Elucidation of the Molecular Consequences of Two Unique p6Gag Mutations Derived from HIV-1 CRF07_BC-infected Patients

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Research

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Abstract

Background We previously observed that individuals infected with HIV-1 CRF07_BC showed slower disease progression than those infected with HIV-1 subtype B or CRF01_AE. CRF07_BC viruses carry two unique mutations in the p6 Gag protein: insertion of PTAPPE sequences downstream of the original Tsg101 binding domain, and deletion of a seven-amino-acid sequence (30 PIDKELY 36) that partially overlaps with the Alix binding domain. To further define the role of these mutations in virus release and replication, we introduced them into the HIV-1 proviral clone pNL4-3 for functional characterization.

Results We found that the seven-amino-acid deletion, but not the PTAPPE insertion, significantly decreased virus release, Gag processing, and virus infectivity. The seven-amino-acid deletion also resulted in a virus replication defect in both T-cell lines and peripheral blood mononuclear cells. We found that these defects were caused by the seven-amino-acid deletion in p6 Gag , especially deletion of Tyr-36 of p6 Gag , not the deletion of the overlapping p6* sequence in the HIV-1 GagPol protein. The p6 Gag deletion mutant was resistant to a dominant-negative Alix fragment, suggesting a loss of binding between p6 Gag and Alix.

Conclusions Our results indicate that the patient-derived seven-amino-acid deletion in p6 Gag of HIV-1 CRF07_BC virus affects virus release, infectivity and replication capacity by disrupting the interaction between HIV-1 p6 Gag and host protein Alix. These results may explain the slower disease progression observed in the subjects infected with HIV-1 CRF07_BC bearing this unique mutation.

Background

CRF07_BC, a circulating recombinant form (CRF) of human immunodeficiency virus type one (HIV-1), was first identified in 2000 and has become one of the most commonly transmitted viruses in China[1–3]. In 2007, Lin et al. described a unique deletion mutation of 7 amino acids (aa) ($_{30}$PIDKELY$_{36}$) in the p6^{Gag} protein of HIV-1 CRF07_BC isolates [4]. This 7-aa region partially overlaps with the host ALG-2 interacting protein X (Alix) binding domain, YPXnL, and the 7-aa deletion variant (Δ7) exclusively exists in HIV-1 CRF07_BC isolates [4, 5]. We previously reported that about 54% of CRF07_BC sequences from the Los Alamos National Laboratory database and 92% of the sequences from CRF07_BC-infected men who have sex with men (MSM) carry this Δ7 mutation[5]. In addition, we found that 26% of CRF07_BC viruses carry another insertion mutation of 6 aa, PTAPPE (insPTAP), in the p6^{Gag} protein downstream of the PT/SAP motif, which serves as the binding site for the host protein tumor susceptibility gene 101 (Tsg101) [5]. CRF07_BC isolates with double mutation of Δ7 and insPTAP have been observed [5].

Previous studies indicated that patients infected with the CRF07_BC Δ7 variant exhibited lower viral loads and slower disease progression compared with individuals infected with HIV-1 subtype B or CRF01_AE [6–10]. Previous in vitro studies showed that HIV-1 isolates and the infectious clone of CRF07_BC with the Δ7 mutation displayed relatively lower replication capacity and slower replication kinetics than HIV-1 subtype B or Thai B’ [7, 8]. Lin et al. found that the Δ7 mutation resulted in a defect in virus particle release and Gag processing, which in turn caused the accumulation of immature virions on the plasma membrane [8]. These results suggest that the Δ7 mutation in p6^{Gag} may affect the late events of the HIV-1 replication cycle and viral infectivity. However, the detailed molecular mechanisms contributing to these defects have not yet been investigated.

The HIV-1 Gag polyprotein precursor Pr55^{Gag} is sufficient for the assembly of virus-like particles (VLP). Once HIV-1 particles are released from infected cells, the Gag polyprotein is cleaved by the viral protease (PR) into several mature viral Gag proteins: matrix (MA), capsid (CA), nucleocapsid (NC), p6, and two spacer peptides, spacer 1 (SP1) and spacer 2 (SP2). PR-mediated Gag processing triggers virus maturation and is essential for the conversion of the immature VLP to the infectious virion [11, 12]. The p6^{Gag} domain is required for virion budding-off from the plasma membrane through the action of its two

highly conserved late domain motifs: PT/SAP and YPXnL [11, 12]. The PT/SAP motif plays a major role in HIV-1 release by binding to Tsg101, a component of the endosomal sorting complex required for transport I (ESCRT-I) [11, 13–15]. Mutation of the PT/SAP motif results in a severe defect in virus budding [16, 17]. The YPXnL motif regulates virus release by directly binding to the ESCRT-associated host protein Alix [18, 19]. Mutations that block YPXnL-Alix binding or overexpression of Alix disrupt virus replication and virion production [18–22]. Recently, Ajasin et al. reported that the CC chemokine ligand 2 (CCL2) can mobilize the Alix protein away from F-actin structures to the cytoplasm to enhance virion release in the presence of the LYPX motif in HIV-1 $p6^{Gag}$ [23]. This new finding may explain the lower replication capacity and slower disease progression in subjects infected with HIV-1 subtype C (HIV-1C) due to the absence of LYPX motif in HIV-1C [24, 25]. In addition, the C-terminal region of p6 overlaps with the N-terminal region of pol, referred to as $p6^{*}$, as a result of a -1 ribosomal frameshift that occurs during translation of the gag open reading frame (ORF) [26–28]. Thus, the 7-aa PIDKELY deletion in $p6^{Gag}$ also results in deletion of amino acids DRQGTVS in $p6^{*}$ (Fig. 1), which is 3 amino acids away from p6*-PR cleavage site. It has been reported that PR activation and/or PR-mediated maturation could be affected by the substitution of four amino acids, SFNF, near the p6*-PR cleavage site [29, 30].

We hypothesize that the two $p6^{Gag}$ late domain-related mutations described above, insPTAP and Δ7, may affect virus release by disrupting the interaction of $p6^{Gag}$ with Tsg101 and Alix, respectively. In addition, the overlapping 7-aa deletion of DRQGTVS in $p6^{*}$ may affect the activity of HIV-1 PR. In this study, we introduced these mutations into a full-length, infectious HIV-1 proviral clone and characterized their role in virus release and replication. We found that the 7-aa deletion in $p6^{Gag}$ (Δ7), but not the PTAP insertion (insPTAP), significantly decreased virus release, Gag processing, virus infectivity and replication. We further demonstrated that the Δ7 mutation in $p6^{Gag}$, especially the deletion of tyrosine 36 (Y36), but not the corresponding deletion mutation in $p6^{*}$, caused the observed defects by disrupting the interaction between HIV-1 $p6^{Gag}$ and Alix. These defects likely contribute to the slower disease progression observed in subjects infected with the HIV-1 CRF07_BC Δ7 variant.

Results

HIV-1 p6 ^{Gag} deletions impair virus release, infectivity and replication

The deletion of the Tsg101-binding motif PTAP (ΔPTAP), insPTAP, Δ7, and PΔ7 $p6^{Gag}$ mutations (Fig. 1) were introduced into the full-length HIV-1 molecular clone pNL4-3. Wild type (WT) and mutant clones were transfected into 293T cells (Fig. 2a). Western blotting (WB) of cell and viral lysates was performed (Fig. 2a), and virus release and Gag processing efficiencies were quantified (Fig. 2b and c). By quantifying the p24 levels in virions relative to total Gag, we determined that deletion of the Tsg101-binding motif PTAP (ΔPTAP) severely impaired virus particle production (Fig. 2b), as reported previously [16, 17]. However, the PTAPPE insertion (insPTAP) downstream of the original PTAP motif did not significantly affect virus release (Fig. 2b) except for a slightly increased tolerance to overexpression of Tsg101 (data not shown). The 7-aa deletion (Δ7) and the double mutation PΔ7 moderately inhibited virus production, to ~ 77% and ~ 62% of the WT level, respectively (Table 1, Fig. 2b). Similar results were obtained when the reverse transcriptase (RT) activity of culture supernatants was measured to determine the efficiency of virus release (Table 1). In addition, the efficiency of Gag processing was ~ 73%, 73%, 52% and 37% for ΔPTAP, insPTAP, Δ7 and PΔ7, respectively (Fig. 2c). These results demonstrate that the 7-aa deletion and the double mutation PΔ7, but not the PTAP duplication in $p6^{Gag}$, cause defects in virus release and Gag processing.

Table 1

Phenotypes of HIV-1 wild-type and p6 mutant virions

Virus[a]	Virus Release[b]		Maturation (p24/Pr55)%	Infectivity[b] RLU[e] (%)	Replication Capacity relative to WT		Reverse Transcription[b]		
	RT[c] (%)	VRE[d] (%)			days of peak relative to WT	peak RT level	Initiation (R-U5) %	Minus-strand DNA (U3-U5/ R-U5) %	Plus-strand DNA (R-5'UTR/ R-U5) %
WT	100	100	100	100	-	-	100	82.0 ± 13.6	79.0 ± 6.5
ΔPTAP	15.9 ± 14.6	1.9 ± 1.4	72.9 ± 40.3	-	delayed in Sup-T1, MT-4 no replication in PBMC	reduced	-	-	-
PΔ7	63.9 ± 26.1	62.1 ± 5.1	36.5 ± 13.9	69.1 ± 18.6	delayed in Sup-T1, MT-4	reduced	87.6 ± 25.3	63.8 ± 6.6	65.8 ± 4.9
insPTAP	108.8 ± 25.3	113.4 ± 15.4	73.4 ± 20.6	113.5 ± 20.9	near-WT level	near-WT level	117.8 ± 51	67.7 ± 17.6	78.0 ± 4.3
Δ7	73.0 ± 11.4	76.9 ± 11.0	52.0 ± 3.2	56.3 ± 3.6	delayed in Sup-T1, MT-4	reduced	57.2 ± 9.8	81.3 ± 13.3	66.2 ± 5.7

[a]WT, wild type; ΔPTAP, deletion of original PTAP motif; insPTAP, insertion of PTAPPE sequences; Δ7, deletion of 7-aa in p6; PΔ7, double mutation of insPTAP and Δ7.

[b]The levels of p6 mutants were expressed as a percentage relative to WT level, which was arbitrarily set as 100%.

[c]RT, reverse transcriptase activity

[d]VRE, virus release efficiency

[e]RLU, relative luciferase unit

We further investigated the infectivity of these $p6^{Gag}$ mutants and found that the insPTAP mutant showed levels of virus infectivity similar to those of WT in the TZM-bl system (Table 1). The infectivity of the mutant Δ7 and the double mutant PΔ7 was ~ 56% and 69% WT level, respectively (Table 1). We next analyzed virus replication kinetics in the SupT1 and MT-4 T-cell lines and in primary human peripheral blood mononuclear cells (PBMCs) from two donors. As expected, the ΔPTAP mutant showed very low-level and delayed replication kinetics in the T-cell lines (Fig. 3a and b), and no replication was observed in PBMCs (Fig. 3c and d). The insPTAP mutant was replication competent in both T-cell lines and PBMCs with no major difference from WT (Fig. 3). However, the Δ7 and PΔ7 mutants were defective in virus replication in SupT1 and MT-4 T cells (Fig. 3a and b, Table 1), while low-level replication was also observed in PBMCs from two different donors (Fig. 3c and d, Table 1). The defects in viral replication were consistent with impairment of reverse transcription, in particular the

initiation of reverse transcription (Fig. 4a), but not elongation (Fig. 4b), compared to WT. We found that the mutant insPTAP did not affect initiation of reverse transcription while the mutants PΔ7 and Δ7 reduced the initiation efficiency to ~ 88% and 57% of WT level, respectively (Fig. 4a). These results demonstrate that the 7-aa deletion and the double mutation PΔ7, but not the PTAP duplication mutation in $p6^{Gag}$, result in defects of virus infectivity and replication.

Deletion mutation in $p6^{Gag}$ but not in p6* is responsible for the defects in virus release and Gag processing

The 7-aa PIDKELY deletion in $p6^{Gag}$ also results in deletion of amino acids DRQGTVS in p6* (Fig. 1). To determine the effects of the deletion mutation in $p6^{Gag}$ and p6* in virus release and Gag processing, we constructed several HIV-1 pNL4-3/KFS clones expressing $p6^{Gag}$ with the 7-aa deletion (GagΔ7) and p6* with the 7-aa deletion (GagPolΔ7). The GagPol construct expresses GagPol but does not express Gag, as the result of a 1-nucleotide insertion in the frameshift region that places gag and pol in the same ORF [31]. The Gag- and GagPol-expressing plasmids were co-transfected into 293T cells at a ratio of 15:1 to generate viral particles with a similar ratio of Gag to GagPol proteins as normal HIV-1 particles [31] (data not shown). We found that the deletion mutation in p6* did not significantly inhibit virus particle production or Gag processing (Fig. 5). In contrast, the deletion in $p6^{Gag}$ resulted in a decrease in virus release and Gag processing efficiency to ~ 47% of the WT level (Fig. 5b and c). Furthermore, the deletions in $p6^{Gag}$ and p6* did not affect the incorporation and processing of GagPol protein (data not shown). These results indicate that the 7-aa deletion in $p6^{Gag}$, but not the deletion in the p6* domain, impairs virus release and Gag processing.

Tyrosine 36 (Y_{36}) in $p6^{Gag}$ is critical for virus release and Gag processing

It has been reported that mutation Y36A in $p6^{Gag}$ markedly impaired virus particle production and Gag processing [20], demonstrating an important role of Y36 in controlling HIV-1 release and Gag processing. Consistent with these results, we observed that deletion of Y_{36} (ΔY) severely impaired virus production and Gag processing (Fig. 6a). The efficiency of virus release and Gag processing was ~ 20% (Fig. 6b) and 29% (Fig. 6c) of the WT level, respectively. In contrast, the level of virus release and Gag processing for the 6-aa ($_{30}$PIDKEL$_{35}$) deletion mutation (Δ6) was ~ 80% (Fig. 6b) and 108% (Fig. 6c) relative to WT, respectively. These data indicate that Y_{36} residue is critical in regulating virus release and maturation. Furthermore, the deletion of 6-aa ($_{30}$PIDKEL$_{35}$) sequences upstream of Y_{36} can partially restore the defects caused by Y_{36} deletion.

The deletion mutation in $p6^{Gag}$ is resistant to overexpression of the Alix V domain

The Alix binding domain $_{36}$YPXnL$_{41}$ in $p6^{Gag}$ promotes virus release through the interaction between HIV-1 $p6^{Gag}$ and host protein Alix [18]. The central, so-called “V” domain of Alix is responsible for binding the $_{36}$YPXnL$_{41}$ in $p6^{Gag}$ [18, 21, 32]. As a result, overexpression of the Gag-binding V domain of Alix (Alix V) potently disrupts particle budding by binding directly to HIV-1 Gag [21, 22, 33]. As expected, virus release of WT HIV-1 was significantly inhibited by overexpressing Alix V in 293T cells. Alix V/F676D protein, an Alix V variant that contains a Phe-to-Asp substitution in Alix residue 676 that abrogates p6 binding [21], did not inhibit particle release (Fig. 6a and b). Notably, overexpression of Alix V did not affect virus release for the three $p6^{Gag}$ mutants analyzed: ΔY, Δ6, or Δ7 (Fig. 6a and b), indicating that these mutations prevent the interaction between $p6^{Gag}$ and Alix. These data suggest that the deletion mutations in $p6^{Gag}$ inhibit virus release by disrupting the binding of $p6^{Gag}$ and Alix protein.

Discussion

HIV-1 CRF07_BC originated from co-infection or superinfection of HIV-1 subtype B’ and C [1, 34, 35]. Now it is becoming increasingly prevalent and is one of the most common CRFs in China [36–39]. Previous studies showed slower disease progression in subjects infected with CRF07_BC than subtype B or CRF01_AE [6, 8, 10, 40]. Further investigation indicated

that the above clinical findings may be associated with the deletion of 7-aa ($_{30}$PIDKELY$_{36}$) in p6^{Gag} [6, 8]. In this study, we characterized the biological significance of two unique patient-derived mutations in p6^{Gag}, i.e., PTAPPE insertion and 7-aa ($_{30}$PIDKELY$_{36}$) deletion, from HIV-1 CRF07_BC-infected subjects. Our results show that the 7-aa deletion, not the PTAPPE insertion, moderately reduce virus release and Gag processing, and result in defects of infectivity and replication in both T-cell lines and PBMCs by disrupting the interaction between p6^{Gag} and the host Alix protein. Our study provides further evidence for the important role of the interaction between HIV-1 p6^{Gag} protein and Alix binding domain in regulating virus release, Gag processing and replication, which in turn may explain at the molecular level the slower disease progression observed in individuals infected with CRF07_BC with the unique 7-aa deletion mutation in p6^{Gag}.

The primary role of HIV-1 p6^{Gag} is to regulate virus budding by recruiting the ESCRT apparatus through the interaction between the late (L) domains of p6^{Gag} and host factors Tsg101 and Alix to catalyze the membrane fission reaction that allows the virus to pinch off from the plasma membrane [11]. The two L domains in HIV-1 p6^{Gag} are the Tsg101-binding site, $_{7}$PTAP$_{10}$, and the Alix-binding site, $_{36}$YPLASL$_{41}$. Insertions into, or duplication of, the PTAP motif could enhance the interaction between Gag and Tsg101. Sharma et al. reported that 94.9% of p6^{Gag} sequences of HIV-1 subtype C carry the duplication of the PTAP motif. They confirmed that duplication of the PTAP motif enhances virus replication fitness, but not virus release of HIV-1, by binding the Tsg101 protein with a higher affinity [41]. PTAP duplication is usually observed in HIV-1 strains with drug resistance mutations [42–45]. Martins et al. demonstrated that PTAP duplication enhances virus infectivity by increasing PR-mediated processing between NC and p6 in the presence of PR mutations and PR inhibitors (PIs) [31]. Martins et al. also found that the PTAP duplication did not increase virus release or the incorporation of pol products in virions. Tamiya et al. demonstrated that the PTAP insertion near Gag cleavage sites could restore the replication competence of multi-PI-resistant HIV-1 variants by enhancing the otherwise compromised enzymatic activity of mutant PR [45]. In this study, we demonstrated that the PTAP duplication alone does not affect virus release, infectivity or replication. These results are consistent with those obtained in previous studies and indicate that the major role of PTAP duplication may be to restore the replication capability in the presence of drug resistance mutations.

The HIV-1 p6^{Gag} protein interacts with the ESCRT-I component Tsg101 and Alix, which in turn recruit ESCRT-III [11, 19]. Although the Tsg101-binding PTAP motif is more critical for HIV-1 release than the Alix-binding YPXL motif, the Alix binding domain is also required for optimal virus budding [18] and replication [20]. Previous studies have defined the critical role of p6^{Gag} residues Y36, L41, and L44 in Alix binding [19, 22, 32, 46]. A point mutation at Y_{36} of p6^{Gag} leads to a severe defect in HIV-1 budding and Gag processing[18–20, 32]. Fujii et al. proposed that, because the Y36A mutation had a more severe phenotype than other p6 Alix-binding site mutations, the Y36A mutation may not simply block the p6-Alix interaction, but could affect p6 folding and the upstream interaction with Tsg101[20]. In our study, we further confirmed the profound impact of deleting Y_{36} on virus release, to about 20% of WT levels. Interestingly, compared with the severe defect exhibited by the Y_{36} deletion mutant, the 7-aa deletion $_{30}$PIDKELY$_{36}$ only moderately affected virus release and replication while deletion of the 6-aa ($_{30}$PIDKEL$_{35}$) did not significantly impair virus budding or Gag processing. Our results indicate that the deletion of the 6-aa $_{30}$PIDKEL$_{35}$ may partially neutralize the defect caused by Y_{36} deletion, and suggest the importance of optimal structure and conformation of the p6 domain of Gag. In fact, it is not uncommon that mutation at Y_{36} and flanking amino acids could rescue viral defects. For example, the mutants Y36S/L44H and Y36S /L44R, exhibit WT levels of virus release in Hela and Jurkat cells, whereas the Y36A mutant impairs particle production by ~ 5 fold [20]. Fujii et al. also found several compensatory mutations in the putative revertant isolates, such as Y36A/L41I mutant [20]. Our study also indicated that PTAP duplication coupled with the 7-aa deletion in p6^{Gag} may further interfere with the global folding of p6 and result in severe defects in particle release, Gag processing and virus replication.

It is noteworthy that deletion mutations in p6^{Gag} also result in amino-acid changes in p6* in the overlapping pol ORF. Several studies have reported that mutations upstream or downstream of the PR region potentially affect PR activity and Gag processing [47–50]. Wondrak et al. demonstrated that insertion of Alanine into the p6-PR cleavage site (from Phe-Pro

to Phe-Ala-Pro) severely impaired the autoprocessing of PR [51]. Chiu et al. reported that removal of the entire $p6^{*}$ region did not affect incorporation of GagPol into virions, but abrogated viral infectivity [52]. In our study, the 7-aa deletion in p6* is 3-aa (Phe-Ser-Phe) away from the p6-PR cleavage site. Our results showed that the corresponding deletion mutation in p6* did not influence virus release, but moderately decreased Gag processing. In addition, the deletion mutations in $p6^{Gag}$ or p6* did not affect the expression and incorporation of virion-associated reverse transcriptase (RT) or integrase (IN) (data not shown). Our results are consistent with those of Chiu et al., and indicated that deletion mutations in $p6^{*}$ have only a modest effect on PR-mediated Gag processing [52].

Conclusion

In summary, in this study we characterized two patient-derived mutations, a PTAPPE insertion and a PIDKELY deletion, in the p6 domain of HIV-1 Gag and the corresponding deletion in HIV-1 p6*. Our results demonstrated defects induced by the 7-aa deletion mutation in $p6^{Gag}$ on virus release, Gag processing, infectivity and replication kinetics, due, at least in part, to disruption of the p6-Alix interaction. Our results provide further evidence about the importance of the intact p6 protein and its optimal conformation in regulating virus replication and infectivity. Our findings help define the molecular mechanism regarding the association between the unique 7-aa deletion mutation in $p6^{Gag}$ and the slower disease progression observed in subjects infected with the 7 mutant CRF07_BC. It is noteworthy that the two patient-derived mutations in $p6^{Gag}$ only moderately affect virus replication, which in turn may confer a selective advantage and increase the prevalence of HIV-1 CRF07_BC with this unique mutation. Further study is needed to evaluate the virus replication capability and virus fitness by using the virus isolates with the 7-aa deletion in $p6^{Gag}$.

Methods

Plasmids

The molecular clone pNL4-3 [53] and the envelope (*env*)(-) derivative pNL4-3/KFS [54] of HIV-1 were obtained through the NIH AIDS Reagent Program. Patient-derived mutations in $p6^{Gag}$ were introduced by polymerase chain reaction (PCR)-based mutagenesis as previously reported [55] into pNL4-3 to generate the following three mutants: PTAPPE insertion (insPTAP), 7-aa ($_{30}$PIDKELY$_{36}$) deletion (D7), and the double mutant (PD7) containing both insPTAP and D7 (Fig. 1a, b). A PTAP motif deletion mutant (DPTAP) was used as a control for measuring defective virus release (Fig. 1b). Three additional mutations were introduced into pNL4-3/KFS: DY, in which Y36 of $p6^{Gag}$ was deleted; and D6 and D7, in which $_{30}$PIDKEL$_{35}$ or $_{30}$PIDKELY$_{36}$ of $p6^{Gag}$, respectively, were deleted. An additional set of mutants was constructed to express Gag and GagPol polyproteins with the D7 mutation in $p6^{Gag}$ or p6*, respectively, by using the plasmids pR7WT-HA and pR7insFS, which express the Gag and GagPol polyproteins, respectively [31]. The D7 mutation in $p6^{Gag}$ and p6* was then introduced into pNL4-3/KFS. All the constructs were characterized by restriction digestion analysis and DNA sequencing. The plasmids that express the V domain of Alix (residues 364-716, Alix V) and the Alix V derivative containing the F676D mutation (Alix V/F676D) have been described [21, 22].

Cell culture and transfection

293T and TZM-bl cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum (FBS), 100 U/ml penicillin, 100 g/mL streptomycin, and 2 mM L-glutamine (Gibco). TZM-bl is a HeLa-derived indicator cell line that expresses luciferase following HIV-1 infection [56]. Sup-T1, MT-4 T-cells, and PBMCs were cultured in RPMI 1640 medium supplemented with 10% (vol/vol) FBS, 100 U/mL penicillin, 100 g/ml streptomycin, and 2 mM L-

glutamine. PBMCs obtained from anonymous, de-identified NIH blood donors were activated in RPMI 1640 medium supplemented with interleukin-2 and phytohemagglutinin (PHA) prior to HIV-1 infection. Adherent cells were transfected with plasmid DNA using Lipofectamine 2000 (Invitrogen Corp. Carlsbad, CA) according to the manufacturer's recommendations. Cells and viruses were harvested 24h post-transfection and used for further analysis.

Virus release and maturation

293T cells were transfected with WT or mutant pNL4-3 molecular clones using Lipofectamine 2000 transfection reagent. At 24h post-transfection, virions were pelleted by ultracentrifugation. Both cell and virus pellets were lysed and immunoblotted with HIV-1 immunoglobulin (HIV-Ig) obtained from the NIH AIDS Reagent Program. Virus release efficiency was calculated as the amount of virion-associated p24 (CA) as a fraction of the total amount of Gag including cell-associated-p24 and $Pr55^{Gag}$ plus virion-associated p24, or the RT activity of culture supernatants relative to WT level [17, 57]. Virus maturation was measured by Gag processing and expressed as a ratio of virion-associated p24 over $Pr55^{Gag}$ levels as described previously [6, 30].

Virus replication and infectivity

Multi-cycle replication assays were performed using the Sup-T1 or MT-4 T-cell lines, and PBMCs. Sup-T1 and MT-4 T cells were transfected using DEAE-dextran reagent [57, 58]. PBMCs from multiple donors were infected with virus supernatants generated by transfecting 293T cells. Virus inputs were normalized by RT activity. Cells were infected by inoculation of HIV-1 viruses for 2h at 37C. Virus replication was monitored by measuring RT activity as described previously [59]. Virus infectivity was monitored by measuring luciferase activity in TZM-bl cells infected with HIV-1 virus supernatants from 293T cells as described previously [31].

Western blotting analysis

293T cells were harvested at 24h post-transfection. Virus-containing supernatants were collected and virus particles were pelleted by ultracentrifugation. Cells and virus pellets were lysed in a buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, and protease inhibitor cocktail (Roche Life Sciences, Basel, Switzerland). After denaturation, proteins were subjected to SDS-PAGE, transferred to a polyvinylidene fluoride (PVDF) membrane, and incubated with HIV-Ig. The membrane was then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies, and the chemiluminescence signal was detected by using Western Pico substrate (Thermo Scientific) or Western Femto substrate (Fdbio Science). Quantification of the protein band intensity was performed using ImageLab software (Bio-Rad).

Quantitative PCR analysis of reverse transcription

Pseudotyped HIV-1 virions were produced by co-transfecting 293T cells with pNL4-3/KFS encoding WT or $p6^{Gag}$-mutant Gag and a vesicular stomatitis virus G (VSV-G)-expressing vector, pHCMV-G [60]. A total of 210^5 293T cells/well were then infected with pseudotyped HIV-1 virions equivalent to 2ng of p24. At 2h post-infection, the culture supernatants were removed and replenished with fresh complete medium. The cells were cultured for another 40-48h. Total DNA was isolated using the QIAamp DNA Mini Kit (Qiagen) and used as the template to analyze initiation (R-U5), minus-strand transfer (U3-

U5), plus-strand transfer (R-5'UTR) of HIV-1 reverse transcription using SYBR-green (TAKARA)-based qPCR and HIV-1 specific primers described previously [61]. The results were obtained from 3 independent experiments.

Statistical analysis

Statistics were calculated using SPSS Statistics 20. Unpaired t tests were performed and two-tailed *P < 0.05, **P < 0.01, and ***P < 0.001 were considered statistically significant.

Abbreviations

HIV-1: human immunodeficiency virus type one; CRF: circulating recombinant form; Alix: ALG-2 interacting protein X; Tsg101: tumor susceptibility gene 101; MSM: men who have sex with men; VSV-G: vesicular stomatitis virus G; ESCRT: endosomal sorting complex required for transport; CCL2: CC chemokine ligand 2; PBMC: peripheral blood mononuclear cell.

Declarations

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Not applicable.

Authors' contributions

ZC: acquisition of data, analysis and interpretation of data, and drafting of manuscript. SA: acquisition of data. HW: conception and acquisition of data. EF: conception, design and finalizing manuscript, and ST: conception, design, drafting and finalizing manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used in the study are available from the corresponding author upon request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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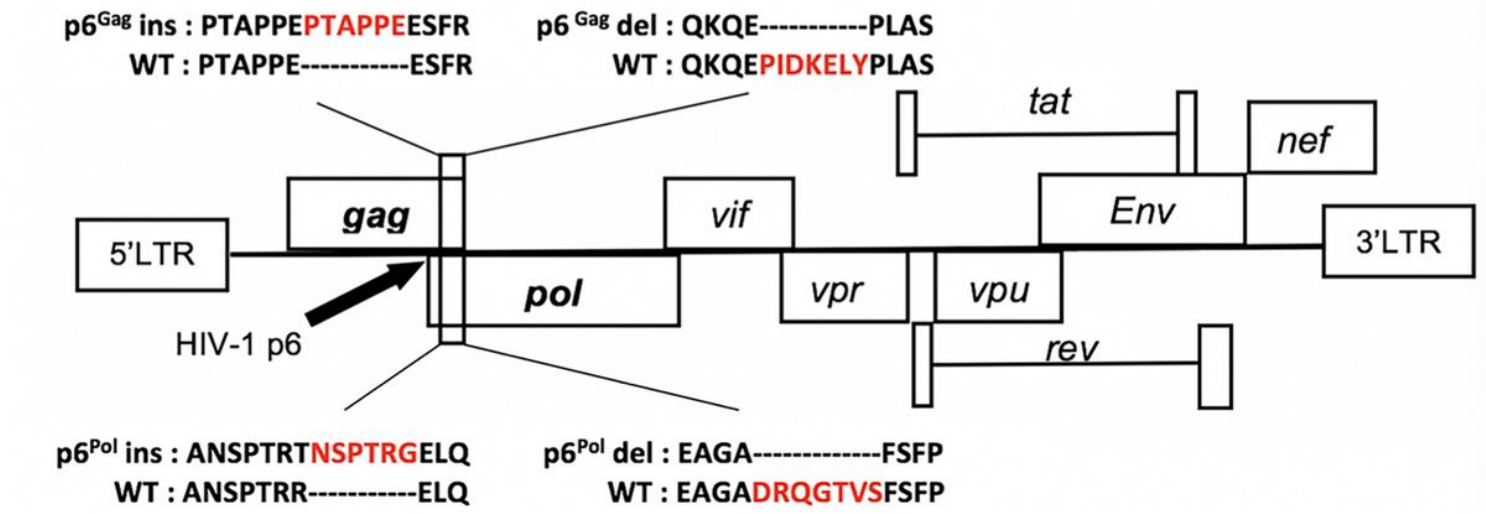
Figures

a



b

P6 Mutants	Amino acid sequences of $p6^{Gag}$
WT	LQSRPEPTAPPE- - - - -ESFRFGEETTTPSQKQE PIDKELY PLASLRSLFGSDPSSQ
ΔPTAP	LQSRPE- - - - PE- - - - -ESFRFGEETTTPSQKQE PIDKELY PLASLRSLFGSDPSSQ
insPTAP	LQSRPEPTAPPEPTAPPEESFRFGEETTTPSQKQE PIDKELY PLASLRSLFGSDPSSQ
Δ7	LQSRPEPTAPPE- - - - -ESFRFGEETTTPSQKQE - - - - - - PLASLRSLFGSDPSSQ
PΔ7	LQSRPEPTAPPEPTAPPEESFRFGEETTTPSQKQE - - - - - - PLASLRSLFGSDPSSQ

Figure 1

Schematic of the HIV-1 genome and p6 mutants. a The HIV-1 genome is shown with an expanded view of p6 mutations including PTAPPE insertion and PIDKELY deletion in p6Gag, as well as NSPTRG insertion and DRQGTVS deletions in p6Pol. b HIV-1 p6Gag mutants. These mutants were generated using the HIV-1 full-length proviral clone pNL4-3, including PTAPPE insertion (insPTAP), PIDKELY deletion (Δ7), and the double mutation insPTAP and Δ7 (PΔ7). A mutant (ΔPTAP) bearing the deletion of the original PTAP motif was constructed and used as a control for deficiency of virus release. Sequences of mutations are highlighted and compared with HIV-1 wild-type (WT).

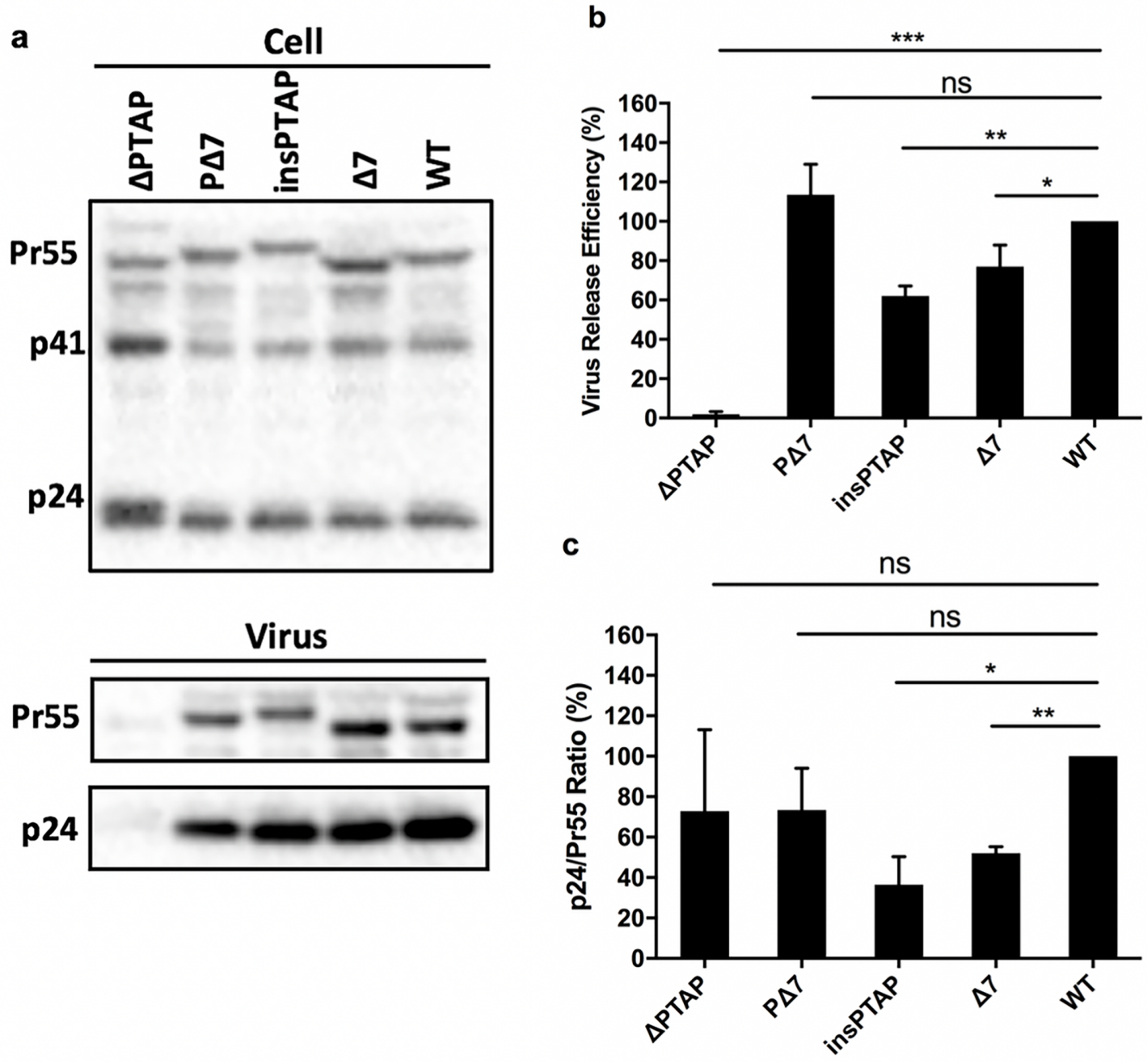


Figure 2

Virus release efficiency and Gag processing. a Western blotting (WB) analysis. 293T cells were transfected with WT pNL4-3 or p6Gag mutants. At 24 h post-transfection, cell and virus lysates were collected and analyzed by WB with HIV immunoglobulin (HIV-Ig). Positions of HIV-1 Gag precursor Pr55Gag, Gag processing intermediate p41 and HIV-1 capsid protein p24 are indicated. b Virus release efficiency. The relative efficiency of virus release was calculated as the amount of virion p24 divided by total Gag (virion p24 + cellular Pr55Gag + cellular p24). c Gag processing. Gag processing was expressed as the ratio of p24 relative to Pr55Gag in virions. The data were plotted in bar graphs. The efficiency of virus release and Gag processing for WT was set as 100%. Error bars indicate the standard deviation from more than three independent experiments; ns, not significant. *P < 0.05, **P < 0.01, and ***P < 0.001.

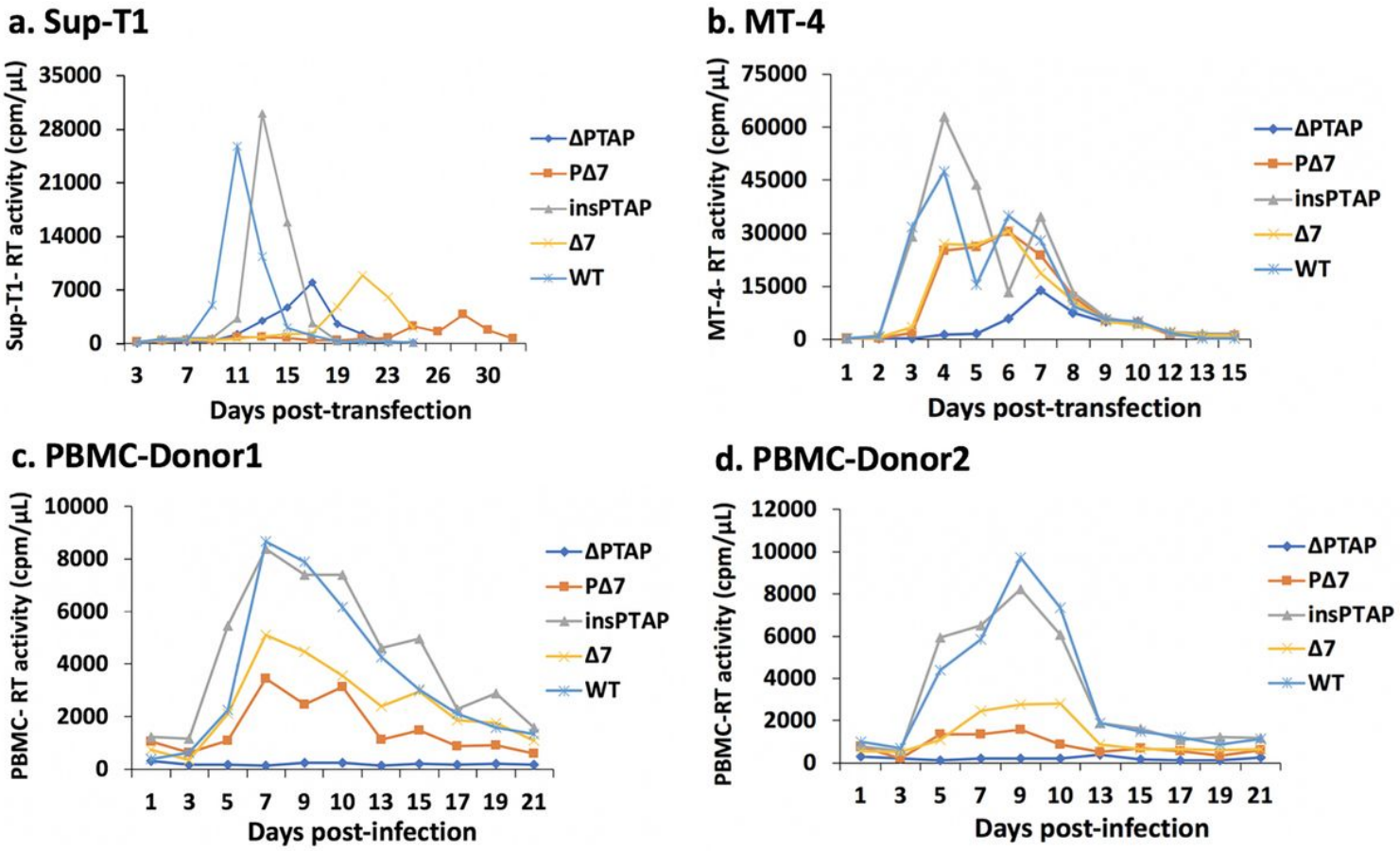


Figure 3

Replication of HIV-1 WT and p6 mutants in Sup-T1 T cells (a), MT-4 T cells (b), and PBMCs (c and d). Both Sup-T1 and MT-4 T-cell lines were transfected with WT or mutant pNL4-3 molecular clones. Cells were split every day or every two days. Culture supernatants were collected for reverse transcriptase (RT) activity analysis. PBMCs from two donors were infected with virus stocks generated in 293T cells. Cells were split, and culture supernatants collected for RT activity analysis every 2–3 days. Virus replication was monitored by RT activity.

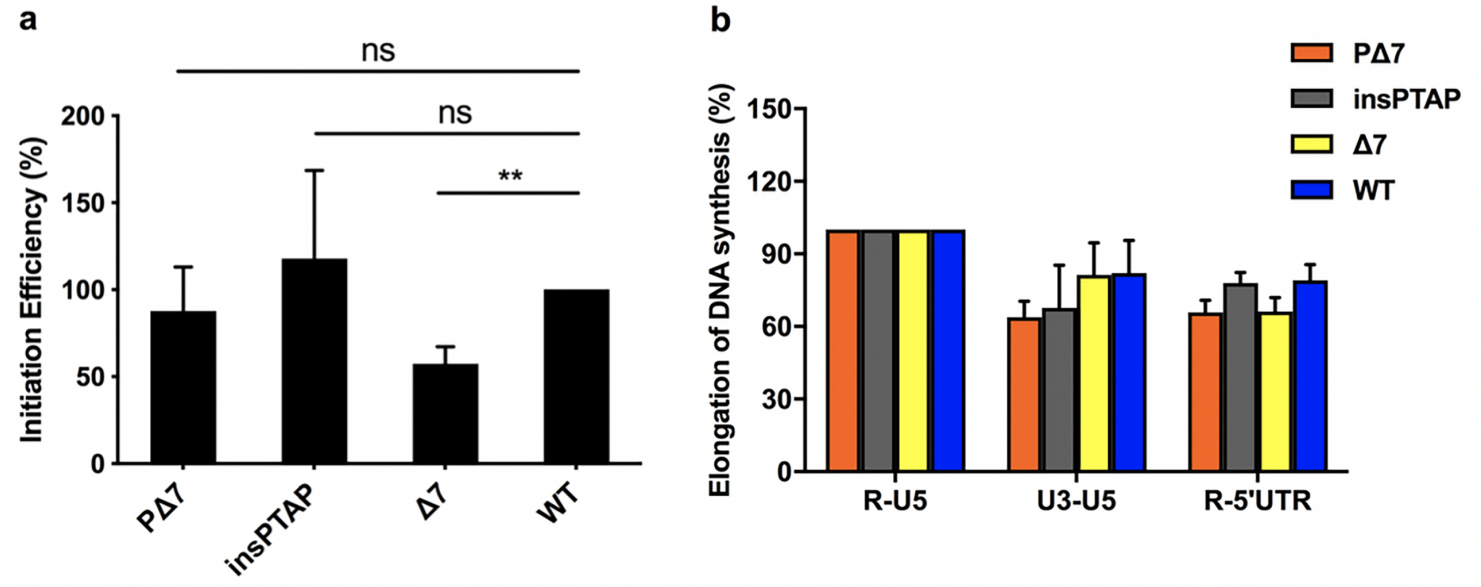


Figure 4

Effects of p6 mutations on the initiation and elongation of HIV-1 reverse transcription. 293T cells were co-transfected with WT or p6 mutant HIV-1 proviral clones with a VSV-G expressing vector. Pseudotyped virus was harvested and used to infect 293T cells. After 48h of infection, cells were lysed followed by DNA extraction. DNA synthesis products R-U5, U3-U5, R-5'UTR were analyzed by real-time quantitative PCR. a Initiation efficiency of reverse transcription in mutant viruses. Initiation efficiency was measured by analyzing the amount of R-U5 DNA. The level of WT virus was set as 100%. b Relative efficiency of DNA elongation. The level of R-U5 product was set as 100%, and the amounts of U3–U5 and R-5'UTR products were expressed relative to the R-U5 level. Results from three independent experiments were summarized; error bars indicate standard deviation (SD); ns, not significant. **P < 0.01.

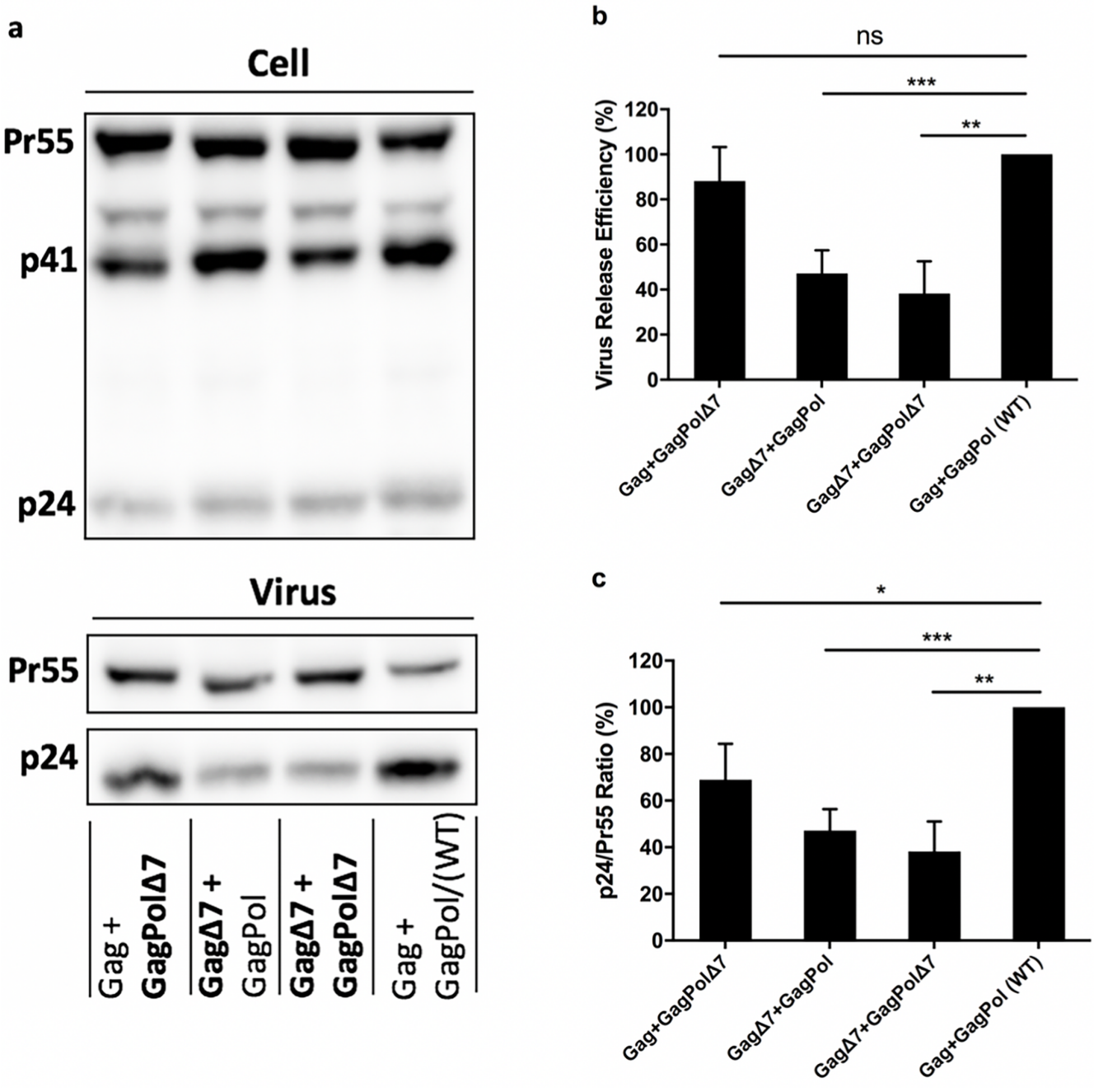


Figure 5

The role of the seven-amino-acid deletion in p6Gag and the overlapping deletion in p6*. Detection of cell- and virion-associated proteins by WB analysis. a 293T cells were co-transfected with HIV-1 proviral clones that encode Gag and GagPol at a ratio of 15:1. Two days post-transfection, virus and cell lysates were harvested and measured by WB. Virus release efficiency (b) and Gag processing (c) were calculated as described in Figure 2. Virus production for WT was set as

100%. Standard deviation was obtained from more than three independent experiments; ns, not significant. *P < 0.05, **P < 0.01, and ***P < 0.001.

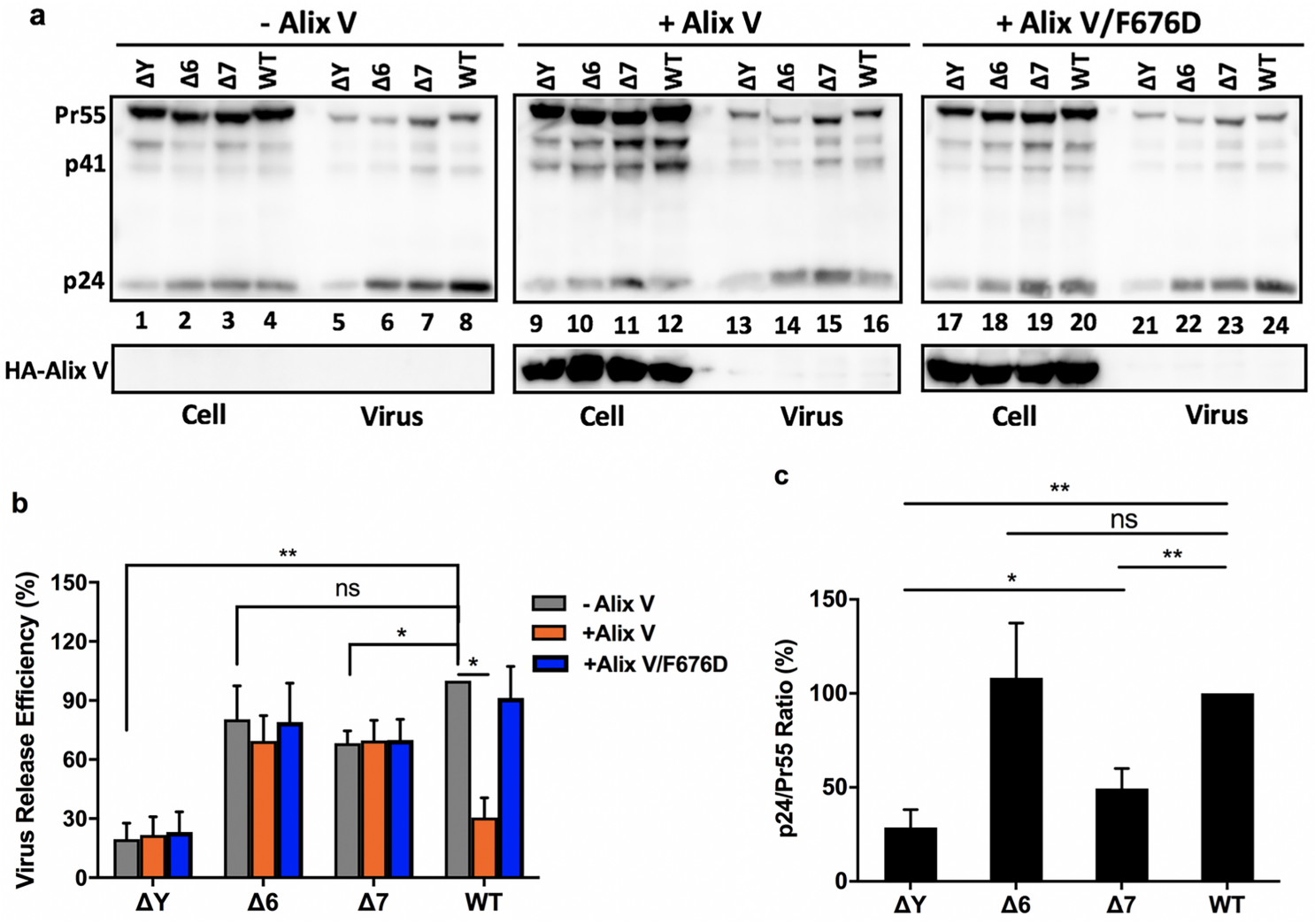


Figure 6

The p6 mutants are resistant to inhibition by Alix V overexpression. a 293T cells were co-transfected with HIV-1 proviral DNA encoding WT Gag or the deletion of the 36th reside (ΔY), the 6-aa deletion (Δ6), or Δ7 mutant in p6Gag, together with control plasmid DNA (pcGNM2-Alix) or HA-Alix V or HA-Alix V/F676D expressing vector. At 24h post-transfection, cell-associated and virus-associated proteins were measured by WB. Pr55Gag, p41, p24 and HA-Alix V are indicated. The efficiency of virus release (b) and Gag processing (c) was calculated as described in Figure 2. The levels of WT virus produced in the absence of Alix V (-Alix) was set as 100%. Error bars show the standard deviation (SD) from 4 experiments. ns, not significant. *P < 0.05, **P < 0.01.