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Foamy Retrovirus Integrase Contains a Pol Dimerization Domain Required for Protease Activation▽

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Unlike orthoretroviruses, foamy retroviruses (FV) synthesize Pol independently of Gag. The FV Pol precursor is cleaved only once between reverse transcriptase (RT) and integrase (IN) by the protease (PR), resulting in a PR-RT and an IN protein. Only the Pol precursor, not the cleaved subunits, is packaged into virions. Like orthoretroviral PRs, FV PR needs to dimerize to be active. Previously, we showed that a Pol mutant lacking IN has defects in PR activity and Pol packaging into virions. We now show that introduction of a leucine zipper (zip) dimerization motif in an IN truncation mutant can restore PR activity, leading to Pol processing in cells. However, these zip mutants neither cleave Gag nor incorporate Pol into virions. We propose that IN is required for Pol dimerization, which is necessary for the creation of a functional PR active site.

The foamy retroviruses (FV) comprise the only genus in a subfamily of *Retroviridae*, the *Spumaretrovirinae*. One of the fundamental differences in the viral replication cycles of *Spumaretrovirinae* and *Orthoretrovirinae* involves synthesis of the Pol protein. Orthoretroviral Pol is expressed from a full-length genomic RNA as a Gag-Pol fusion protein that coassembles into virus particles through self-assembling domains in Gag (7). However, FV Pol is expressed independent of Gag from a low-abundance spliced mRNA (22, 36). This implies that regulation of Pol expression and packaging into virions differs in spumaretroviruses and orthoretroviruses. Two genomic RNA sequences, called Pol encapsidation sequences (PES), are required for FV Pol packaging (27). Previously, we showed that the C terminus of Gag contains determinants required for Pol packaging, suggesting either that Pol first binds to Gag and the resulting complex binds to RNA or that Pol binds to a complex of Gag and RNA for incorporation into virions (19).

FV Pol is synthesized as a precursor protein consisting of protease (PR), reverse transcriptase (RT), and integrase (IN). Compared with orthoretroviral PRs, FV PR makes limited cleavages: once in Pol, producing a PR-RT fusion and IN (17), and once in Gag near the C terminus, releasing a small peptide, p3 (6). FV PR is absolutely required for processing and viral infectivity (18). Like orthoretroviral PRs, FV PR is an aspartyl protease that is active only as a homodimer in which each subunit contributes half of the catalytic site (26, 34). While orthoretroviral PRs form stable dimers (25, 34), conflicting data have been published on the monomer/dimer status of FV PR (9, 28). Nuclear magnetic resonance (NMR) spectroscopy showed that the macaque simian foamy virus PR, SFVmac PR, which was monomeric in solution, exhibited proteolytic activity at high salt concentrations, presumably because, at high salt, some dimers were formed (11). Recently, Hartl et al. (10) proposed that prototype FV (PFV) PR could form weak transient dimers in a small fraction of the total protein, which could have been missed in the published biochemical analyses. These results suggest that FV PR is a weak dimer and that efficient dimerization of PR *in vivo* requires other viral or cellular components.

Retroviral INs catalyze the specific and efficient integration of viral DNA into host genomic DNA (reviewed in reference 4). Retroviral INs form dimers or higher-order complexes (21). Recent structural analysis shows that FV IN forms a dimer on each end of the viral DNA and that the dimers associate to form tetramers, bringing the two ends of the viral DNA together for integration (8). We previously showed that mutations around the FV Pol cleavage site did not greatly affect the production of infectious particles from transfected cells but prevented replication in subsequent rounds of infection (30), suggesting that IN is not active as a PR-RT-IN fusion protein but that PR and RT are active before IN is cleaved.

Previously, we showed that a Pol mutant lacking IN was deficient in both the proteolytic cleavage of Gag and the packaging of Pol into virions (30). In this report, we provide evidence that the C terminus of IN contains a domain(s) that is required for Pol encapsidation and efficient PR dimerization. This dimerization is essential for the processing of Pol.

MATERIALS AND METHODS

DNA mutagenesis and cloning. The PFV used in this study is a chimpanzee FV isolated from a human-derived cell culture, which was previously designated human FV (HFV). Mutations in IN (see Fig. 1B) were generated using a

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full-length proviral clone containing a cytomegalovirus (CMV) immediate early promoter (pcPFV) (32). The IN stop mutants had a premature termination codon introduced at the position encoding amino acid (aa) 90, 120, 150, 210, 270, or 330 (90st mutant, 120st mutant, etc.). In order to insert a leucine zipper (zip) motif from the human CREB protein (23), the wt-AK mutant (see Fig. 2A) was constructed by engineering two unique restriction sites (AgeI and KpnI) after the codon for aa 120, resulting in the insertion of 5 additional residues (Thr-Gly-Ala-Gly-Thr) between aa 120 and 121. The 44-aa zip motif was cloned into the AgeI/KpnI sites of the wt-AK derivative of the full-length proviral vector and, in parallel, into a CMV-driven Pol expression vector (pCiPS) (33). Two mutant versions of the zip motif (Ezip and Kzip) were also inserted at this site. Ezip and Kzip have substitution mutations that introduce either 5 Glu or 7 Lys residues, respectively; these charged residues destabilize the zip structure and prevent homodimerization (23, 38).

Venus protein is a derivative of yellow fluorescent protein (YFP) and is composed of 238 aa that are split into two fragments (31). N-terminal Venus (Vn) contains the first 173 aa of the parental protein, and C-terminal Venus (Vc) contains 84 aa from aa 155 to 238. DNA segments encoding the split Venus proteins were introduced into the pCiPS Pol expression vector so that the split Venus proteins would be fused to the C terminus of wild-type (wt) IN or the truncated INs. pCiPS Pol-Venus fusion protein expression plasmids were constructed to express either active PR (PR^+) or inactive PR (PR^-) (which has a D24A mutation at the active site). All of the site-directed mutations in IN were generated by two rounds of PCR using four oligonucleotides as previously described (19). The two outer oligonucleotides were designed to anneal to the 5′ or 3′ end of the *pol* gene and had unique restriction sites at each end. The two inner mutagenic oligonucleotides (in either forward or reverse orientation) were designed to be complementary to the *pol* sequences except for the desired mutations. Each mutant construct was sequenced to confirm the presence of the correct mutations. Primer sequences will be supplied upon request.

Cell cultures and transfections. 293T cells and FAB cells (37) were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% bovine growth serum and 1% penicillin and streptomycin. Transient transfection was done using 1 mg/ml polyethyleneimine (Polysciences, Warrington, PA) as previously described (5). For FV 4-vector transfection, the pCiGS, pCiES, pCiPS, and pCiΔΦ plasmids (33), respectively, contain the PFV *gag*, *env*, or *pol* gene or express genomic RNA. Expression is driven by the CMV immediate early promoter of the pCI vector (Promega). These four plasmids were transiently transfected into 293T cells at a plasmid/weight ratio of 16:1:16:2 for pCiGS, pCiES, pCiΔΦ, and pCiPS, as suggested by Trobridge et al. (33). To measure the viral infectivity, a foamy virus-activated β-galactosidase (β-Gal) expression assay was performed as previously described (37). Briefly, the FAB indicator cell line has a single integrated copy of a long terminal repeat (LTR)-driven β-Gal gene. Infection of these cells causes the PFV LTR promoter to be transactivated by the viral Tas protein.

Western blot analysis. Cells and viral supernatants were harvested between 45 and 48 h posttransfection, unless otherwise indicated, and prepared as previously described (19). Cell lysates and viral pellets were resuspended in 1× SDS sample buffer (12.5% 4× Tris-HCl–SDS [pH 6.8], 10% glycerol, 2% SDS, 1% 2-mercaptoethanol, 0.01% bromophenol blue) prior to loading onto SDS-10% polyacrylamide gels. Western blot analyses were performed as previously described (1), using a 1:5,000 dilution of polyclonal rabbit anti-Gag antibody (1), a 1:800 dilution of monoclonal mouse anti-Pol antibody (32), or a 1:2,000 dilution of monoclonal mouse anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Proteins were visualized with an Odyssey detection system (Li-Cor, Lincoln, NE), according to the manufacturer's protocol. The Odyssey detection system was shown to have an 8-fold linear range (30). Intracellular protein levels were normalized to the level of GAPDH protein as an internal control.

RT-PCR. Forty-eight hours after transfection, equal amounts of virus-containing supernatants were treated with RNase-free DNase I, and then a QIAamp viral RNA minikit was used to isolate viral RNA (Qiagen). Extracted viral RNA (0.7 μg) was reverse transcribed using a poly(A) · poly$(dT)_{12}$ primer and ThermoScript RT (Invitrogen), according to the manufacturer's protocol. PCR amplification was performed using the same forward primer for both the unspliced *gag* and the spliced *pol* mRNA and a reverse primer specific for each RNA, giving PCR products of 501 nucleotides (nt) and 391 nt, respectively.

BiFC assay and flow cytometry analysis. 293T cells were grown in six-well plates and were transfected with a total of 3 μg of the bimolecular fluorescence complementation (BiFC) construct DNAs. For each set of construct DNAs, two sets of transfections were done. At 30 h posttransfection, fluorescent images were taken with one set of the transfected cells using a Nikon Eclipse TE300 inverted fluorescent microscope and processed with Metamorph software (Universal Imaging, Downingtown, PA). Subsequent to the image capture, cells were used for flow cytometry analysis. The cells were washed once with phosphate-buffered saline (PBS), detached with trypsin containing 2.5 mM EDTA, and resuspended in PBS. A minimum of 10,000 live events were acquired on a BD FACS Canto analytical platform (Becton Dickinson, San Jose, CA) and analyzed with FlowJo software (Treestar, San Carlos, CA) for yellow fluorescence. The other set of transfected cells were prepared for Western blot analysis.

Light scattering. The recombinant PFV PR-RT proteins were expressed in *Escherichia coli* and purified as previously described (29). Six histidine residues were added to the C terminus of the RNase H domain for protein purification. The purity was >97% as determined by SDS-PAGE and Coomassie staining. The native molecular masses of the recombinant proteins were measured by multiangle light scattering. Samples (50 μl) were diluted 1:3 in buffer containing 20 mM HEPES (pH 7.0), 300 mM NaCl, 100 mM imidazole, and 1 mM EDTA to a concentration of 1.25 mg/ml. The samples were injected into a 1.0-cm by 30-cm Superdex 200 column (GE Healthcare), which was run at 0.3 ml/min using a Rainin high-performance liquid chromatography (HPLC) pump. The column outlet was connected to a Rainin Dynamax UV-1 detector set at 280 nm (Varian); a Dawn EOS multiangle, static, light scattering detector (Wyatt Technology Corporation, Santa Barbara, CA); and an Optilab DSP interferometric refractometer (Wyatt Technology Corporation). Average molar mass measurements were determined from aligned elution profiles within ASTRA for Windows software (version 5.3.4.16; Wyatt), using the Debye plot. Detectors 5 (34.8°) to 16 (142.5°) were used. The monomeric form of bovine serum albumin (BSA) (Sigma) was used to align and fit peaks, normalize detectors, and validate the system. The value for the refractive index increment (*dn*/*dc*) used for calculations was 0.185 ml/g. Other constants used were 1.33 for the refractive index of the solvent and 681 nm for the laser wavelength.

RESULTS AND DISCUSSION

FV PR-RT protein is primarily monomeric in solution. We previously reported the development of *E. coli* expression plasmids that induced the synthesis of a recombinant PFV PR-RT protein in which PR was inactivated by an amino acid substitution at the active site (D24A) (29). This recombinant PR-RT protein had RNA-dependent polymerase, DNA-dependent polymerase, and RNase H enzymatic activities, but, as expected, it did not have PR activity, because of the mutation at the active site (2). High-performance liquid chromatography followed by light scattering showed that the majority of the material had an average molecular mass of 87,500 Da, which corresponds to the monomeric form of PFV PR-RT (Fig. 1A), confirming the results obtained with SFVmac PR-RT (11) and PFV PR-RT (9). These results suggest that other viral or cellular components are needed for efficient dimerization of PR and, by extension, for PR enzymatic activity.

C-terminal deletion of the IN domain disrupts PR proteolytic activity in cells and packaging of Pol into virions. We previously showed that a Pol mutant lacking the IN domain was deficient in both the proteolytic cleavage of Gag and the packaging of Pol into virions (30). In order to test the possibility that IN contains regions important for PR activity and Pol packaging, a series of C-terminal IN truncation proteins were expressed by introducing a stop codon into the full-length proviral genome (Fig. 1B). The IN truncation constructs were transiently transfected into 293T cells, and equal amounts of cell lysates and pelleted viral supernatants were analyzed by Western blotting (Fig. 1C and D). All of the IN truncation mutants expressed similar levels of Gag in cells and efficiently released Gag particles into the medium, except for the 270st mutant, which appears to be deficient in particle release (Fig. 1C). FV PR partially cleaves the wt Gag proteins. Both the p68 and p71 Gag bands were detected in both the wt and an IN

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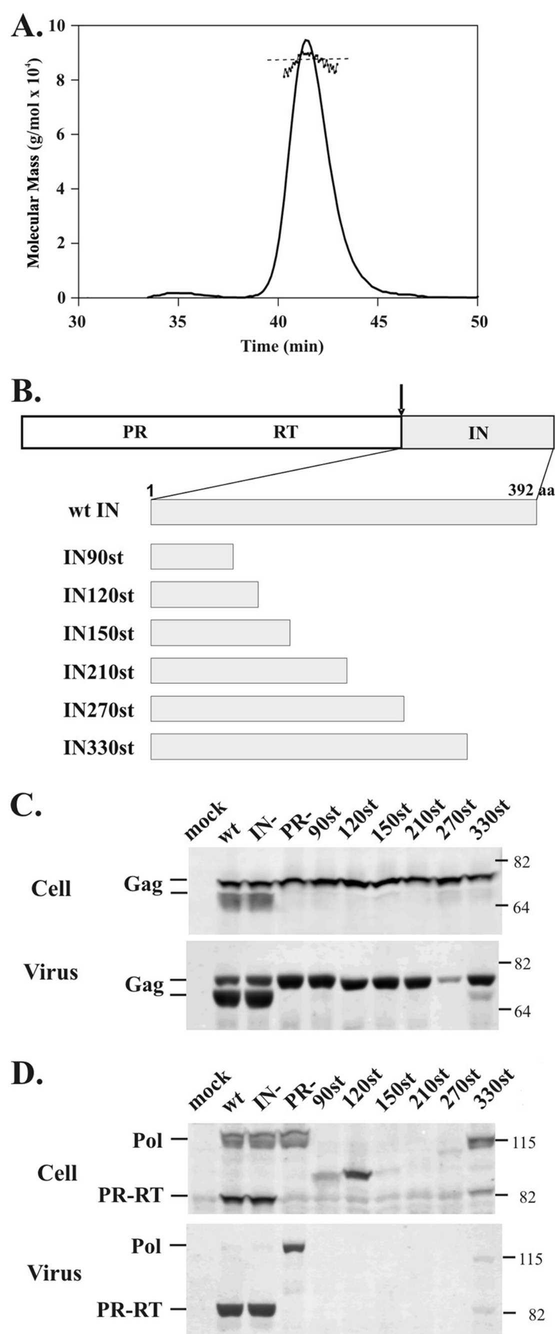

FIG. 1. The native molecular mass of FV PR-RT was determined by light scattering. (A) Purified recombinant PR-RT was prepared and loaded onto a 1.0-cm by 30-cm Superdex 200 column connected to an Optilab DSP interferometric refractometer followed by a Dawn EOS multiangle light scattering detector, as described in Materials and Methods. The majority of the protein elutes from the column as a single peak at approximately 42 min (solid line). Light scattering measurements were taken at various times as protein eluted from the column. The molecular masses obtained from these light scattering measurements are plotted as a series of points across the top the of the protein peak; the average molecular mass is given as a dotted line. (B) IN truncation mutant constructs. The FV Pol polyprotein consists of protease (PR), reverse transcriptase (RT), and integrase (IN) and is cleaved once between RT and IN by PR, as shown by the arrow. IN contains 392 aa. IN truncation constructs were made in the full-length proviral vector under a CMV immediate early promoter (pcPFV) (32). Each IN truncation mutation was engineered by inserting a premature termination codon at the position encoding aa 90, 120, 150, 210, 270, or 330. (C and D) The DNA constructs shown in panel B were transfected into 293T cells, and 45 to 48 h posttransfection, cell lysates and pelleted virions were collected and separated on 9% SDS-polyacrylamide gels. Western blots were prepared and probed with either anti-Gag antibody (C) or anti-Pol antibody (D). Equal amounts of the cell lysates were loaded onto the gel based on the levels of GAPDH (data not shown). The Odyssey system was used for detection. Two control DNAs were used; IN$^-$ encodes a Pol protein with a point mutation in the second aspartic acid in the D,D-35-E motif of the IN active site (24), and PR$^-$ encodes a Pol protein with a D24A mutation in the active site of PR. Molecular mass markers in kilodaltons are shown.

mutant that contained a point mutation in the active site (IN-, Fig. 1C), whereas only uncleaved Gag was found in a PR active site mutant (PR-, Fig. 1C). Most of the IN truncation mutants produced only the Gag precursor, although the virions of the 330st mutant contained low levels of cleaved Gag. Cells expressing the 120st and 330st mutants contained less Pol than the wt (Fig. 1D). Cells expressing most of the IN mutants contained low or undetectable levels of Pol. In cells, the wt Pol protein was partially cleaved between RT and IN, producing p85 PR-RT. The 120st mutant failed to cleave Pol, whereas the cells expressing the 330st mutant processed Pol inefficiently. A background band migrates at the same position as PR-RT (Fig. 1D, cell). The 120st Pol was not incorporated into virions, and the amount of Pol packaged by the 330st mutant was strongly reduced compared to that for the wt. Thus, 120st was deficient in Pol cleavage in cells and Pol packaging into virions, suggesting that IN sequences that are not present in 120st play a role in both of these functions.

The C terminus of IN contains a domain required for PR activity. To test the possibility that IN sequences missing in the 120st mutant facilitate Pol dimerization, enhancing PR activity, we introduced a leucine zipper (zip) dimerization motif from the human CREB protein (23) downstream of 120st IN in the context of the full-length viral genome (Fig. 2A). As a control, a "pseudo-wt" virus was constructed with two unique restriction sites (AgeI and KpnI) after aa 120, resulting in the insertion of 5 additional residues (Thr-Gly-Ala-Gly-Thr); this virus (wt-AK) replicates about 50-fold less well than the wt (Fig. 2A and Table 1). Wild-type zip (wtzip) or the two mutant zips (Ezip and Kzip), both of which have substitution mutations that introduce multiple charged residues in adjoining, mostly hydrophilic residues to prevent the formation of homodimers (23, 38), were cloned into the AgeI/KpnI sites of wt-AK, followed by a stop codon. The zip constructs were transfected into 293T cells, and Western blot analysis was performed to examine the proteolytic processing of Gag and Pol and the encapsidation of Pol into virions (Fig. 2B and C). The levels of Gag in cells and supernatants were approximately the same for wt-AK, 120st-wtzip, 120st-Ezip, and 120st-Kzip (Fig. 2B). Although the Gag protein of wt-AK was processed as well as that of the wt both in cells and in virions, there was no Gag cleavage with 120st-wtzip or the two mutant zips. However, 120st-wtzip showed some Pol processing in cells (Fig. 2C). In contrast, neither of the two mutant zips was able to cleave Pol. Neither 120st-wtzip nor the mutant zips packaged Pol protein into virions. The infectivity data are summarized in Table 1. The wt-AK virus was infectious, but the titer was reduced compared

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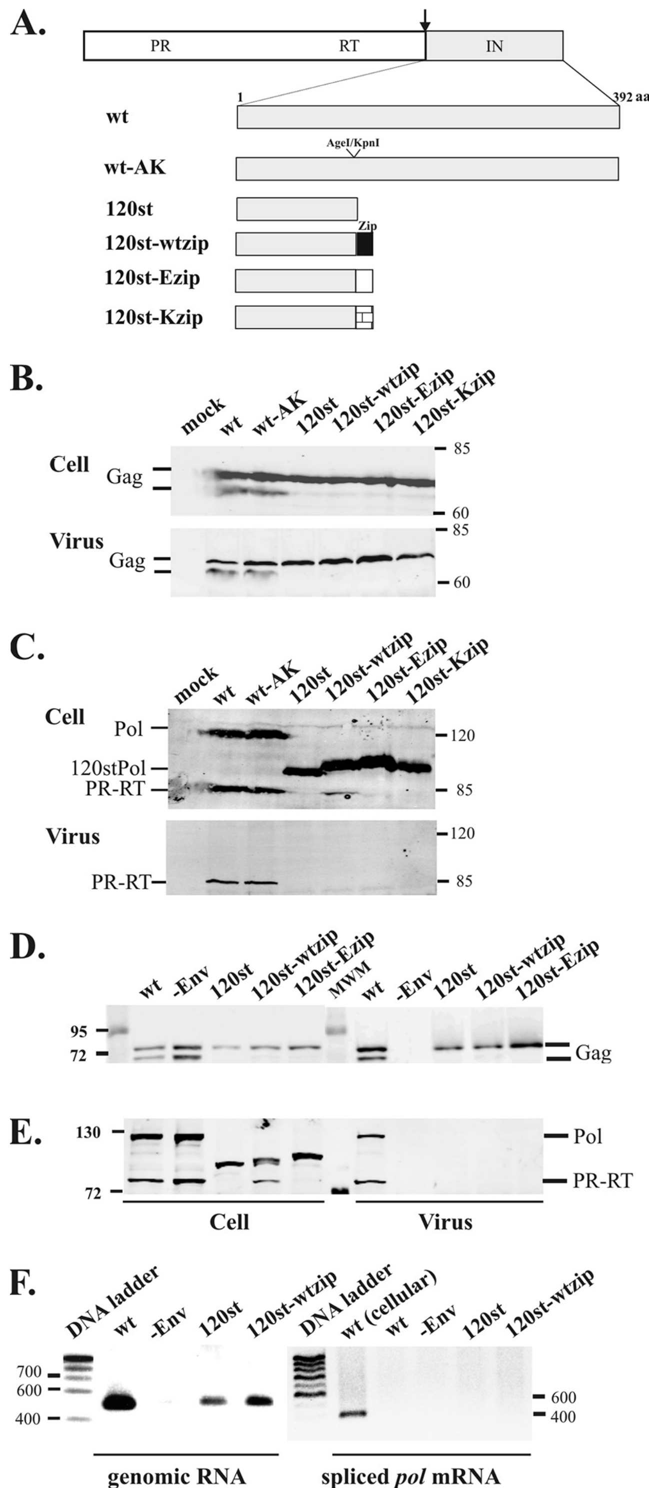

FIG. 2. Leucine zipper motif Pol constructs and analyses of Pol protein expression in cells and packaging into virions. (A) wt-AK has unique restriction endonuclease sites inserted near the codon for aa 120 to allow the insertion of sequences encoding a leucine zipper (zip) dimerization motif or two mutant zips that cannot dimerize (Ezip and Kzip). 293T cells were transfected with construct DNAs, and 45 to 48 h posttransfection, cell lysates and pelleted virions were obtained and separated on 9% SDS-polyacrylamide gels. (B to E) Western blots were probed with either anti-Gag antibody (B and D) or anti-Pol antibody (C and E). Equal amounts of the cell lysates were loaded onto the gel after normalization based on the levels of GAPDH (data not shown). The Odyssey system was used for detection. For experiments with results shown in panels B and C, the IN mutations were introduced into the full-length proviral vector. In the 4-vector transfection system (D to F), the IN mutations were constructed in the Pol expression plasmid (pCiPS) (33). In the control for viral release, −Env, the parental pCI vector was used in place of the Env expression vector (pCiES) (33). Molecular mass markers in kilodaltons are shown. (F) An RT-PCR assay was performed using RNA samples extracted from virions as described in Materials and Methods. The RNA extracted from cells transfected with the wt was used as a positive control for detection of spliced RNA. The sizes of the DNAs in the DNA ladders are given in base pairs.

TABLE 1. Properties of PFV IN mutants

Construct	Proteolytic processing[a]		Pol packaging[b]	Infectivity[c] (IU/ml)
	Gag	Pol		
wt	++	++	++	$(4.9 \pm 1.0) \times 10^5$
wt-AK	++	++	++	$(1.0 \pm 0.3) \times 10^4$
120st	−	−	−	<1
120st-wtzip	−	+	−	11 ± 3
120st-Ezip	−	−	−	<1
120st-Kzip	−	−	−	<1
330st	+	+	+	$(5.0 \pm 0.6) \times 10^2$

[a] The ability of PR to process Gag and Pol proteins is summarized from Western blot analysis of both cell lysates and pelleted virions (Fig. 1 and 2) from four independent assays. ++, wt activity; +, inefficient processing; −, no processing.

[b] The Pol packaging data were obtained using pelleted virions (Fig. 1 and 2) from four experiments. ++, wt activity; +, inefficient processing; −, no processing.

[c] Infectivity was measured by the foamy virus-activated β-galactosidase (β-Gal) expression assay as previously described (37). Values are the averages ± standard deviations from four independent assays.

to that of the wt. The 330st mutant retained a low level of infectivity, 1,000-fold less than that of the wt. However, neither 120st-wtzip nor the mutant zips 120st-Ezip and 120st-Kzip were infectious. The results show that the IN mutants, which lack PR activity and fail to package Pol, are not infectious.

Genomic RNA is required for Pol packaging into virions (12). Two *cis*-acting RNA sequences, called Pol encapsidation sequences (PES), have been identified. One PES is located at the 3′ end of the *pol* gene (27). This PES overlaps with one of the two *cis*-acting sequences, called CASII, that are important for genomic RNA packaging. In order to exclude the possibility that the deficiency in Pol packaging observed with the 120st mutants was due to the absence of sequences required for encapsidation or to an effect on the PES, we used a 4-vector FV system. The 4-vector system separately expresses the components needed for virus assembly: Gag, Pol, Env, and genomic RNA. The Pol proteins containing the mutated INs were expressed from the Pol expression vector pCiPS (33). 293T cells were transiently transfected with the four vectors, and 48 h posttransfection, cell lysates and pelleted supernatants were prepared and used for Western blot analysis (Fig. 2D and E). Gag proteins from all samples were expressed in cells, and with the exception of the control lacking Env (−Env), viruses were released into the supernatants (Fig. 2D). There was efficient Pol processing when 120st-wtzip was ex-

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pressed as part of the 4-vector system (Fig. 2E). However, neither Gag cleavage nor Pol packaging into virions was found with any of the 120st derivatives. These results are consistent with the results obtained with the full-length proviral vector. RT-PCR was used to measure the levels of genomic RNA and spliced *pol* mRNA in virions (Fig. 2F). No viral RNA was detected in the −Env control. The levels of genomic RNA in the virions produced with 120st and 120st-wtzip were reduced relative to that for the wt; however, the overall levels of genomic RNA packaging should be normalized with the reduced levels of intracellular and extracellular Gag compared to the wt (Fig. 2D). As expected, the spliced *pol* mRNA was not detected in any of the viral samples. Thus, addition of a dimerization motif to the 120st mutant can restore the ability of PR to process Pol. However, the addition of a dimerization motif to the truncated Pol was not sufficient to restore either Gag processing or Pol packaging, despite the fact that the virions could package viral RNA. This result suggests that the C terminus of IN is required for Pol to associate (either directly or indirectly) with Gag. Previously, we showed that the C terminus of Gag contains determinants required for Pol packaging, which suggests that Pol binds to Gag or RNA (or both) for incorporation into virions (19).

IN sequences deleted in the 120st mutant contain a Pol interaction domain that enhances PR activity. In order to determine whether the addition of the wtzip motif to the C terminus of 120st leads to Pol dimer formation, we performed bimolecular fluorescence complementation (BiFC) assays to measure protein interactions in living cells. This approach is based on complementation between two split fragments of Venus protein, a yellow fluorescent protein (YFP) variant, which are fused to the two proteins of interest (13, 16, 31). If the two proteins of interest interact, the two halves of the Venus protein are brought together, reconstituting the fluorophore. The Pol-Venus fusion proteins were made by attaching the Venus fragments to the C terminus of the various Pol proteins (Fig. 3A). When we expressed the Pol-Venus fusions in a full-length provirus, we saw relatively few fluorescent cells (data not shown); this is presumably because FV Pol is normally present only at low levels in infected cells (22, 36). To get higher levels of the Pol-Venus fusions, we expressed the proteins under the control of a CMV immediate early promoter. The Pol-Venus fusion constructs were transfected into 293T cells either individually or in pairs. Western blotting results showed that the transfected cells contained similar amounts of each of the fusion proteins (Fig. 3B). Besides the Pol-Venus fusion proteins, there is a protein band that corresponds in size to PR-RT. The fusion proteins (but not the cleaved PR-RT) appear as doublets in the blots; it is possible that the Venus portion of the fusion proteins was subject to posttranslational modification. The wt Pol fusion was cleaved reasonably efficiently, and the various Venus fusions were cleaved to various extents. These data show that PR has some activity in all of the fusions, which demonstrates that even the 120st fusion proteins can form weak dimers, suggesting that overexpression of the fusion proteins enhances dimerization.

The cells were analyzed by both fluorescence microscopy and fluorescence-activated cell sorting (FACS) (Fig. 3C and Table 2). CD8α is known to form homodimers, and CD8α-Venus fusions have been used as controls in the BiFC assay to study HIV-1 assembly (3). Cells cotransfected with CD8α-Vn and PFV Gag-Vc were completely negative for fluorescence, as were cells transfected with only one of the Vn or Vc fusion constructs (data not shown). There was strong fluorescence from cells cotransfected with CD8α-Vn and CD8α-Vc. The fluorescence data obtained by expressing the Pol fusion proteins with wt PR reflect the fact that the cells contain a mixture of the cleaved and uncleaved proteins. To address this problem, the experiments were repeated with a PR^- Pol expression construct (Fig. 3D). Expression of both the PR^+ and the PR^- version of full-length (wt) Pol showed similar high levels of fluorescence. This suggests that both the uncleaved Pol-Venus fusions and the cleaved wt IN-Venus fusions formed good dimers. In contrast, only the PR^- version of the 120st Pol-Venus fusions showed good fluorescence; the fluorescence in the PR^+ experiment was much weaker. This suggests that even when expressed at a high level, the cleaved 120st IN-Venus fusions dimerized only very weakly, while the uncleaved 120st Pol-Venus fusions formed good dimers. The presence of the additional PR, RT, and Venus sequences in the 120st Pol fusion allowed it to dimerize if the fusion protein was present in a high enough concentration. In contrast, 120st-wtzip showed good fluorescence both in the presence and in the absence of an active PR, showing that the wtzip domain caused both the uncleaved 120st Pol-wtzip-Venus fusions and the cleaved 120st IN-wtzip-Venus fusions to dimerize. The 120st-Ezip-Venus fusions did not show any fluorescence.

The BiFC data on the 120st mutant appear to contradict the apparent lack of PR activity when the 120st Pol was expressed using the proviral transfection system. However, there are two important differences in these two assays: (i) the Venus fusion proteins were expressed at a much higher level in the CMV-driven BiFC system than is normal for Pol expressed from a PFV provirus (36), and (ii) the presence of the Venus protein could stabilize a weak interaction of the truncated Pol protein. Kinetic studies of the BiFC formation demonstrated that the initial interactions, mediated by contacts between the proteins fused to the split Venus fragments, are stabilized by association of the two Venus fragments (14, 31). Thus, the BiFC system may detect (and stabilize) PFV PR dimers that form transiently and are normally present at low levels (10). Taken together, the data suggest that although the PR-RT protein is almost all monomeric, it has a limited ability to dimerize. Our data suggest that the addition of IN promotes dimerization of even the low levels of PR-RT present during a PFV infection in cells.

Correct temporal and spatial regulation of protease activity is crucial for the retroviral life cycle. In the assembled HIV-1 virion, PR cleaves itself from the Gag-Pol polyprotein (15, 35). Before the virion assembles, PR activity in the HIV-1 Gag-Pol precursor is low, and the Gag and Pol proteins are not significantly cleaved. When PR is cleaved to its mature form after the virion assembles, Gag-Pol and Gag are processed, leading to the formation of infectious mature virions. In the case of FV, we propose that when PR is part of Pol, Pol (and, by extension PR) can form dimers because of IN-IN interactions. The dimerization of Pol creates a PR active site, leading to PR cleavage of Gag and Pol. Although the exact order of the Gag and Pol cleavages is not known, it is likely that Gag cleavage occurs prior to Pol cleavage, because Pol cleavage releases IN,

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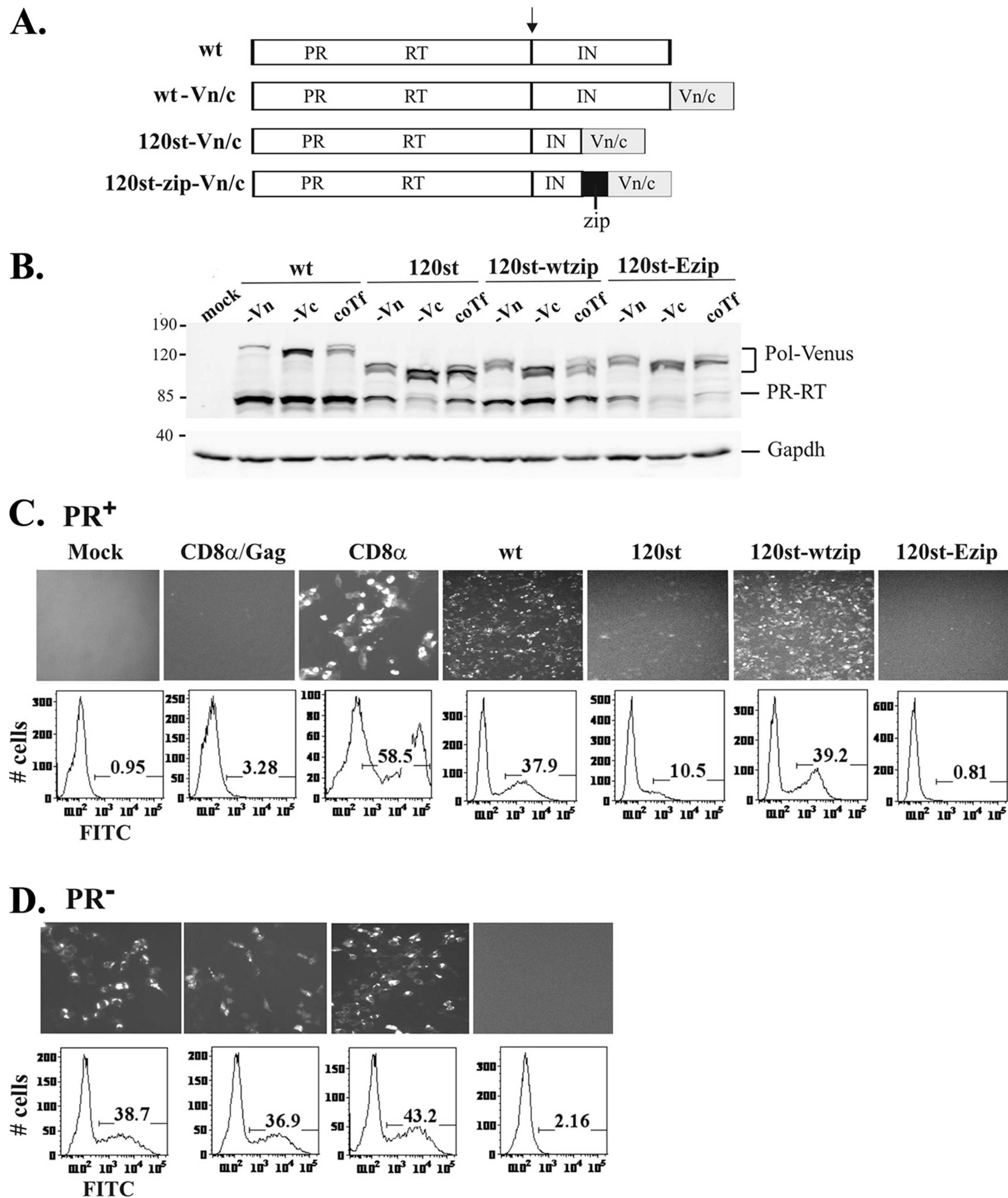

FIG. 3. BiFC assay for protein dimerization. (A) Two split Venus fragments, Vn and Vc, were fused to the C terminus of Pol in the wt and in the 120st, 120st-wtzip, and 120st-Ezip mutants. BiFC constructs were made in the pCiPS vector. The Pol polyprotein is normally cleaved at the site between RT and IN by PR, as shown by the arrow. (B) Western blot analysis for protein expression of BiFC constructs in the presence of an active PR. BiFC construct DNAs were transfected into 293T cells, and 30 h posttransfection, cell lysates were prepared and separated on 10% SDS-polyacrylamide gels. Blots were probed with anti-Pol antibody to detect the Pol-Venus fusions. GAPDH was detected by antibody and served as an internal control. The Odyssey detection system was used. Lanes labeled -Vn or -Vc show lysates from cells transfected with the individual split Venus fusion constructs, and coTf indicates cells transfected with the indicated Vn and Vc pairs. The intensities of both precursor and PR processed bands of the fusion proteins were quantitated by using Image J software. The expression levels in all of the mutants ranged from 0.8- to 1.2-fold of the expression level in cells transfected with wt Vn alone. (C and D) The images of fluorescent cells were captured using an inverted fluorescence microscope at 30 h posttransfection. Cells were then collected and analyzed by FACS. A minimum of 10,000 cells were examined, and all samples were gated at the same window using the mock sample. The percentage of fluorescence-positive cells in a typical experiment is indicated in each graph. CD8α-Venus and PFV Gag-Venus fusions were used as controls for BiFC. BiFC assays were performed in the presence of active PR (C) and inactive PR (D). FITC, fluorescein isothiocyanate.

resulting in the production of a PR-RT protein that is predominantly a monomer (Fig. 1) (9). After cleavage of Pol, free IN is active (30), and PR-RT has very high levels of reverse transcriptase and RNase H activity (2, 29). It is possible that weak dimers of PR-RT, present at a low level in virions, account for the report of low levels of PFV Gag cleavage in newly infected cells (20). We propose that FV PR activity depends on the dimerization of Pol during the assembly process and is then

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TABLE 2. Percentages of fluorescent cells, as measured by FACS analysis[a]

Construct	% of fluorescent cells	
	PR+	PR−
wt	100	100
120st	18 ± 8	89 ± 12
120st-wtzip	123 ± 28	117 ± 34
120st-Ezip	0.4 ± 0.3	2.6 ± 1.2

[a] Two sets of BiFC constructs were made, with active PR (PR+) and inactive PR (PR−). The percentages of fluorescent cells (relative to that for the wt) are shown as the averages ± standard deviations from four independent assays. The pairs CD8α-Vn and CD8α-Vc or CD8α-Vn and PFV Gag-Vc were used as controls. The relative levels of fluorescent cells were 174% ± 40% for CD8α-Vn and CD8α-Vc and 4.9% ± 3.1% for CD8α-Vn and PFV Gag-Vc.

downregulated after assembly is complete, thus protecting critical virion components (including Gag) from unwanted PR cleavages that might occur if FV PR remained dimeric and active in mature virions.

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