ~50% of the control in PML-knockdown cells (Fig. 5D). Furthermore, we performed rescue experiments by introducing expression vector for PML VI together with siRNA. As shown in Fig. 5E, PML VI expression gave higher cDNA production compared to the control suggesting again the positive effect of PML protein on the cDNA production. Totally, these results suggested that the reduction in two-LTR circle cDNA formation and integration by the siRNA might be a simple reflection

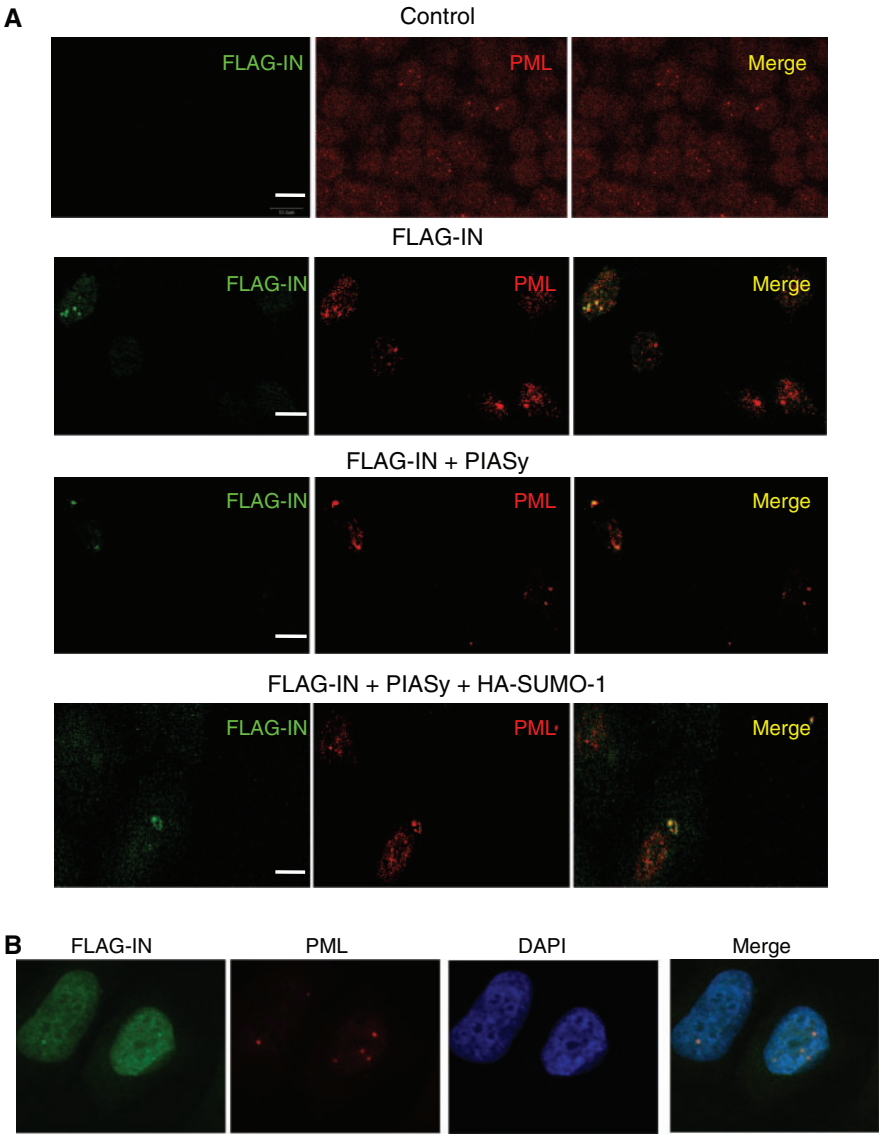

Fig. 4 Co-localization of IN and PML. (A) Hela cells were transfected with expression plasmids for FLAG-IN (phCMV2/FLAG-MLV IN), PIASy and HA-SUMO-1. Pictures were taken by using laser scanning microscopy (Olympus, FV300). (B) Interaction between IN and PML in nucleus. Nuclei were stained with DAPI (Olympus, FV1000).

of the reduced reverse transcription. For 5 days after the viral infection, growth retardation possibly by siRNA was not observed.

In these siRNA experiments, we were able to demonstrate that PML plays important roles in viral life cycle. However, these results do not mean the importance of the interaction between PML and IN. In the binding experiments, we were able to show clear interaction between IN and PML. However, we could not create mutated IN which loses only interacting ability with PML since the interaction required broad region containing catalytic domain of IN. Therefore, the importance of the interaction in viral life cycle seemed to be still obscure. Further study will be required to clarify this point.

RT also interacts with PML

Since RT seemed to be activated by PML proteins, we investigated their physical interaction. FLAG-tagged RT was transiently expressed in 293FT cells and immunoprecipitated by the anti-FLAG antibody. As shown in Fig. 6A, endogenous PML proteins were precipitated with FLAG-tagged RT. This result indicated that RT also interacted with PML proteins. PML VI was mainly precipitated by the anti-FLAG antibody. In the experiment shown in Fig. 6A, a different band pattern of PML proteins was obtained compared to that of Fig. 1A, although the same cell line and antibody were used for detection of PML isoforms. The difference might be caused by different blocking conditions: PBS containing 2% skim milk was used for immunoreaction in Fig. 1A and the commercial immunoreaction enhancer solution (Can Get Signal, Toyobo) was used in Fig. 6A to detect weak signals. In fact, when using PBS with 2% skim milk, lower molecular isoforms were preferentially detected, while higher molecular weight isoforms were detected as dense bands with Can Get Signal (Supplementary Fig. S1). Although band patterns in Figs 1A and 6A seemed to be different, major species of PML isoforms were detected in both

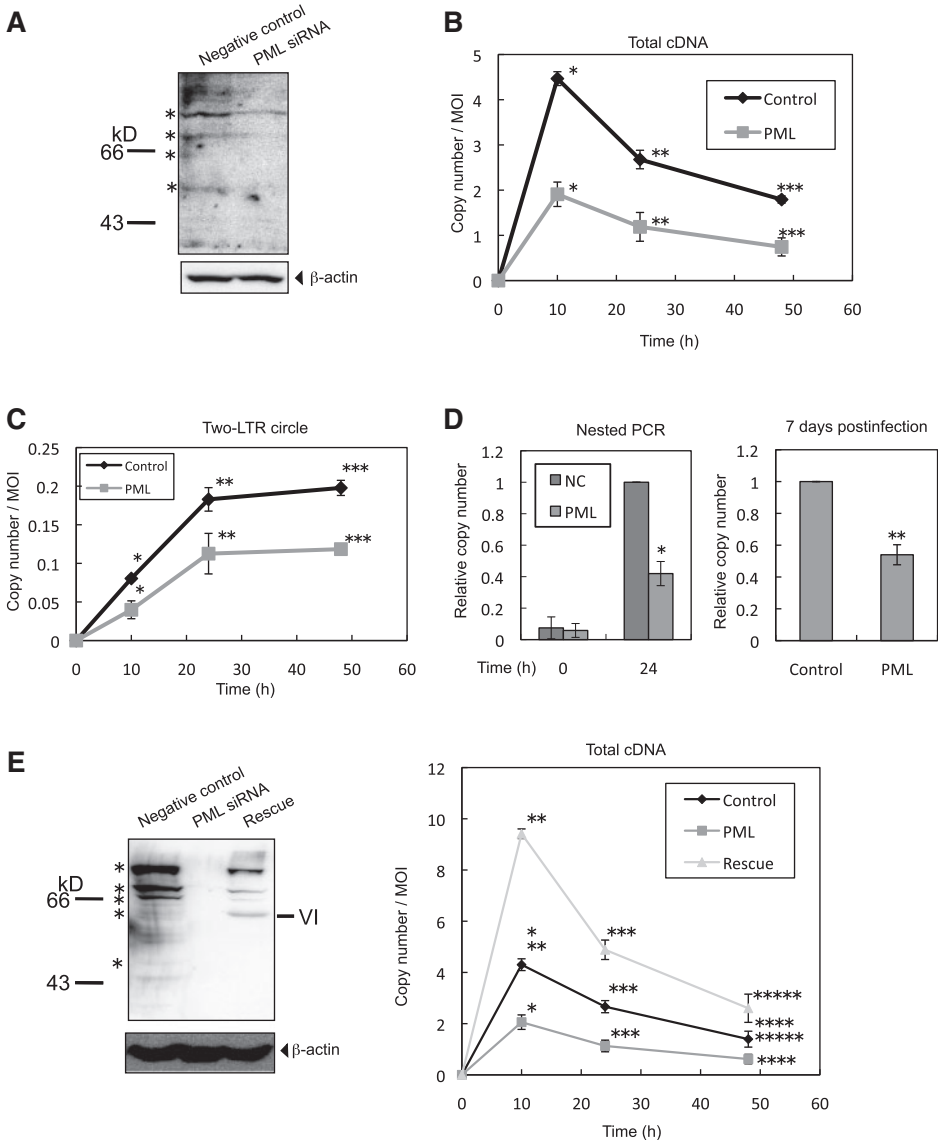

Fig. 5 PML knockdown reduces total viral cDNA and integrated provirus. (A) Knockdown of PML by siRNA was confirmed by western blotting. Can Get Signal was used in the western blot. PML isoforms were marked with asterisks. (B and C) The amounts of total cDNA (B) and two-LTR circle cDNA (C) are shown. NIH3T3 cells were transfected twice with PML siRNA and then infected with pQEGFP at MOI of 0.5–1.0. The values of copy number per MOI were normalized with that of GAPDH (glyceraldehyde-3-phosphate dehydrogenase). Error bars represent the standard deviations of the results of three experiments. Asterisks indicate that values of PML-siRNA cells compared to those of scrambled-siRNA cells were significantly different (*, **, ***, $P<0.001$ in panel B, *, ** $P<0.01$, ***, $P<0.001$ in panel C). (D) The integrated form of viral cDNA was quantified by nested PCR 24 h post-infection (left panel) and real time PCR 7 days post-infection (right panel) and normalized as described in (B) and (C). Error bars represent the standard deviations of the results of three experiments. The values corresponding to scrambled- siRNA-transfected cells were arbitrarily set to 1. Asterisks indicate that values of PML-siRNA compared to those of scrambled-RNA were significantly different (*, **, $P<0.001$). (E) Rescue experiments by over-expression of PML VI. PML VI expression was confirmed by western blotting. Asterisks show PML isoforms (left panel). Can Get Signal was used in the western analysis. The amounts of viral cDNA were analysed (right panel). Asterisks indicate that values of PML-siRNA and PML-siRNA plus PML VI-expression plasmid compared to those of scrambled-RNA were significantly different (*, *****, $P<0.05$, ***, **** $P<0.001$, **, $P<0.0001$). In this experiment, levels of other PML species also increased by unknown reason.

figures. The interaction was also confirmed by the *in vitro* binding assay. For this, purified FLAG-tagged RT and purified His-tagged PML VI protein were used (Fig. 6B and C). The purity of these proteins was confirmed by silver staining (data not shown).

Discussion

In previous reports, PML and INI1 were recruited to cellular PIC and RTCs upon retroviral infection (*2*, *7*). In the present study, we confirmed that IN and PML proteins interacted physically, which may contribute to the recruitment of PML proteins to PIC. We also found that RT interacted with PML, which also supports the finding that the RTC contains PML (*2*). *In vivo*, PML proteins activated the RT reaction. We could assume two mechanisms for this activation. First, interaction with PML directly affected RT activity. Second, IN facilitates the RT reaction through physical interaction as reported previously (*22*, *23*),

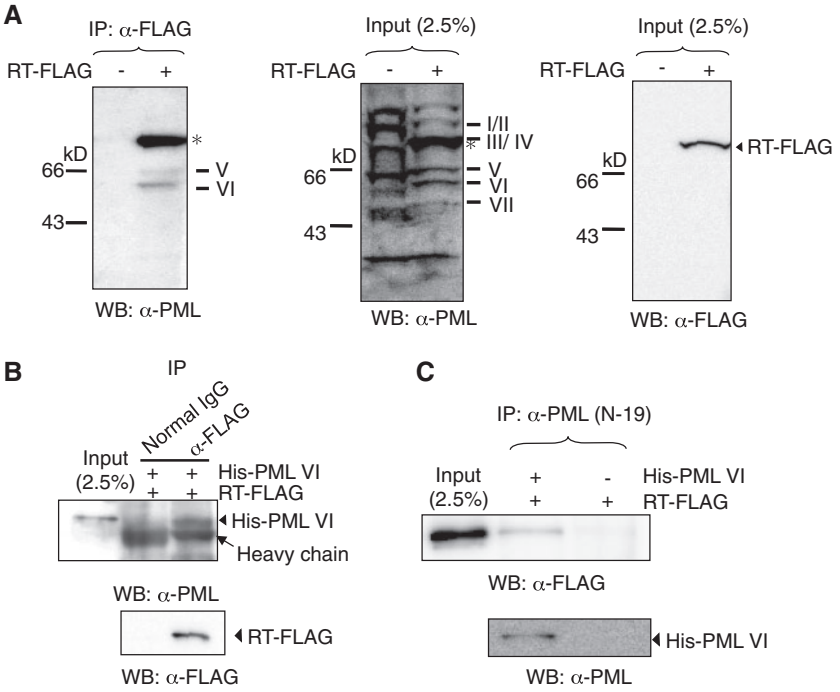

Fig. 6 PML protein interacts with MoMLV RT. (A) Result of co-immunoprecipitation of endogenous PML protein and RT-FLAG expressed in 293FT cells. RT was precipitated by FLAG M2 beads, and then PML protein was detected with the anti-PML antibody (left panel). Input (2.5%) was also analysed with the anti-PML and -FLAG antibodies (center and right panels). Artifact band detected by the anti-PML antibody was possibly RT-FLAG since the anti-FLAG antibody detected the band that showed the same mobility (*, left and centre panels). We also confirmed purified RT-FLAG reacted this anti-PML antibody (H-238) (data not shown). (B) Physical interaction between purified MoMLV RT-FLAG and His-PML VI proteins. Upper panel: *In vitro* immunoprecipitation using purified RT-FLAG and His-PML VI, immunoprecipitation was performed with anti-FLAG antibody. Lower panel: input RT-FLAG was detected with the anti-FLAG antibody. (C) *In vitro* immunoprecipitation using purified RT-FLAG and His-PML VI. Immunoprecipitation was performed with anti-PML antibody (SantaCruz, N-19). The anti-PML antibody was confirmed not to react with RT protein. RT-FLAG was detected with the anti-FLAG antibody. A result representative of several experiments is presented. Can Get Signal was used in these western analyses.

and PML modulates this interaction. In fact, we reported a similar but opposite influence of YY1; IN-YY1 interaction may inhibit RT (*9*). It is also possible that SUMOylation of IN affects this interaction.

It was reported that HIV-1 IN is SUMOylated, and SUMO conjugation facilitates a step of the early viral life cycle between after reverse transcription and before integration. In the report, SUMO-1, −2 and −3 all conjugated to IN (*15*). In the present article, we found that MoMLV IN was SUMOylated, possibly by the actions of PIASy as an E3 ligase. Transient expression of PIASy and SUMO proteins in cultured cells showed that SUMO-1, −2, or −3 conjugated MoMLV IN could be detected. A previous report demonstrated that MoMLV capsid is SUMOylated by PIASy and that the SUMOylation of capsid is important between after reverse transcription and before nuclear entry (*24*). Thus, it is rational that SUMOylation of IN is catalysed by PIASy.

We found that exogenously expressed IN co-localized with PML proteins in speckle-like structures. In the previous report, over-expressed HIV-1 IN was reported to localize in the nuclei (*15*). Zhang *et al.* also reported that over-expressed HIV-1 IN was co-localized in a nuclear structure with TTRAP, one of the proteins detected in the PML body. Furthermore, they indicated that TTRAP facilitates integration of viral cDNA in the host chromosome (*25*). Together, these results including ours suggested that transiently expressed IN might localize in the PML body. Over-expressed proteins often accumulate in the PML body possibly for degradation (*12*). Thus, we cannot approve the physiological meaning of the localization in the PML body, although IN interacted with PML proteins in a viral context and PML possibly activated RT.

For the PML-body's formation, physical interaction between SUMO and SIM is believed to be crucial. However, SUMOylation of partner proteins of the PML body such as Sp100 and p53 is not crucial (*26*, *27*). We found that non-SUMO conjugated IN and PML interacted physically, but we cannot rule out the possibility that SUMOylated form of these proteins interacted with each other since SUMO-conjugated proteins are usually unstable and de-SUMOylation occurs by the actions of SUMO-specific proteinases. Thus, further study of the SUMOylation of PML and IN and its effect on their physical interaction may help clarify the meaning of SUMOylation and PML–IN interaction.

Supplementary Data

Supplementary Data are available at *JB* Online.

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Conflict of interest
None declared.

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