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Resistance to Rabies Virus Infection Conferred by the PMLIV Isoform▽

Danielle Blondel,[1]† Sabrina Kheddache,[2]† Xavier Lahaye,[1] Laurent Dianoux,[2] and Mounira K. Chelbi-Alix[2]*

UPR 3296 CNRS, 91198 Gif sur Yvette, France,[1] and CNRS, FRE 3235, Université Paris Descartes, 75006 Paris, France[2]

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Various reports implicate PML and PML nuclear bodies (NBs) in an intrinsic antiviral response targeting diverse cytoplasmic replicating RNA viruses. PML conjugation to the small ubiquitin-like modifier (SUMO) is required for its localization within NBs. PML displays antiviral effects *in vivo*, as PML deficiency renders mice more susceptible to infection with the rhabdovirus vesicular stomatitis virus (VSV). Cells derived from these mice are also more sensitive to infection with rabies virus, another member of the rhabdovirus family. Alternative splicing from a single gene results in the synthesis of several PML isoforms, and these are classified into seven groups, designated PMLI to -VII. We report here that expression of PMLIV or PMLIVa, which is missing exon 5, inhibited viral mRNA and protein synthesis, leading to a reduction in viral replication. However, the expression of other nuclear isoforms (PMLI to -VI) and cytoplasmic PMLVIIb failed to impair viral production. This antiviral effect required PMLIV SUMOylation, as it was not observed with PMLIV 3KR, in which the lysines involved in SUMO conjugation were mutated. Thus, PMLIV and PMLIVa may exert this isoform-specific function through interaction with specific NB protein partners via their common C-terminal region.

The PML (promyelocytic leukemia) protein, also known as TRIM19, functions as the organizer of PML nuclear bodies (NBs), which are dynamic structures harboring numerous transiently and permanently localized proteins (21). The RBCC/TRIM motif, which contains a C3HC4 (RING finger) zinc-binding domain, two cysteine/histidine-rich motifs (the B boxes B1 and B2), and an α-helical coiled-coil region, is embedded within the PML protein and is required for PML NB formation (16). Posttranslational modification of PML by SUMO (small ubiquitin-like modifier), a ubiquitin-like protein of 11 kDa, is another requirement for the formation of PML NBs and the recruitment of PML NB-associated proteins (15, 37). SUMO is covalently coupled to PML through its lysines 65, 160, and 490 via a process called SUMOylation (17, 18).

Several PML isoforms result from alternative splicing from a single gene, and these isoforms are classified into seven groups, designated PMLI to -VII (reviewed in reference 16). They share the N-terminal region (exons 1 to 3), which encodes the RBCC motif, whereas they differ in their C-terminal regions. A further subgroup classification, i.e., a, b, or c, represents PML isoforms without exon 5, exons 5 and 6, or exons 4, 5, and 6, respectively. The b and c variants are likely to be cytoplasmic, as they lack the nuclear localization signal (NLS). The PML isoform PMLVIIb also lacks the NLS and is indeed cytoplasmic.

The PML gene is directly induced by type I and type II interferons (IFNs) through identified ISRE (-GAGAATCGA AACT-) and GAS (-TTTACCGTAAG-) elements in its promoter (31), leading to an increase in various PML isoforms and a marked increase in the number and the size of PML NBs (6).

Several pathways have been found to be involved in resistance to viral infection in IFN-treated cells, one of which implicates PML and PML NBs (10, 27). PML demonstrates intrinsic antiviral properties, targeting DNA viruses and cytoplasmic replicating RNA viruses (10, 28, 32). PMLIII expression in human cells confers resistance to vesicular stomatitis virus (VSV), influenza virus, and foamy virus independently of p53 (7, 28) and confers p53-dependent resistance to poliovirus by inducing PML-dependent p53 activation, which leads to apoptosis in infected cells (23). The PML antiviral effect has also been observed *in vivo*, as PML knockout mice are more sensitive to infections with the arenavirus lymphocytic choriomeningitis virus (LCMV) and the rhabdovirus VSV (3). Some RNA viruses that replicate in the cytoplasm and are inhibited by PML have developed different strategies to counteract PML NBs (8, 27). LCMV infection results in PML NB modification, mediated by a small nonstructural protein, Z. The RING finger protein Z associates with PML NBs and delocalizes PML to the cytoplasm, where both proteins interact with the elongation factor eIF-4E, reducing its affinity for the 5′ mRNA cap structure and inhibiting cellular translation (4, 19). The rabies virus protein P, which has been shown to be a nucleocytoplasmic protein (24), also directly binds via its C-terminal region to the PML RING finger, causing the redistribution of PML into the cytoplasm, where both proteins colocalize (2).

Rabies virus, the prototype of the *Lyssavirus* genus, which belongs to the *Rhabdoviridae* family (*Mononegavirales* order), causes a fatal disease associated with intense viral replication in the central nervous system. Its single-stranded negative-sense RNA genome (about 12 kb), encoding five viral proteins,

* Corresponding author. Mailing address: CNRS, FRE 3235, Université Paris Descartes, 75006 Paris, France. Phone: 33 1 42 86 20 43. Fax: 33 1 42 86 38 10. E-mail: mounira.chelbi-alix@parisdescartes.fr.
† These authors contributed equally to this work.
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is encapsidated by the nucleoprotein N to form the nucleocapsid, which is associated with the RNA-dependent RNA polymerase L and its cofactor the phosphoprotein P. Inside the viral particle, this nucleocapsid has a tightly coiled helical structure that is associated with the matrix protein M and is surrounded by a membrane containing a unique glycoprotein, G. Both L and P are involved in transcription and replication. A positive-stranded leader RNA and five mRNAs are synthesized during transcription. The replication process yields nucleocapsids containing full-length antisense genome RNA molecules, which in turn serve as templates for the synthesis of sense genome RNA. It has been recently shown that viral transcription and replication take place in cytoplasmic inclusions, called Negri bodies, formed during viral infection (20).

Fibroblasts derived from PML knockout mice ($PML^{-/-}$ mouse embryo fibroblasts [MEFs]) are more sensitive to infection with rabies virus (2). Therefore, we studied the roles of various PML isoforms during rabies virus infection. We show that only cells stably expressing PMLIV or PMLIVa (without exon 5) confer resistance to rabies virus infection, whereas the nuclear PML isoforms PMLI, -II, -III, -V, and -VI and the cytoplasmic PMLVIIb isoform failed to inhibit this virus. This protective effect required PMLIV SUMOylation, as it was not observed with PMLIV 3KR, in which lysines involved in conjugation to SUMO were mutated.

MATERIALS AND METHODS

IFN and antibodies. Murine recombinant IFN-α was purchased from R&D Systems. The following antibodies were used: mouse monoclonal anti-PML (clone PGM3) (used for immunofluorescence microscopy); rabbit polyclonal anti-PML (clone H-238) (used for Western blotting); rabbit polyclonal antitubulin (clone C-11), anti-PKR, and anti-Stat1 antibodies (from Santa-Cruz Biotechnology); and anti-P and anti-N antibodies, produced as previously described (26).

Virus stocks and cell infection. The CVS strain of rabies virus was grown in BSR cells. Virus titers were determined by standard plaque assays with BSR cells. U373MG cells were grown on glass coverslips in six-well plates (from 50 to 80% confluence) and were infected with rabies virus at various multiplicities of infection (MOIs) and at various times postinfection (p.i.).

Cell cultures and treatments. U373MG cells, MEFs from wild-type (wt) or knockout ($PML^{-/-}$) mouse embryos (35) (a gift from P. P. Pandolfi) immortalized with the simian virus 40 (SV40) T antigen, and MEFs deficient in PKR, RNase L, and Mx1 (a gift from R. H. Silverman) were grown at 37°C in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum. U373MG cells transfected with empty vector or stably expressing PMLIII (U373MG-PMLIII) (7), PMLI, PMLII, PMLIV, or PMLVI were kept in medium supplemented with 0.5 mg/ml of neomycin. Cells stably expressing PMLIVa were kept in medium supplemented with 0.2 mg/ml of zeomycin.

Constructs and expression vectors. All cDNA construct sequences were confirmed by DNA sequencing.

(i) PML expression constructs. cDNAs of the PML isoforms (PMLI, PMLII, PMLIII, PMLIV, PMLV, PMLVI, and PMLVIIb) were amplified by PCR from pBluescript SK(+) for insertion into appropriate vectors. To generate untagged expression constructs, PML PCR fragments of each isoform were restriction digested with BamHI and XhoI for PMLIII, -IV, -V, -VI, and -VIIb; with BamHI and XbaI for PMLI; and with HindIII and XhoI for PMLII (the PML_BamHI_ATG sense primer was used as the sense primer for all isoforms; a specific antisense primer, PML X _Stop_Enz name, was designed for each isoform, where "X" is the number of the isoform and "Enz name" is the name of the enzyme used to cut cDNA in the 3′ direction). Restricted PCR fragments were then inserted into the BamHI/XhoI site of either pcDNA3.1(+) or the HIV-1-based lentiviral pTRIP vector for stable expression (25).

(ii) Primers. The sense primer was PML_BamHI_ATG sense (5′TGTCTAAGCTTGCTAGCGGATCCACACCATGGAGCCTGCACCCGCCCGATCTCCG3′). Antisense primers were PMLI Stop XbaI (5′AATACGGTACCTCTAGAACTCAGCTCTGCTGGGAGGCCCTCTC3′). PMLII_Stop_XhoI (5′AATACCTCGAGTCTAGATATCAGAGGCCTGCTTGACGGGCGCCTGGGAC3′), PMLIII_Stop_XhoI (5′ATCCGCCTCGAGCAGAATCAGCGGGCTGGTGGGGAGGCCAAGC3′), PMLIV_Stop_XhoI (5′AATACCTCGAGTCTAGATACTAAATTAGAAAGGGGTGGGGGTAGCCCCAGG3′), PMLV_Stop_XhoI (5′AATACCTCGAGTCTAGATATCAATGCCTCACTGGAAAATTCCCCAGGCGC3′), PMLVI_Stop_XhoI (5′AATACCTCGAGTCTAGATATCACCACAACGCGTTCCTCTCCCTACCTGCC3′), and PMLVIIb Stop_XhoI (5′GTACCTCGAGTATTAATGAGTGCTACTCTGTGCAGGGCCTGTAAGAGCATGGGCTGGAGGAGGCACCAGGTCAACGTCAATAGGGTCCCTGGGAGTGC3′).

Stable expression of PML isoforms. Stable PMLI-, PMLII-, PMLIV-, and PMLVI-expressing U373MG cells were obtained via transfection with pcDNA3.1 PMLI, pcDNA3.1 PMLII, pcDNA3.1 PMLIV, and pcDNA3 PMLVI constructs and subsequent neomycin selection (final concentration, 0.5 mg/ml). U373MG cells stably expressing PMLIVa were generated via transfection with pcDNA4.1 PMLIVa (a gift from P. G. Pelicci) and zeomycin selection (0.2 mg/ml). Control cells were generated in the same way using the empty vectors.

Stable transduction of PML isoforms. Stable expression of PML constructs was obtained by use of a lentivirus-based strategy, as previously described (36). Efficiency of the HIV-1 based lentiviral pTRIP vector, into which the PML constructs were inserted, was associated with the presence of a triple-stranded DNA structure that acts as a *cis* determinant for HIV-1 DNA import. The stable integration of transgenes into host DNA allows efficient and long-term transgene expression without clone selection. Virus stock production and infection were performed to transduce and express PML variants (PMLIII, PMLIV, PMLIV 3KR mutant, PMLV, and PMLVIIb) in the U373MG cell line, as previously described (25).

Immunofluorescence analysis. Infected cells grown on glass coverslips were fixed for 20 min with 4% (wt/vol) paraformaldehyde (PFA) in phosphate-buffered saline (PBS) and permeabilized for 5 min in 0.1% Triton X-100 in PBS. Cells were then prepared for double-immunofluorescence staining and analyzed by confocal microscopy. The intracellular distribution of viral antigens P and N was analyzed by using the specific monoclonal antibodies (MAbs) at dilutions of between 1/500 and 1/1,000, followed by incubation with the corresponding IgG antibody conjugated to Alexa 568 or Alexa 488 diluted 1/1,000. The PML protein was detected with a rabbit anti-PML antibody diluted 1/400 and the corresponding anti-rabbit IgG antibody conjugated to Alexa 568 or Alexa 488. The cells were mounted onto glass slides using Immu-Mount (Shandon) containing 4,6-diamidino-2-phenylindole (DAPI) to stain nuclei. Confocal laser microscopy was performed on a Leica SP2 microscope (63× oil-immersion objective) using UV excitation at 351 nm (DAPI), blue laser excitation at 488 nm (Alexa 488), and green laser excitation at 545 nm (Alexa 568) in sequential recording mode.

Northern blot analysis. RNA was isolated from cells with the RNA NOW kit (Ozyme). Total RNA was separated on a 1.5% agarose gel under denaturing conditions and blotted onto nylon membranes (Roche Molecular Biochemicals). Hybridizations were performed with digoxigenin (DIG)-labeled cDNAs recognizing the rabies virus N or P gene sequence and by incubation with anti-DIG antibody conjugated to alkaline phosphatase followed by CDP Star.

Western blot analysis. Cells were washed and resuspended in PBS, lysed in hot Laemmli sample buffer, and boiled for 5 min. About 20 μg of protein was analyzed on a 10% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. The proteins were blocked on the membranes with 10% skim milk in Tris-buffered saline (TBS) for 2 h and incubated overnight at 4°C with rabbit polyclonal anti-PML, anti-P, anti-N, or antitubulin antibodies. The blots were then washed extensively in PBS-Tween and incubated for 1 h with the appropriate peroxidase-coupled secondary antibodies (Amersham). All of the blots were then subjected to chemiluminescence (ECL Amersham).

RESULTS

MEFs from triply PKR-, RNase L-, and Mx1-deficient mice and MEFs from PML-deficient mice are protected against rabies virus infection in the presence of IFN. The kinase PKR, the endoribonuclease RNase L, and the Mx proteins are known mediators implicated in IFN-induced antiviral defense (12, 14, 30). To determine the effect of the absence of these mediators on rabies virus growth, MEFs derived from triply PKR-, RNase L-, and Mx1-deficient mice (38) were treated for

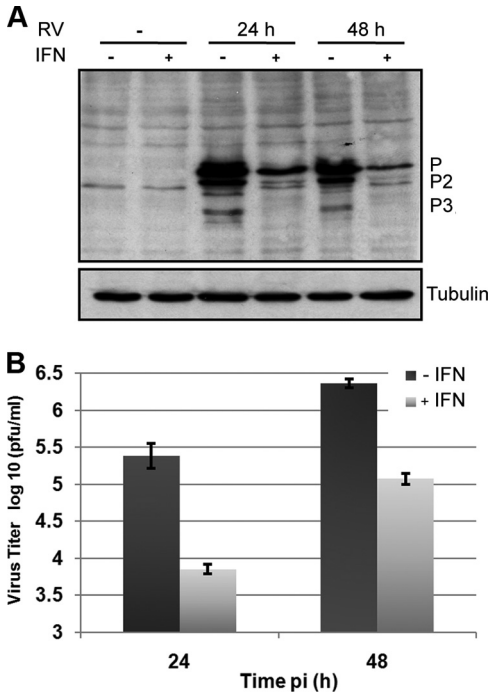

FIG. 1. IFN inhibits rabies virus replication in MEFs from triply deficient mice. MEFs from mice deficient in PKR, RNase L, and Mx1 were treated for 1 day with IFN-α and were uninfected (RV −) or infected with rabies virus (RV +) at an MOI of 1 for 24 h or 48 h. (A) Cell extracts were analyzed by Western blotting using anti-P and antitubulin antibodies. P and truncated P products P2 and P3 were detected as expected (9). (B) Viral titers were determined as described in Materials and Methods. Error bars indicate standard deviations.

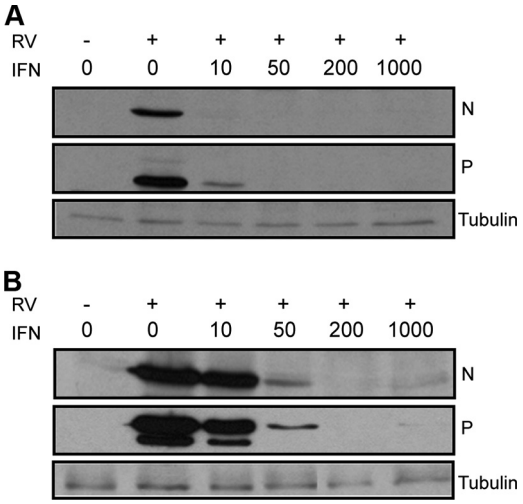

FIG. 2. Effect of IFN on rabies virus replication in wt MEFs and PML$^{-/-}$ MEFs. wt MEFs (A) and PML$^{-/-}$ MEFs (B) were treated for 24 h with various doses of IFN and then left uninfected (RV −) or infected with rabies virus (RV +) at an MOI of 1 for another day. Cells were extracted and analyzed by Western blotting using anti-N, anti-P, and antitubulin antibodies.

1 day with IFN-α and were then infected for 1 or 2 days with rabies virus at a multiplicity of infection (MOI) of 1. Analysis of the amounts of viral protein P by Western blotting and determination of viral titers (Fig. 1) showed that IFN-α conferred resistance to rabies virus in the absence of PKR, RNase L, and Mx1. This suggests that other mechanisms are also implicated in resistance against this virus in response to IFN.

Next, we analyzed the effect of IFN on rabies virus in wild-type (wt) MEFs and PML$^{-/-}$ MEFs. These cells were treated for 1 day with various concentrations of IFN-α before infection with rabies virus at an MOI of 1 for 1 day. Consistent with our previous results (2), rabies virus replication and viral protein expression were higher in PML$^{-/-}$ MEFs than in wt MEFs (Fig. 2). IFN was still active in MEFs in the absence of PML, but its capacity to confer resistance to rabies virus was reduced; i.e., 200 units/ml versus 50 units/ml was needed to observe similar levels of inhibition of both viral protein expression and viral growth in PML$^{-/-}$ MEFs compared to wt MEFs (Fig. 2 and data not shown). Taken together, these results show that PML is one of the IFN mediators against rabies virus.

Effect of the different PML isoforms on rabies virus infection. To determine whether some PML isoforms are capable of inhibiting rabies replication, we stably expressed various PML isoforms (PMLI, PMLII, PMLIII, PMLIV, PMLV, PMLVI, or PMLVIIb) either in stable transfected or in transduced U373MG cells. The structures of these PML isoforms are presented in Fig. 3. All the nuclear isoforms presented the characteristic dots within the PML NBs (Fig. 3B; see Fig. 5A for PMLIV), whereas, as expected, PMLVIIb was expressed in the cytoplasm (Fig. 3B). U373MG cells stably expressing PMLI, PMLII, PMLIII, PMLIV, or PMLVI or transduced with PMLIII, PMLIV, PMLV, or PMLVIIb constructs were infected with rabies virus for 1 day. Extracts from these infected cells were analyzed by Western blotting using anti-N, anti-P, and antitubulin antibodies (Fig. 4). The profiles of the viral proteins N and P were similar in control U373MG cells (transfected with the empty vector) and cells expressing the nuclear isoforms PMLI (Fig. 4A), PMLII (Fig. 4A), PMLIII (Fig. 4B), PMLV (Fig. 4D), and PMLVI (Fig. 4C) and the cytoplasmic isoform PMLVII (Fig. 4D), indicating that none of these PML isoforms were involved in inhibiting rabies virus infection. In contrast, the viral N and P proteins were undetectable in extracts from cells stably expressing PMLIV, revealing that PMLIV inhibited the expression of viral proteins (Fig. 4E, left panel). Similar results were obtained with U373MG cells stably expressing PMLIVa missing exon 5 (Fig. 4E, left panel). The level of PML in cells expressing PMLIV or PMLIVa is shown in Fig. 4E (right panel). We also studied the effects of PMLIII and PMLIV in transduced cells. U373MG cells transduced with PMLIV also inhibited rabies virus protein expression, whereas PMLIII did not impair protein synthesis (Fig. 4F), demonstrating that the protective effects of PMLIV against rabies virus were observed in either stably transfected or transduced cells.

PMLIV or PMLIVa expression inhibits viral mRNA and protein synthesis. To confirm the antiviral effect of PMLIV, U373MG cells stably expressing PMLIV and U373MG control cells were infected with rabies virus at an MOI of 0.3 for 1 day. Double-immunofluorescence staining, performed for PML and viral antigen expression, demonstrates that rabies virus antigens that were highly expressed in control cells were undetectable in cells stably expressing PMLIV (Fig. 5A). Next, we

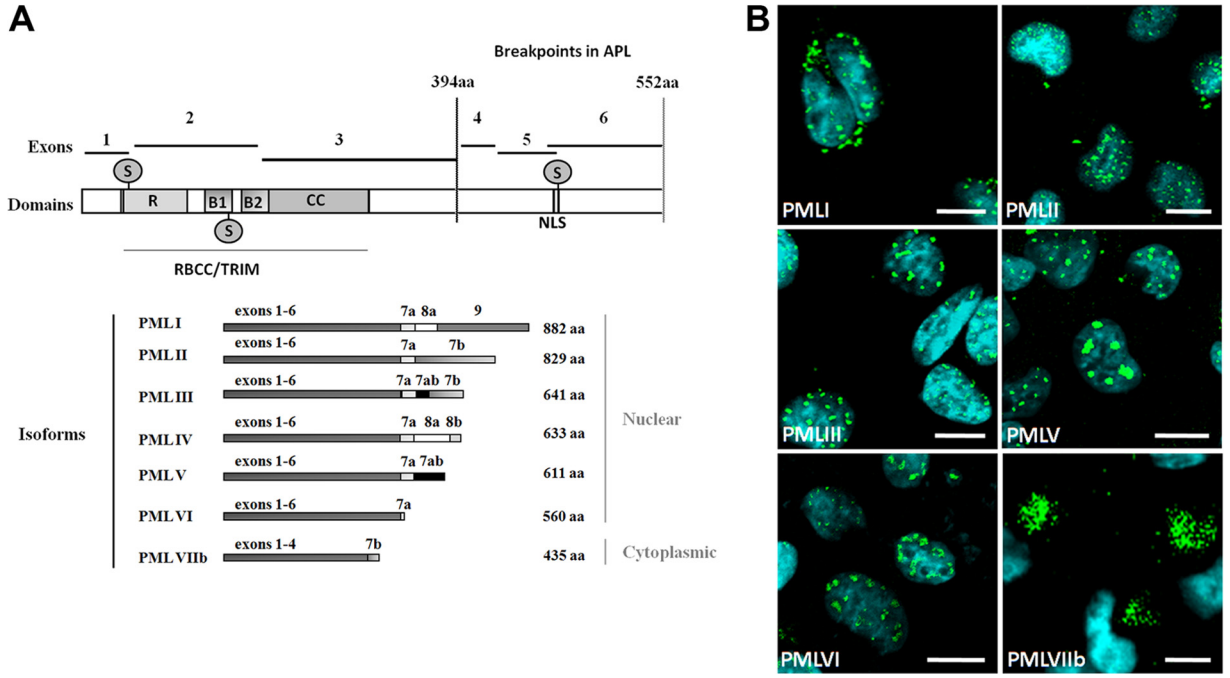

FIG. 3. Structure and localization of PML isoforms. (A) Schematic representation of the domain structures of PML isoforms. All PML isoforms share an N terminus but differ in their C termini due to the alternative splicing of exons 7 to 9; protein domains of all isoforms include the RING finger (R), the B1 and B2 boxes, the coiled-coil (CC) motif, the nuclear localization domain (NLS), and three SUMOylation sites. (B) PML isoform localization. U373MG cells expressing PMLI, PMLII, PMLIII, PMLVI, PMLV, or PMLVIIb were analyzed by immunofluorescence staining with anti-PML antibodies. The scale bars correspond to 20 μm.

tested whether the inhibition of viral protein synthesis observed in PMLIV- or PMLIVa-expressing cells was due to inhibition of viral mRNA synthesis. U373MG control, U373MG-PMLIV, and U373MG-PMLIVa cells were infected with rabies virus at an MOI of 0.3. Total RNA preparations were analyzed for the presence of rabies virus N mRNA by Northern blot analysis. Significant amounts of viral N mRNA were present in infected U373MG control cells, whereas viral N mRNA was undetectable in infected U373MG cells stably expressing PMLIV or PMLIVa (Fig. 5B). These results demonstrate that PMLIV and PMLIVa had a strong inhibitory effect on viral mRNA synthesis.

PMLIV or PMLIVa confers resistance to viral replication. PMLIV or PMLIVa expression inhibited viral mRNA and protein synthesis in infected cells. To quantify the level of viral inhibition, supernatants from infected U373MG control, U373MG-PMLIV, and U373MG-PMLIVa cells were used for viral titration. No difference in virus yield was found between parental U373MG and U373MG cells transfected with the empty vector (data not shown).

Virus production in U373MG-PMLIVa and U373MG-PMLIV cells infected with rabies virus was about 200-fold lower than that in control cells (Fig. 6A). This suggests that the expression of PMLIV or PMLIVa induces IFN secretion, which could in turn inhibit viral replication. However, resistance in PMLIV-expressing cells was not due to higher IFN production in cells expressing the PMLIV isoform during rabies virus infection, for various reasons. Culture supernatants from U373MG cells transfected with the empty vector (S1) and from U373MG-PMLIV cells (S2) infected with rabies virus were subjected to IFN titer determination using HeLa cells; the IFN titers were 8 units for both supernatant samples (data not shown). We then tested whether these supernatants induce the expression of PKR and STAT1, two IFN-induced proteins, in U373MG cells. The two culture supernatants displayed similar but higher levels of Stat1 and PKR than control uninfected cells (Fig. 6B, upper panel). We also tested whether these supernatants inhibit VSV protein synthesis. For this, U373MG cells treated with medium (C) or S1 or S2 supernatant for 24 h were infected with VSV for 6 h. VSV protein expression analysis in these cell extracts by Western blotting revealed that VSV proteins were similarly expressed in extracts from cells treated with S1 and S2 (Fig. 6B, lower panel). We observed slightly lower viral protein expression in extracts from cells treated with S1 or S2 than in those from control cells.

Taken together, these results show that PMLIV-expressing cells, in contrast to cells transfected with empty vector, did not produce sufficient IFN to ensure a protective effect and that PMLIV displayed intrinsic antiviral activity against rabies virus.

SUMOylation of PMLIV is required for its antiviral property. PML is covalently linked to SUMO via lysine 65, 160, and 490 (17). This modification is required for the localization of PML within NBs. To evaluate the role of SUMOylation in PMLIV-induced rabies virus inhibition, we generated PMLIV 3KR, in which the SUMO target lysines 65, 160, and 490 are mutated into arginines, and we stably transduced U373MG cells with wild-type PMLIV and PMLIV 3KR. The expression of PMLIV and PMLIV 3KR in transduced U373MG cells was verified by immunofluorescence (Fig. 7A). PML expression

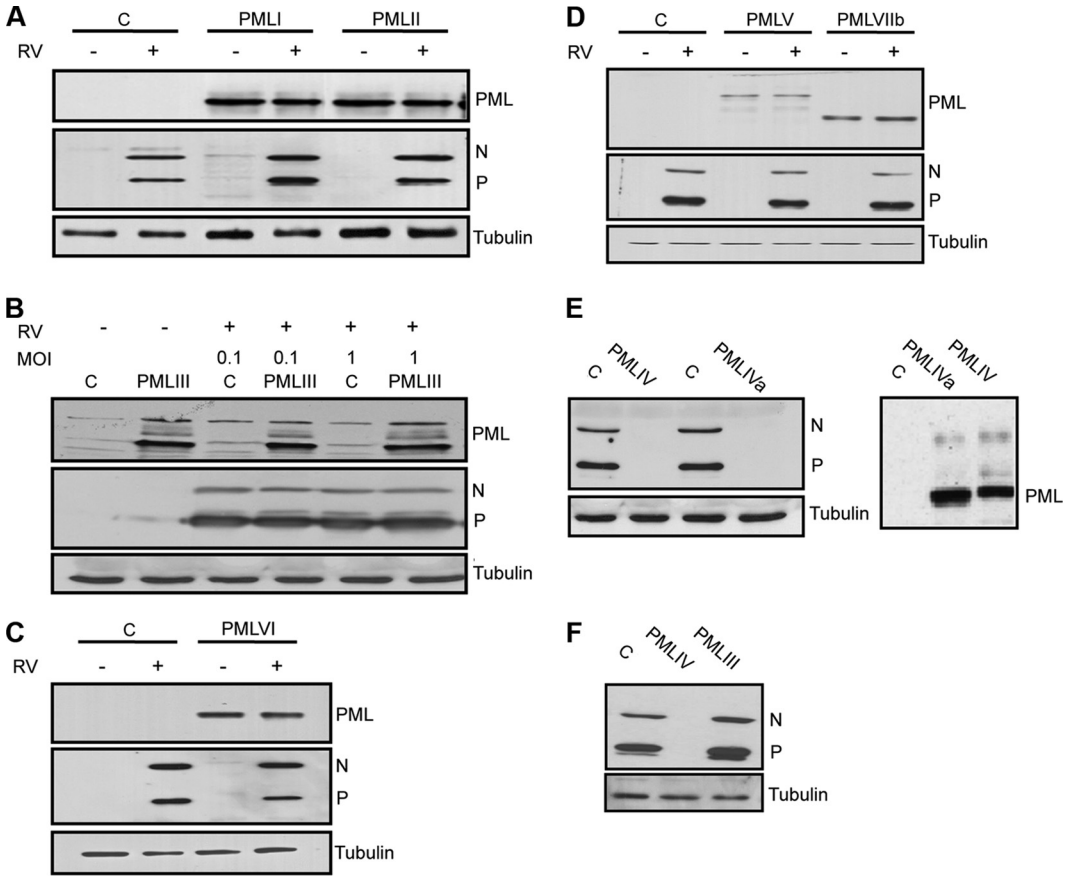

FIG. 4. PMLIV is the only isoform that inhibits rabies virus replication. (A to D) U373MG cells stably expressing PMLI or PMLII (A), PMLIII (B) PMLV (D), PMLVI (C), or PMLVIIb (D) constructs were uninfected (RV −) or infected with rabies virus (RV +) at an MOI of 0.3 for 1 day (in the case of PMLIII, the MOIs used were 0.1 and 1). Cell extracts from these cells were analyzed by Western blotting with anti-PML, anti-N, anti-P, and antitubulin antibodies. Control cells transfected with the empty vector (C) were uninfected or infected as described above. (E) Extracts from control cells and U373MG-PMLIV or U373MG-PMLIVa cells infected with rabies virus at an MOI of 0.3 were analyzed by Western blotting using anti-N, anti-P, and antitubulin antibodies (left panel). The levels of PMLIVa and PMLIV are also shown (right panel). (F) Extracts from control and U373MG cells transduced with PMLIII or PLIV and infected as for panel E were analyzed by Western blotting using anti-N, anti-P, and antitubulin antibodies.

analysis by Western blotting showed that PML and its SUMOylated form were detected in cells transduced with wild-type PMLIV, and, as expected, only the unmodified form was detected in cells transduced with PMLIV 3KR (Fig. 7B).

Next, we analyzed the effects of PMLIV and PMLIV 3KR on viral protein expression. U373MG control, U373MG-PMLIV, or U373MG-PMLIV 3KR cells were infected with rabies virus, and cell extracts were analyzed by Western blotting. N and P protein production was inhibited by PMLIV expression and was not altered by that of PMLIV 3KR (Fig. 7B).

To confirm these results, U373MG control cells, expressing PMLIV or PMLIV 3KR, were infected with rabies virus at MOIs of 0.3 and 1 for 1 day and were then used to determine virus yields. Viral multiplication was inhibited in cells expressing PMLIV and not in cells expressing PMLIV 3KR (Fig. 7C).

Taken together, these results demonstrate that PMLIV SUMOylation is required for resistance against rabies virus and suggest that PML NB formation is required for this effect.

DISCUSSION

An antiviral effect of PML against rhabdoviruses has been observed *in vivo*, as PML deficiency renders mice more susceptible to VSV infections (3) and cells derived from these mice are more sensitive to infection with rabies virus (2). We showed here that IFN inhibits rabies viral replication in $PML^{-/-}$ MEFs, indicating that PML is not the sole mediator and that other IFN-induced proteins are involved in this process. Among the various PML isoforms tested (nuclear PMLI, PMLII, PMLIII, PMLIV, PMLV, and PMLVI and cytoplasmic PMLVIIb), only stable expression of PMLIV and PMLIVa (missing exon 5) conferred resistance to rabies virus. The antiviral property of PMLIV was shown in either transduced or stably transfected cells. This inhibitory effect was observed at the level of virus mRNA and protein synthesis, resulting in a 200-fold-lower rabies virus yield than in control cells. We demonstrated that resistance to rabies virus in PMLIV-expressing cells was not due to higher IFN production during infection. Indeed, culture media from infected control and U373MG-

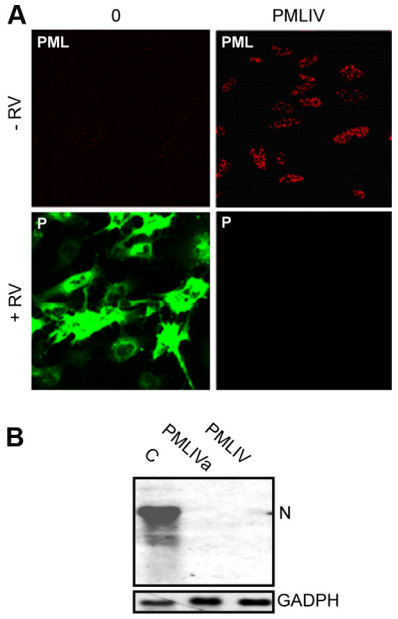

FIG. 5. PMLIV or PMLIVa inhibits viral mRNA and protein synthesis. (A) U373MG-PMLIV and control cells transfected with the empty vector (C) were infected for 1 day with rabies virus at an MOI of 0.3. Double-immunofluorescence staining was performed with anti-PML and anti-P antibodies. (B) Total RNA was extracted from cells infected for 16 h as described for panel A. Samples (20 μg of RNA per lane) were analyzed for the presence of rabies N mRNA and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA, as described in Materials and Methods.

PMLIV cells had similar capacities to induce IFN-stimulated gene products and to inhibit VSV antigens. These findings demonstrate that PMLIV displays intrinsic antiviral properties against rabies virus.

Moreover, SUMOylation of PML is necessary for PML NB

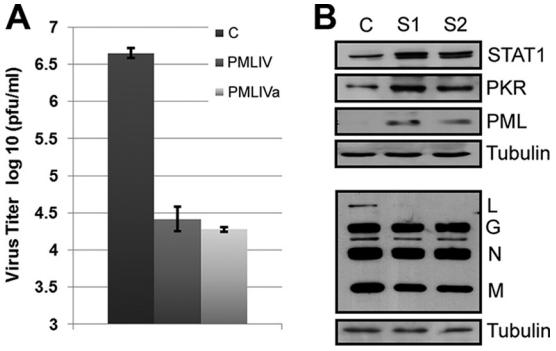

FIG. 6. PMLIV and PMLIVa inhibits rabies virus replication. (A) Viral titers of culture media from infected U373MG-PMLIV cells and control cells were determined as described in Materials and Methods. Error bars indicate standard deviations. (B) PMLIV-expressing cells do not produce larger amounts of IFN than control cells. U373MG cells prepared in duplicate were untreated (C) or treated for 24 h with culture media from control U373MG (S1) and U373MG-PMLIV (S2) cells. One series was analyzed by Western blotting using anti-STAT1, anti-PKR, and anti-PML antibodies (upper panel). Cells from the second series were infected with VSV at an MOI of 1 for 6 h and then were analyzed by Western blotting using anti-VSV antibodies to detect viral L, G, N, and M proteins (lower panel).

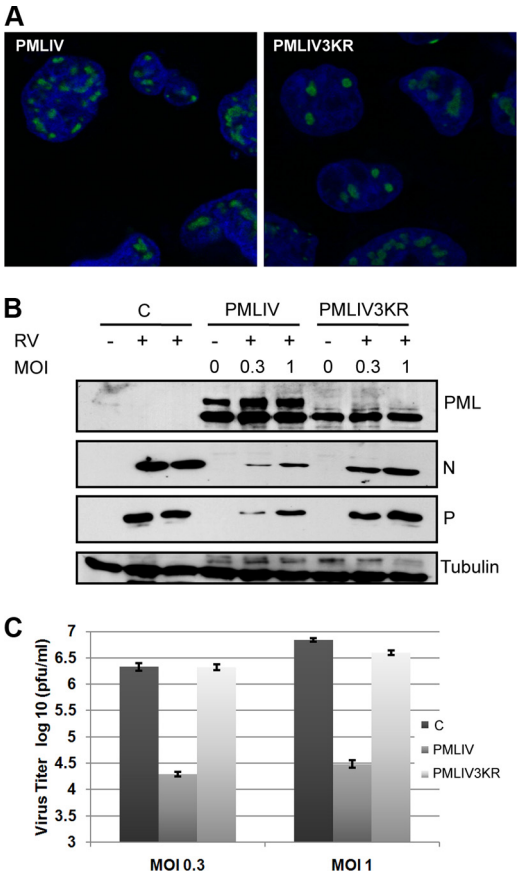

FIG. 7. PMLIV SUMOylation is required for resistance against rabies virus. (A) Localization of PMLIV, PMLIV 3KR, and PMLVIIb. U373MG cells transduced with PMLIV or PMLIV 3KR were analyzed by immunofluorescence for PML staining. (B) The capacity of PMLIV to confer viral resistance necessitates its SUMOylation. U373MG control cells and cells transduced with PMLIV and PMLIV 3KR were uninfected (RV −) or infected (RV +) at MOIs of 0.3 and 1. Cell extracts were analyzed by Western blotting using anti-PML, anti-N, anti-P, and antitubulin antibodies. (C) Viral titers were determined as described in Materials and Methods. Error bars indicate standard deviations.

formation and the recruitment of various proteins (15, 37). Interestingly, PMLIV 3KR, which is mutated in the three SUMOylation sites, failed to inhibit rabies virus infection, demonstrating that SUMOylation of PMLIV and PML NB integrity are required for these antiviral effects.

PMLIII interacts with the rabies virus P protein in cotransfected and infected cells via the C-terminal domain of P and the RING motif of PML, which is shared by all PML isoforms (2). PMLIV also interacts with P in cotransfected cells (data not shown), but no interaction could be detected in infected cells due to the inhibition of viral replication by PMLIV expression. Furthermore, rabies virus infection of cells stably expressing PMLIII induces a modification of PML NBs, which become larger and appear as dense aggregates when analyzed by confocal or electron microscopy, respectively (2). Again, this result cannot be observed in cells expressing PMLIV, as the infection is inhibited. In addition, although in transfected cells the nucleocytoplasmic P protein interacts with both

PMLIII and PMLIV, it sequesters only the PMLIII isoform into the cytoplasm (reference 2 and data not shown).

Indeed, we showed that viral transcription is inhibited by PMLIV, and as the P protein is involved in viral transcription, we suspect that primary transcription of viral proteins in the presence of the incoming P protein is affected by PMLIV. Interestingly, the viral P protein is a multifunctional protein that mediates inhibition of the IFN system by different pathways: it alters PML NBs, and it inhibits IFN production by impairing IFN regulatory factor-3 phosphorylation and IFN signaling by blocking nuclear transport of Stat1 (5, 33, 34).

The mechanism by which nuclear PMLIV confers resistance to rabies virus, which replicates in the cytoplasm, is unknown. Resistance to viral infection is specific to PMLIV and PMLIVa. PML isoforms share an N-terminal region but differ in their C termini. In addition, variability in the C-terminal part of PML isoforms is important for the recruitment of interacting partners and their functions (1). PMLIV and PMLIVa share the same C-terminal region and may recruit within PML NBs, cellular factors required for rabies virus replication. Indeed, variability in the C-terminal part of PML isoforms is important for the recruitment of partners that interact with PML and therefore for its function. As an example, PMLIV harbors binding motifs for p53 (11, 13) and MDM2 (1), which are required for PMLIV-induced apoptosis. It has been also shown that PMLIV functions as a regulator of telomerase activity by interacting with the telomerase reverse transcriptase (22). It should be noted that the antiviral property of PMLIV against rabies virus occurs in a p53-independent way, because the p53 gene is mutated in U373MG cells (29).

The PMLIII isoform has been shown to use various mechanisms to confer resistance to other RNA viruses whose replication takes place in the cytoplasm. PMLIII confers resistance to poliovirus in a p53-dependent manner and confers resistance to VSV independently of p53 (7, 23). Indeed, PMLIII expression reduces viral mRNA and protein synthesis, leading to inhibition of VSV production. The protective effect of PMLIII against VSV can occur outside the NBs, whereas that of PMLIV against rabies virus required PML localization within the NBs. Also, a cytoplasmic PMLIII mutant has been shown to confer resistance to VSV infection (7), whereas the cytoplasmic PMLVIIb isoform failed to inhibit rabies virus (this paper). Taken together, these findings suggest that PML uses alternate ways to inhibit two members of the rhabdovirus family.

In conclusion, our comparative study between all PML isoforms demonstrated that resistance to rabies virus is conferred by the SUMOylated PMLIV isoform, PMLIV, via its C-terminal region and may exert this isoform-specific function through interaction with specific protein partners within the NBs.

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