Elucidation of the Molecular Mechanism Driving Duplication of the HIV-1 PTAP Late Domain

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ABSTRACT

HIV-1 uses cellular machinery to bud from infected cells. This cellular machinery is comprised of several multiprotein complexes known as endosomal sorting complexes required for transport (ESCRTs). A conserved late domain motif, Pro-Thr-Ala-Pro (PTAP), located in the p6 region of Gag (p6^{Gag}), plays a central role in ESCRT recruitment to the site of virus budding. Previous studies have demonstrated that PTAP duplications are selected in HIV-1-infected patients during antiretroviral therapy; however, the consequences of these duplications for HIV-1 biology and drug resistance are unclear. To address these questions, we constructed viruses carrying a patient-derived PTAP duplication with and without drug resistance mutations in the viral protease. We evaluated the effect of the PTAP duplication on viral release efficiency, viral infectivity, replication capacity, drug susceptibility, and Gag processing. In the presence of protease inhibitors, we observed that the PTAP duplication in p6^{Gag} significantly increased the infectivity and replication capacity of the virus compared to those of viruses bearing only resistance mutations in protease. Our biochemical analysis showed that the PTAP duplication, in combination with mutations in protease, enhances processing between the nucleocapsid and p6 domains of Gag, resulting in more complete Gag cleavage in the presence of protease inhibitors. These results demonstrate that duplication of the PTAP motif in p6^{Gag} confers a selective advantage in viral replication by increasing Gag processing efficiency in the context of protease inhibitor treatment, thereby enhancing the drug resistance of the virus. These findings highlight the interconnected role of PTAP duplications and protease mutations in the development of resistance to antiretroviral therapy.

IMPORTANCE

Resistance to current drug therapy limits treatment options in many HIV-1-infected patients. Duplications in a Pro-Thr-Ala-Pro (PTAP) motif in the p6 domain of Gag are frequently observed in viruses derived from patients on protease inhibitor (PI) therapy. However, the reason that these duplications arise and their consequences for virus replication remain to be established. In this study, we examined the effect of PTAP duplication on PI resistance in the context of wild-type protease or protease bearing PI resistance mutations. We observe that PTAP duplication markedly enhances resistance to a panel of PIs. Biochemical analysis reveals that the PTAP duplication reverses a Gag processing defect imposed by the PI resistance mutations in the context of PI treatment. The results provide a long-sought explanation for why PTAP duplications arise in PI-treated patients.

The assembly of human immunodeficiency virus type 1 (HIV-1) particles is driven by the Gag precursor protein Pr55^{Gag} (1–3). Domains within Pr55^{Gag} function in concert to promote virus assembly and release: the matrix (MA) domain directs Pr55^{Gag} to the inner leaflet of the plasma membrane and participates in the incorporation of the viral envelope (Env) glycoproteins into the assembling virion; the capsid (CA) is largely responsible for Gag multimerization at the plasma membrane; the nucleocapsid (NC) recruits the viral RNA genome and also contributes, through its capacity to bind nucleic acid, to the assembly process; and the p6 domain of Gag (p6^{Gag}) is required for virus budding (4). Pr55^{Gag} also contains two spacer peptides: SP1, located between the CA and NC domains, and SP2, located between NC and p6^{Gag}. A second polyprotein precursor, Pr160^{GagPol}, is synthesized as the result of a ribosomal frameshifting event during Gag translation. Pr160^{GagPol}, which is expressed at levels ~5% of those at which Pr55^{Gag} is expressed, is copackaged with Pr55^{Gag} in virus particles and contains the domains for the viral enzymes protease (PR), reverse transcriptase (RT), and integrase (IN).

Mutational analyses demonstrated that a Pro-Thr-Ala-Pro (here referred to as PTAP) motif near the N terminus of p6^{Gag} is

primarily responsible for the ability of p6^{Gag} to promote virus release (5, 6). The PTAP motif was subsequently shown to interact directly with the Tsg101 subunit of the endosomal sorting complex required for transport I (ESCRT-I) (7–10). The ESCRT machinery is a cellular apparatus that catalyzes several membrane fission events; retroviruses have evolved to hijack this machinery to bud off from the infected cell (11).

As the nascent virion is released from the cell surface, PR cleaves Pr55^{Gag} and Pr160^{GagPol} between the individual Gag and Pol domains to release the mature Gag proteins and the viral en-

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FIG 1 Position of duplications in p6^{Gag} and p6^{Pol} and drug resistance mutations in PR. The organization of Gag and Pol is shown, and the Gag domains (MA, CA, SP1, NC, SP2, and p6^{Gag}) and Pol domains (p6^{Pol}, PR, RT, IN) are indicated. At the top are shown the WT sequence at the N terminus of p6^{Gag} and the duplication in the Gag clone, highlighted in red. At the lower left are shown the WT sequence of p6^{Pol} and the duplication (p6^{Pol}-ins) that results from the PTAP duplication in p6^{Gag}, highlighted in blue. At the lower right are shown the sequences of the indicated amino acid residues in WT PR and the PI-resistant PR clone. The GagPR clone contains both the duplications in p6^{Gag}/p6^{Pol} and the mutations in PR.

zymes (2, 12, 13). Differences in the primary amino acid sequences at Gag and GagPol cleavage sites contribute to the wide-ranging efficiencies with which PR cleaves each site (14–18); this differential processing efficiency in turn results in a highly ordered cascade of cleavage events (19–22). The consequence of this highly ordered cleavage cascade is virus maturation, during which the CA protein reorganizes to form the condensed conical core typical of the mature lentiviral particle.

There are currently over two dozen antiretroviral drugs approved for clinical use (<http://www.who.int/hiv/topics/treatment/en/>). These fall into several broad classes: inhibitors that block the activity of the viral enzymes RT, PR, and IN and drugs that interfere with virus fusion and entry. These compounds are administered in various combinations; the resulting combined antiretroviral therapy (cART; also known as highly active antiretroviral therapy) is effective in suppressing viral loads in infected patients. However, cART is not curative and thus must be administered for the lifetime of the infected patient. This long-term therapy is associated with issues of toxicity and compliance, and resistance to currently available antiretroviral drugs may become a significant problem for many patients (23–26). Understanding the basis for drug resistance is thus key to the sustained success of antiretroviral therapy.

Resistance to the available antiretroviral drugs is well established, and cross-resistance to drugs within a drug class is often observed (23). Resistance to RT and IN inhibitors typically results from mutations within the enzymes themselves. In the case of PR inhibitors (PIs), resistance is associated with mutations not only within the enzyme but also in the Gag substrate both proximal to and distal from PR cleavage sites (27–38). Mutations in Gag can increase resistance by enhancing the ability of the PI-resistant PR to bind and cleave the substrate (39). Mutations within the cytoplasmic tail of gp41 have also been reported to contribute to PI resistance (40), most likely stemming from the fact that the fusogenic activity of the Env glycoprotein complex is suppressed in immature virions and that this suppression is linked to the gp41 cytoplasmic tail (41, 42).

A number of studies have observed full or partial duplications of the PTAP motif in viral sequences derived from infected patients. However, the basis for such duplications has not been

clearly defined. Some groups have reported no association between the frequency of PTAP duplications and cART (43, 44), whereas other studies have documented a significant increase in the frequency of PTAP duplications in patients on therapy (45–47). Tamiya et al. reported that amino acid insertions near Gag cleavage sites, including PTAP duplications, increased processing by PR mutants bearing PI resistance changes; however, the role of PIs in this phenomenon was not investigated (48). Alternatively, it has also been suggested that PTAP duplications improve viral fitness by enhancing virus assembly and increasing Pr160^{GagPol} packaging into virions (47). Because the p6^{Gag} open reading frame overlaps with the N-terminal region of the *pol* open reading frame (referred to as p6^{Pol} or transframe protein p6*), duplications in the PTAP motif in p6^{Gag} also lead to concomitant insertions in p6^{Pol} (Fig. 1). Thus, it is possible that it is the insertions in p6^{Pol} rather than those in p6^{Gag} that drive the emergence of the duplications. Indeed, it has been reported that duplication of PTAPP to PTAPPAPP in p6^{Gag} leads to a Ser-Pro-Thr (SPT) duplication in p6^{Pol} that provides escape from the host cytotoxic T-lymphocyte (CTL) response (49).

In this study, we sought to examine the consequences of PTAP duplication at the virological and biochemical level. We observed that a full PTAP duplication had little effect on virus replication kinetics or particle infectivity in the context of wild-type (WT) PR or in the context of a mutant PR bearing PI resistance mutations in the absence of a PI. However, in the context of the mutant PR, PTAP duplication significantly increased PI resistance, resulting in markedly improved virus replication kinetics and particle infectivity in the presence of PIs. The enhanced infectivity could be attributed to the PTAP duplication in p6^{Gag} rather than the overlapping duplication in p6^{Pol}. Biochemical analysis revealed that the PTAP duplication significantly increased cleavage of the NC-SP2-p6 (p15^{Gag}) processing intermediate to mature NC and p6^{Gag}. These results suggest that enhanced resistance to PIs linked to more complete Gag processing is likely to be a major contributor to the selection of PTAP duplications *in vivo*.

MATERIALS AND METHODS

Plasmids. The molecular clone pR7-GFP (50) was obtained through the NIH AIDS Reagent Program. A patient-derived PTAP duplication (LEPT

APSR) (51) together with mutations in PR derived from the same patient (PI major resistance mutations I54V and V82L; PI minor resistance mutations K20R, L24I, E35D, M36I, R41K, I62V, L63P, and I64V) (<http://hivdb.stanford.edu/>) was introduced into the pR7-GFP clone (Fig. 1). PR mutations were introduced in two steps. First, two BstEII restriction sites were introduced by site-directed mutagenesis, using a QuikChange site-directed mutagenesis kit (Agilent Technologies), into the 3' end of the p6^{Gag}-coding region and the 5' end of the RT-coding region. Upon digestion with BstEII and ligation using T4 ligase (New England BioLabs), the pR7ΔPR intermediate clone was obtained. In the second step (52), nested PCR was used to amplify the PR-coding region using the cDNA from the HIV-1 strain isolated from the clinical sample; this fragment was introduced into the pR7ΔPR clone using an Infusion kit (Clontech). To generate the molecular clones pR7Gag (carrying only the PTAP duplication) and pR7GagPR (carrying both the PTAP duplication and the drug resistance mutations in PR), the original pR7-GFP and pR7PR clones, respectively, were used. The PTAP duplication was inserted upstream of the native PTAP domain as it was found in the patient's isolate. To study the effect of the insertions in p6^{Gag} or in the overlapping open reading frame, p6^{Pol}, a second set of vectors was constructed. These vectors expressed Gag or Gag-Pol. Gag- and PR-coding regions from pR7-GFP, pR7Gag, and pR7PR were subcloned into pBluescript SK(+) using the SpeI and BclI enzymes. The hemagglutinin (HA) tag, followed by a stop codon, was introduced after p6^{Gag} to generate clones pR7WT-HA and pR7Gag-HA expressing only Gag. For the clones expressing Gag-Pol, a 1-nucleotide insertion in the frameshift (FS) region was introduced (insFS), thereby placing the gag and pol coding regions in the same open reading frame. pR7WTinsFS, pR7GaginsFS, pR7PRinsFS, and pR7GagPRinsFS were generated after cloning back from pBluescript SK(+) to the pR7 backbone. To construct R7/NL4-3 chimeras, the region of the R7 clones corresponding to the region from the SpeI site in the CA-coding region (pNL4-3 nucleotide 1508) to the SbfI site in the RT-coding region (pNL4-3 nucleotide 2844) was introduced into pNL4-3.

Cell culture, transfections, and PIs. 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 μg/ml streptomycin, and 2 mM L-glutamine (Gibco). TZM-bl (obtained from J. Kappes through the NIH AIDS Reagent Program) is a HeLa cell-derived indicator cell line that expresses luciferase under the control of the HIV long terminal repeat (53). Jurkat cells, MT-4 cells, and peripheral blood mononuclear cells (PBMCs) were cultured in RPMI 1640 medium supplemented with 10% (vol/vol) FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. PBMCs obtained from anonymous, deidentified NIH blood donors were activated in RPMI 1640 medium supplemented with interleukin-2 and phytohemagglutinin P prior to HIV-1 infection. Adherent cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. Virus supernatants were harvested at 24 h posttransfection. PIs, obtained through the NIH AIDS Reagent Program, were reconstituted to a concentration of 20 mM in dimethyl sulfoxide, aliquoted, and stored at -20°C. Dilutions for working solutions of 200 μM in the medium were newly prepared before each experiment.

Virus replication and infectivity assays. Multicycle replication assays were performed using Jurkat cells, MT-4 cells, and PBMCs. Jurkat and MT-4 cells were transfected by using DEAE-dextran in the presence or absence of 1 μM ritonavir (RTV) or 1 μM nelfinavir (NFV). PBMCs from multiple donors were infected in the presence or absence of 1 μM RTV. Virus inputs were normalized by RT activity, and cells were infected by spinoculation at 1,200 × g for 2 h. Virus replication was examined by measurement of RT activity as previously described (54). TZM-bl cells were infected with virus supernatants generated by transfection of 293T cells in the presence or absence of PIs. Virus inputs were normalized by RT activity. Briefly, TZM-bl cells were plated in 96-well plates 1 day before infection, and after serial dilution, virus supernatants were used to infect the cells. Infections were performed in the presence of DEAE-dextran for

2 h at 37°C. At 2 days postinfection, cells were lysed with luciferase lysis buffer (Promega), and luciferase activity was measured using Britelite Plus substrate (Perkin-Elmer).

Virus release assays. 293T cells were transfected with WT or mutant HIV-1 molecular clones using the Lipofectamine 2000 transfection reagent. At 1 day posttransfection, cells were metabolically labeled for 4 h or overnight with [³⁵S]Met-Cys, and virions were pelleted by centrifugation. Virus-encoded proteins in cell and virus lysates were immunoprecipitated with HIV immunoglobulin (HIV-Ig; obtained from the NIH AIDS Reagent Program) and analyzed by SDS-PAGE followed by fluorography (55). The virus release efficiency was calculated as the amount of virion-associated p24(CA) as a fraction of the total amount (cell- plus virion-associated) of Gag. Virion-associated p66(RT) and p32(IN) were quantified, and the amount was normalized to the amount of virion p24(CA).

Western blotting. 293T cells were transfected with WT or mutant R7/NL4-3 chimeras. After overnight incubation, cells were treated with a PI (5 μM RTV or 1 μM NFV). Virions were collected after 24 h, pelleted as described above, and lysed in Triton X-100 lysis buffer. After denaturation, proteins were subjected to SDS-PAGE, transferred to a polyvinylidene fluoride (PVDF) membrane, and incubated with goat antiserum against NCp7 (a gift from Robert Gorelick, AIDS and Cancer Virus Program, Frederick National Laboratory for Cancer Research, Frederick, MD). The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies, and the chemiluminescence signal was detected by using Western Pico substrate (Thermo Scientific). Quantification of the protein band intensity was performed using ImageLab software (Bio-Rad).

Phenotypic drug susceptibility testing. PI susceptibility was determined using the PhenoSense HIV assay, a single-cycle replication assay (56). Briefly, 1.5 kb of the HIV-1 sequence from pR7, pR7Gag, pR7PR, and pR7GagPR was amplified and transferred into a pNL4-3-based HIV-1 genomic vector containing a luciferase reporter gene to generate resistant test vectors. Pseudotyped virus stocks were produced by cotransfecting 293T cells with resistant test vector DNA and an amphotropic murine leukemia virus envelope glycoprotein expression vector. Virus-producing cells were cultured in the absence or presence of serial dilutions of PIs, and virus stocks were harvested 48 h after transfection and used to infect target 293T. The target cells were lysed 72 h after inoculation, and luciferase activity was measured to assess virus infectivity. Susceptibility to PIs was calculated by plotting the percent inhibition of virus infection versus the drug concentration to derive the drug concentration required to inhibit virus replication by 50% (IC₅₀). The fold change in drug susceptibility was calculated by comparing the IC₅₀ for the test virus to the IC₅₀ for a WT reference strain, NL4-3.

RESULTS

PTAP duplication markedly increases virus replication kinetics in the context of PIs and drug resistance mutations in PR. To evaluate the effect of PTAP duplication on HIV-1 replication, we constructed R7-based molecular clones (50) containing mutations in p6 and PR derived from the same patient (51) (Fig. 1). The clone denoted "Gag" contains a PTAP duplication just upstream of the native PTAP motif in p6^{Gag}, the clone denoted "PR" contains 10 mutations in PR that confer resistance to PIs, and the clone denoted "GagPR" contains both the PTAP duplication and the changes in PR. These molecular clones, along with the parental R7 clone, were used to transfect the Jurkat T-cell line. Virus replication was monitored by measuring RT activity in the culture supernatants in the presence or absence of clinically achievable concentrations (57–59) of the PIs RTV and NFV (Fig. 2). Although RTV is no longer used as a PI, it was selected for use here because the patient from whom the Gag and PR mutations were derived had been treated with a regimen that included RTV as the sole PI (51). In the absence of a PI, the WT and Gag clones repli-

FIG 2 Effect of PTAP duplication on HIV-1 replication in T-cell lines. (A) The Jurkat T-cell line was infected with RT-normalized virus stocks prepared in 293T cells by transfection with WT R7, Gag, PR, and GagPR molecular clones. Cultures were treated with 1 μ M RTV or were left untreated. (B and C) The MT-4 T-cell line was transfected with the indicated molecular clones, and the transfected cells were left untreated or were treated with 1 μ M RTV (B) or 1 μ M NFV (C). Virus replication was monitored by RT activity.

cated with similar kinetics. Consistent with the fact that PI resistance mutations in PR reduce viral fitness (60, 61), the clones with the mutations in PR (PR and GagPR) replicated with a delay. As expected, in the presence of a PI, the WT and Gag clones failed to replicate. Interestingly, in the presence of a PI, the GagPR clone replicated markedly better than the PR clone (Fig. 2A). Likewise, in the MT-4 T-cell line, a replication advantage was observed for the GagPR clone versus the PR clone in the presence of either RTV or NFV (Fig. 2B and C). To corroborate these findings in primary human cells, PBMCs were infected with RT-normalized inocula of parental strain R7 and the Gag, PR, and GagPR clones. The analysis was performed in cells from three different donors (Fig. 3). As observed in T-cell lines, in the absence of a PI, the clones containing mutations in PR (PR and GagPR) showed delayed replication. In contrast, in the presence of 1 μ M RTV, the PTAP duplication clearly enhanced virus replication kinetics (compare the results for the PR clone versus those for the GagPR clone in Fig. 3). These results demonstrate that the PTAP duplication increases PI resistance in both T-cell lines and primary PBMCs.

PTAP duplication does not increase virus release efficiency or the levels of *pol* products in virions. As mentioned in the introduction, the PTAP motif plays a central role in recruiting the ESCRT machinery to the site of virus budding and is thus required

for efficient particle release. Mutation of the PTAP motif has also been reported to reduce the levels of *pol* products that are packaged into virions (6, 62). It therefore seemed possible that the PTAP duplication could enhance virus replication by stimulating virus budding and/or increasing the incorporation or retention of Pr160^{GagPol} into virus particles. To evaluate these possibilities, 293T cells were transfected with the WT R7 clone and the Gag, PR, and GagPR derivatives. The transfected cells were metabolically radiolabeled; cell and virus lysates were immunoprecipitated with HIV-Ig. Virus release efficiency was determined by quantifying the amount of virion-associated CA relative to the total amount (cell- plus virus-associated) of Gag by phosphorimager analysis. The results indicated that the presence of the PTAP duplication did not significantly affect the virus release efficiency (Fig. 4A and B). This analysis allowed us to measure in parallel the levels of the *pol* products in virions produced by the R7, Gag, PR, and GagPR clones. By quantifying the levels of RT (p66) and IN in virions by phosphorimager analysis and calculating the ratios of RT to CA (Fig. 4C) and IN to CA (Fig. 4D), we concluded that the PTAP duplication did not significantly affect the levels of *pol* products in virions.

PTAP duplication significantly increases PI resistance in single-cycle infectivity assays. The data presented above indicate

FIG 3 Effect of PTAP duplication on HIV-1 replication in primary T cells. PMBCs from three different donors were infected with RT-normalized virus stocks prepared in 293T cells by transfection with WT R7, Gag, PR, and GagPR molecular clones. Cultures were treated with 1 μ M RTV or were left untreated. Virus replication was monitored by determination of RT activity.

that duplication of the PTAP motif markedly improves virus replication capacity in the presence of a PI but does not affect virus release efficiency or the levels of *pol* products in virions. A prediction based on these results is that the PTAP duplication would increase PI resistance in single-cycle infectivity assays. To test this prediction, the WT, Gag, PR, and GagPR molecular clones were used to transfect 293T cells, which were then either treated or not treated with RTV or NFV. Virus-containing supernatants, normalized for RT activity, were used to infect the TZM-bl indicator cell line (53), and luciferase activity was measured at 2 days postinfection. When produced in the absence of a PI, the PR and GagPR clones showed modestly reduced infectivity relative to the infectivity of the WT (Fig. 5). In contrast and consistent with the multiround virus replication data presented above, in the presence of either 5 μ M RTV or 0.5 μ M NFV, the GagPR clone showed significantly higher levels of infectivity than the other clones (Fig. 5). These results demonstrate that PTAP duplication confers markedly enhanced PI resistance in single-cycle virus infectivity.

PTAP duplication increases resistance to a number of PIs. We next used the PhenoSense assay (see Materials and Methods) to determine whether the reduced sensitivity to PIs conferred by the PTAP duplication could also be observed with other inhibitors. This assay measures the single-round virus infectivity of an HIV-1-based luciferase reporter virus pseudotyped with the am-

photropic murine leukemia virus envelope glycoprotein. The data are tabulated as the fold change in the IC_{50} s of the inhibitors for the test virus relative to the IC_{50} for the NL4-3 reference strain. The results demonstrated that the IC_{50} s of most, though not all, of the PIs tested were increased by the PTAP duplication relative to those for the virus containing only resistance mutations in PR (Table 1). These results confirmed and extended the data presented above by showing that PTAP duplication increases resistance to a broad panel of PIs that are currently used to treat HIV-1-infected patients.

To determine if the increased PI resistance observed with this PTAP duplication could also be observed with other PTAP region insertions, we constructed a series of four clones that contained mutations in PR with or without a partial PTAP region insertion, APP. The PhenoSense assay was performed with these clones, as described above. In this case, we did not observe an effect of the APP insertion on the levels of PI resistance (data not shown).

The PTAP duplication in p6^{Gag} but not the corresponding insertion in p6^{Pol} enhances virus infectivity in the presence of a PI. The p6^{Gag} open reading frame overlaps with the *pol* open reading frame such that PTAP duplications in p6^{Gag} also result in a duplication in p6^{Pol} (Fig. 1). To examine whether the insertion in p6^{Gag} or p6^{Pol} is responsible for the above-described increases in PI resistance, we applied a system in which we could analyze each

FIG 4 Duplication of PTAP has no impact on HIV-1 release and virion-associated levels of the *pol* products RT and IN. (A) At 1 day posttransfection with WT or mutant R7 molecular clones, cells were metabolically labeled for 14 h and virions were pelleted by ultracentrifugation. Cell and virus lysates were immunoprecipitated with HIV-Ig and subjected to SDS-PAGE followed by fluorography. The envelope glycoproteins gp160 and gp120, the Gag precursor Pr55^{Gag}, p24 (CA), p66 (RT), and p32 (IN) are labeled on the left. (Top) Cell lysates; (bottom three panels) virus lysates. (B) Relative virus release efficiency (Rel VRE) was calculated as the amount of virion-associated p24 (CA) relative to the total amount (cell- plus virion-associated) of Gag. (C and D) The levels of RT (p66 subunit) (C) and IN (p32) (D) in the virions were quantified and normalized to the level of p24 in the virions; this ratio was set equal to 100% for the WT. Data were obtained from three independent experiments and represent the mean $\pm$ SD.

insertion independently of the other. We generated molecular clones that expressed Pr55^{Gag} but not Pr160^{GagPol} or expressed Pr160^{GagPol} but not Pr55^{Gag} (see Materials and Methods). We then introduced the PTAP duplication into the Gag-expressing clone and the p6^{Pol} insertion (p6^{Pol}-ins) into the GagPol-expressing clone. The genome of the GagPol-expressing clone also encoded the mutant PR containing the PI resistance mutations (Fig. 1). The Gag- and GagPol-expressing clones were transfected into 293T cells at a 20:1 ratio to generate particles with the appropriate ratio of Gag to Pol proteins (63) and were cultured with or without a PI. Supernatants were then harvested and used to infect TZM-bl cells. We calculated the fold reduction in infectivity in the presence versus the absence of a PI (Fig. 6). The results indicated that the insertion in p6^{Pol} had no effect on PI resistance. In contrast, in the presence of PI resistance mutations in PR, the PTAP duplication in p6^{Gag} significantly decreased the susceptibility of the virus to the PI. These results show that the PTAP duplication in p6^{Gag} but not the overlapping insertion in p6^{Pol} is responsible for the enhanced PI resistance demonstrated above.

PTAP duplication enhances PR-mediated processing between NC and p6 in the context of PR mutations and PIs. To understand the mechanistic basis for the increased resistance to PIs conferred by the PTAP duplication, we analyzed the Gag processing pattern in the absence and presence of PIs. Initial experi-

ments indicated that anti-p6 antibody from several sources reacted poorly with p6 from the R7 strain (data not shown). For this reason and also to avoid the possibility that the PTAP duplication in p6 might affect anti-p6 antibody reactivity and thus complicate interpretation of the results, we used an anti-NC antibody for this analysis. Because the R7 molecular clone produces virus less efficiently than NL4-3 (data not shown), we constructed R7/NL4-3 chimeras containing the CA to RT region from R7 and the remaining viral sequences from NL4-3 (see Materials and Methods). These R7/NL4-3 chimeras produced levels of virus comparable to those obtained with NL4-3 (data not shown) and, importantly, retained the effect of the PTAP duplication on PI resistance that was observed with the parental R7 clones (Fig. 7A and B). To determine whether these effects on PI resistance, observed in Fig. 7A and B with RTV and NFV, extended to other PIs, we performed the same analysis with two additional PIs, amprenavir (AMP) and atazanavir (ATV). The results again indicated that the PTAP duplication significantly increased the PI resistance conferred by the changes in PR (Fig. 7C).

To examine Gag processing efficiency in the context of mutations in PR with or without PTAP duplications, cells were transfected with WT R7/NL4-3 chimeras or derivatives containing the PTAP duplication in p6 (the Gag clone), mutations in PR (the PR clone), or both the PTAP duplication and mutations in PR (the

FIG 5 PTAP duplication increases PI resistance. 293T cells were transfected with the indicated R7 clones and then not treated or treated with 5 μM RTV (A) or 0.5 μM NFV (B). Virus supernatants were normalized for RT activity and used to infect the TZM-bl indicator cell line. Luciferase activity was measured at 2 days postinfection. Data from the infectivity assays shown on the left were used to calculate the fold reduction in infectivity induced by the PI (the ratio of the infectivity of virions prepared in the absence [untreated] to that of virions prepared in the presence [drug treated] of a PI) shown on the right. Data were evaluated for statistical significance by using the unpaired Student *t* test. *, *P* = 0.02 (*n* = 3); **, *P* < 0.001 (*n* = 3); ***, *P* < 0.0001 (*n* = 3).

GagPR clone). Transfected cells were treated or not treated with a PI (RTV or NFV), and virus-containing supernatants were harvested and analyzed by Western blotting with an anti-NC antibody. In the absence of PIs, the processing of Pr55^{Gag} was nearly complete, resulting in >90% of Pr55^{Gag} being processed to NC (Fig. 8). In the presence of PIs, Pr55^{Gag} processing to NC was severely inhibited in the context of the WT and the PTAP duplication alone (the Gag clone). The PI resistance mutations in PR largely prevented the PI-imposed block to Pr55^{Gag} processing. Interestingly, however, in the context of PR mutations alone, an accumulation of the NC-SP2-p6 processing intermediate (referred to as p15) was observed (Fig. 8A, PR lanes). This incomplete Gag processing phenotype was alleviated by the PTAP duplication (Fig. 8A, GagPR lanes). This effect was quantified from multiple

independent experiments by calculating the ratio of NC-SP2-p7 (p15) to p15 plus p7 (Fig. 8B). Again, the GagPR clone, which contains the PTAP region duplication, showed a significantly reduced accumulation of the NC-SP2-p6 (p15) Gag processing intermediate relative to that seen for the PR clone. The processing of CA-SP1 to mature CA was not affected by the PTAP duplication (Fig. 8A). These results demonstrate that the PTAP duplication enhanced the ability of the mutant PR to cleave the NC-SP2-p6 processing intermediate to mature NC and p6. To determine whether these effects on NC-SP2-p6 processing could be confirmed with other PIs, we performed experiments similar to those whose results are presented in Fig. 8A and B with two additional PIs: AMP and ATV. We again observed higher levels of NC-SP2-p6 (p15) accumulation in the GagPR clones than in the PR

TABLE 1 PTAP duplication increases resistance to the majority of PIs

Virus ^b	Fold change in IC ₅₀ ^a								
	ATV	DRV	AMP	IDV	LPV	NFV	RTV	SQV	TPV
WT	1.03	0.88	0.97	1.06	1.06	1.02	1.14	1.02	1.10
Gag	1.18	1.08	1.09	1.14	1.46	1.26	1.55	1.03	1.08
PR	4.13	1.02	1.77	3.10	7.37	6.46	63.04	1.14	1.52
GagPR	13.60	1.39	7.28	6.74	18.09	11.24	115.0	1.88	2.63

^a The fold change in IC₅₀ represents the IC₅₀ for the test virus relative to the IC₅₀ for the NL4-3 reference strain. The PIs are abbreviated as follows: ATV, atazanavir; DRV, darunavir; AMP, amprenavir; IDV, indinavir; LPV, lopinavir; NFV, nelfinavir; RTV, ritonavir; SQV, saquinavir; TPV, tipranavir.
^b Test viruses (Fig. 1) were as follows: WT, the R7 molecular clone; Gag, the R7 clone with a PTAP duplication in p6; PR, the R7 clone with mutations in PR; GagPR, the R7 clone with a PTAP duplication in p6 and PI resistance mutations in PR.

FIG 6 The increased PI resistance of the GagPR clone is a result of the PTAP duplication in p6^{Gag} and not the overlapping insertion in p6^{Pol}. 293T cells were transfected with a 20:1 ratio of Gag-expressing R7-based clones to GagPol-expressing R7-based clones. Gag clones contained either WT p6^{Gag} or the version containing the PTAP region duplication (PTAPdup) shown in Fig. 1. The GagPol vector contained the PI resistance mutation in PR (PR in Fig. 1) and p6^{Pol} that either was the WT or contained the duplication (p6^{Pol}-ins). Transfected cultures were treated or not treated with 5 μM RTV. At 2 days posttransfection, virus supernatants were harvested, normalized for RT activity, and used to infect TZM-bl cells. At 2 days postinfection, luciferase activity was measured. The fold reduction in infectivity resulting from RTV treatment was calculated. Note that the PTAP duplication in p6^{Gag} significantly reduces sensitivity to RTV, while the overlapping insertion in p6^{Pol} has no effect. Data were evaluated for statistical significance by using the unpaired Student *t* test. *, *P* < 0.05 (*n* = 3).

clones (Fig. 8C), reinforcing the conclusion that the PTAP duplication increases resistance by allowing more complete processing of NC-SP2-p6 (p15) in the presence of PIs.

DISCUSSION

Although duplications of the PTAP motif in p6^{Gag} have been noted in many studies, the factors that drive the acquisition of these duplications and the consequences of the duplications for HIV-1 replication have not been clearly established. A number of models have been proposed in previous studies, including effects on virus release efficiency, GagPol incorporation into virions, and Gag processing efficiency (see the introduction). Here we demonstrate that a patient-derived PTAP region duplication has no significant effect on virus replication kinetics or specific particle infectivity in the absence of PIs, but in the context of PI-resistant PR and in the presence of inhibitors, it markedly enhances virus replication and infectivity. We show that the PTAP duplication has no effect on the efficiency of virus release but in the presence of clinically relevant concentrations of PIs (57–59) significantly increases the efficiency of NC-SP2-p6 processing. The ability of the PTAP duplication to reduce the susceptibility of virus to PIs was seen across a range of cell types, including primary PBMCs, and was seen with the majority of the PIs tested. Our data clearly show that it is the duplication in p6^{Gag} and not the duplication in the overlapping p6^{Pol} sequence which is responsible for these effects.

There is precedent for mutations in Gag arising together with primary resistance mutations in PR during PI therapy (reviewed

FIG 7 PTAP duplication increases PI resistance in the context of R7/NL4-3 chimeric clones. (A) 293T cells were transfected with the indicated R7/NL4-3 chimeric clones and then not treated or treated with 5 μM RTV or 0.5 μM NFV. Virus supernatants were normalized for RT activity and used to infect the TZM-bl indicator cell line. Luciferase activity was measured at 2 days postinfection. (B) On the basis of the data in panel A, the fold reduction in infectivity for PR and GagPR clones in the presence of PIs was calculated (the ratio of the infectivity of virions prepared in the absence [untreated] to that of virions prepared in the presence [drug treated] of a PI). Data were evaluated for statistical significance by using the unpaired Student *t* test. *, *P* < 0.05 (*n* = 4); **, *P* < 0.01 (*n* = 4). (C) The analysis described above in the legends to panels A and B was repeated with 1 μM AMP or 1 μM ATV. Data were evaluated for statistical significance by using the unpaired Student *t* test. *, *P* < 0.05 (*n* = 3).

FIG 8 PTAP duplication overcomes defects in PR-mediated processing of p15 (NC-SP2-p6) in the presence of PIs. (A) 293T cells were transfected with WT or mutant R7/NL4-3 chimeras and were treated with the indicated concentrations of PIs. Virus-containing supernatants were harvested, and virions were pelleted by ultracentrifugation. Virus lysates were subjected to Western blot analysis with goat anti-NCp7 antiserum. The Gag precursor Pr55^{Gag}, Gag processing intermediates Pr41^{Gag} and p15 (NC-SP2-p6), and mature p7 (NC) are labeled on the right. (B) Defects in NC-SP2-p6 (p15) processing in the presence of PIs were calculated as the ratio of the amount of virion-associated NC-SP2-p6 (p15) to the amount of NC-SP2-p6 (p15) plus p7 (NC). Data were evaluated for statistical significance by using the paired Student *t* test. *, *P* < 0.05 (*n* = 3). (C) The analysis described above in the legends to panels A and B was repeated with 1 μM AMP or 1 μM ATV. Data were evaluated for statistical significance by using the unpaired Student *t* test. *, *P* < 0.05 (*n* = 3); **, *P* < 0.01 (*n* = 3).

in reference 64). An early study (28) identified mutations in the cleavage sites flanking SP2, between NC and p6 (Fig. 1), an observation confirmed in subsequent reports (e.g., see references 30, 33, 36, and 65 to 68). These cleavage site mutations improved cleavage at their respective sites by the PR bearing PI resistance mutations (30), and in some cases, specific Gag mutations appeared to be associated with particular substitutions in PR (38). Gag cleavage site mutations have been demonstrated to not only correct for the fitness defects imposed by PI resistance mutations in PR but also to contribute to drug resistance (27, 28, 33, 34, 37, 39, 69). The Gag mutations, which can be in or outside cleavage site recognition sequences, generally improve PR binding, accessibility, and processing (39).

Although there is some discrepancy in the literature as to the importance of the cleavage between NC and SP2 (27, 70–72), there is strong support for the critical requirement of SP2-p6 cleavage in virus maturation and infectivity (70, 71). Failure to cleave at the SP2-p6 site leads to a marked increase in the percentage of particles with irregular cores and corresponding decreases in particle infectivity and the proportion of virions with normal, conical cores (71). There is also evidence that uncleaved NC-SP2-p6 is defective, relative to mature NC, in nucleic acid condensation and NC-mediated nucleic acid chaperone activity (73, 74). Thus, the accumulation of NC-SP2-p6 observed here in the presence of a PI and drug-resistant PR would be expected to significantly reduce particle infectivity. Although this processing defect is not complete, several studies have demonstrated dominant negative effects of Gag processing intermediates on particle infectivity (72,

75, 76). The ability of the PTAP region duplication in p6^{Gag} to reverse this NC-SP2-p6 processing defect correlates with strongly improved virus replication kinetics and single-cycle infectivity in the context of PI treatment.

As discussed above, a number of studies have demonstrated the ability of cleavage site mutations in the C-terminal region of Gag to enhance viral fitness in the context of drug-resistant PR. This may occur as a result of mutations in Gag restoring a contact with PR abolished by PI resistance mutations in PR (39). PTAP duplications would be predicted to reside outside the portion of the SP1-p6 cleavage site that fits within the substrate-binding groove of PR, but they could well change the interaction of the Gag substrate with the mutant enzyme (the so-called substrate envelope [77]) in such a way as to reverse a processing defect conferred by the PI resistance mutations. It is noteworthy that in our study we did not observe an effect of the PTAP duplication on virus replication kinetics or particle infectivity in the absence of a PI. This result is in concordance with that of our biochemical analysis, which indicated that in the absence of a PI no accumulation of NC-SP2-p6 was observed. Tamiya et al. (48) observed that an insertion just downstream of the native PTAP motif enhanced virus replication and Gag processing efficiency in the absence of a PI. Although the effect of the insertion in the presence of a PI was not tested in that study, it seems likely that the specific sequence and position of the insertion could influence the phenotype of the insert-containing virus. We not only examined the large PTAP region insertion LEPTAPSR (Fig. 1) but also evaluated the effect of a much smaller insertion, APP, on PI resistance. In this case, we

observed that the APP insertion did not increase the PI resistance conferred by changes in PR. Thus, it would appear that not all PTAP region insertions confer enhanced PI resistance. However, given the high frequency at which PTAP region duplications are observed in patients on PI therapy (46), it is likely that increased PI resistance is not unique to the LEPTAPSR insertion studied in detail here.

In summary, the results presented here demonstrate that duplication of the PTAP motif acts not by stimulating virus budding but, rather, by reversing a defect in NC-SP2-p6 processing conferred by the mutations in PR in the context of PIs. The results highlight the interconnected nature of PR and the Gag substrate and the multiplicity of pathways that HIV-1 can follow to enhance drug resistance.

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REFERENCES

1. Bieniasz PD. 2009. The cell biology of HIV-1 virion genesis. *Cell Host Microbe* 5:550–558. <http://dx.doi.org/10.1016/j.chom.2009.05.015>.
2. Freed EO. 2015. HIV-1 assembly, release and maturation. *Nat Rev Microbiol* 13:484–496. <http://dx.doi.org/10.1038/nrmicro3490>.
3. Sundquist WI, Krausslich HG. 2012. HIV-1 assembly, budding, and maturation. *Cold Spring Harb Perspect Med* 2:a006924. <http://dx.doi.org/10.1101/cshperspect.a006924>.
4. Gottlinger HG, Dorfman T, Sodroski JG, Haseltine WA. 1991. Effect of mutations affecting the p6 Gag protein on human immunodeficiency virus particle release. *Proc Natl Acad Sci U S A* 88:3195–3199. <http://dx.doi.org/10.1073/pnas.88.8.3195>.
5. Huang M, Orenstein JM, Martin MA, Freed EO. 1995. p6Gag is required for particle production from full-length human immunodeficiency virus type 1 molecular clones expressing protease. *J Virol* 69:6810–6818.
6. Demirov DG, Orenstein JM, Freed EO. 2002. The late domain of human immunodeficiency virus type 1 p6 promotes virus release in a cell type-dependent manner. *J Virol* 76:105–117. <http://dx.doi.org/10.1128/JVI.76.1.105-117.2002>.
7. Demirov DG, Ono A, Orenstein JM, Freed EO. 2002. Overexpression of the N-terminal domain of TSG101 inhibits HIV-1 budding by blocking late domain function. *Proc Natl Acad Sci U S A* 99:955–960. <http://dx.doi.org/10.1073/pnas.032511899>.
8. Garrus JE, von Schwedler UK, Pornillos OW, Morham SG, Zavitz KH, Wang HE, Wettstein DA, Stray KM, Cote M, Rich RL, Myszkowski DG, Sundquist WI. 2001. Tsg101 and the vacuolar protein sorting pathway are essential for HIV-1 budding. *Cell* 107:55–65. [http://dx.doi.org/10.1016/S0092-8674\(01\)00506-2](http://dx.doi.org/10.1016/S0092-8674(01)00506-2).
9. Martin-Serrano J, Zang T, Bieniasz PD. 2001. HIV-1 and Ebola virus encode small peptide motifs that recruit Tsg101 to sites of particle assembly to facilitate egress. *Nat Med* 7:1313–1319. <http://dx.doi.org/10.1038/nm1201-1313>.
10. VerPlank L, Bouamr F, LaGrassa TJ, Agresta B, Kikonyogo A, Leis J, Carter CA. 2001. Tsg101, a homologue of ubiquitin-conjugating (E2) enzymes, binds the L domain in HIV type 1 Pr55(Gag). *Proc Natl Acad Sci U S A* 98:7724–7729. <http://dx.doi.org/10.1073/pnas.131059198>.
11. Votteler J, Sundquist WI. 2013. Virus budding and the ESCRT pathway. *Cell Host Microbe* 14:232–241. <http://dx.doi.org/10.1016/j.chom.2013.08.012>.
12. Konvalinka J, Krausslich HG, Muller B. 2015. Retroviral proteases and their roles in virion maturation. *Virology* 479-480:403–417. <http://dx.doi.org/10.1016/j.virol.2015.03.021>.
13. Lee SK, Potempa M, Swanstrom R. 2012. The choreography of HIV-1 proteolytic processing and virion assembly. *J Biol Chem* 287:40867–40874. <http://dx.doi.org/10.1074/jbc.R112.399444>.
14. Billich S, Knoop MT, Hansen J, Strop P, Sedlacek J, Mertz R, Moelling K. 1988. Synthetic peptides as substrates and inhibitors of human immune deficiency virus-1 protease. *J Biol Chem* 263:17905–17908.
15. Erickson-Viitanen S, Manfredi J, Viitanen P, Tribe DE, Tritch R, Hutchison CA, III, Loeb DD, Swanstrom R. 1989. Cleavage of HIV-1 gag polyprotein synthesized in vitro: sequential cleavage by the viral protease. *AIDS Res Hum Retroviruses* 5:577–591. <http://dx.doi.org/10.1089/aid.1989.5.577>.
16. Krausslich HG, Ingraham RH, Skoog MT, Wimmer E, Pallai PV, Carter CA. 1989. Activity of purified biosynthetic proteinase of human immunodeficiency virus on natural substrates and synthetic peptides. *Proc Natl Acad Sci U S A* 86:807–811. <http://dx.doi.org/10.1073/pnas.86.3.807>.
17. Pettit SC, Moody MD, Wehbie RS, Kaplan AH, Nantermet PV, Klein CA, Swanstrom R. 1994. The p2 domain of human immunodeficiency virus type 1 Gag regulates sequential proteolytic processing and is required to produce fully infectious virions. *J Virol* 68:8017–8027.
18. Tozser J, Blaha I, Copeland TD, Wondrak EM, Oroszlan S. 1991. Comparison of the HIV-1 and HIV-2 proteinases using oligopeptide substrates representing cleavage sites in Gag and Gag-Pol polyproteins. *FEBS Lett* 281:77–80. [http://dx.doi.org/10.1016/0014-5793\(91\)80362-7](http://dx.doi.org/10.1016/0014-5793(91)80362-7).
19. Gowda SD, Stein BS, Engleman EG. 1989. Identification of protein intermediates in the processing of the p55 HIV-1 gag precursor in cells infected with recombinant vaccinia virus. *J Biol Chem* 264:8459–8462.
20. Gowda SD, Stein BS, Steimer KS, Engleman EG. 1989. Expression and processing of human immunodeficiency virus type 1 gag and pol genes by cells infected with a recombinant vaccinia virus. *J Virol* 63:1451–1454.
21. Mervis RJ, Ahmad N, Lillehoj EP, Raum MG, Salazar FH, Chan HW, Venkatesan S. 1988. The gag gene products of human immunodeficiency virus type 1: alignment within the gag open reading frame, identification of posttranslational modifications, and evidence for alternative gag precursors. *J Virol* 62:3993–4002.
22. Wieggers K, Rutter G, Kottler H, Tessmer U, Hohenberg H, Krausslich HG. 1998. Sequential steps in human immunodeficiency virus particle maturation revealed by alterations of individual Gag polyprotein cleavage sites. *J Virol* 72:2846–2854.
23. Iyidogan P, Anderson KS. 2014. Current perspectives on HIV-1 antiretroviral drug resistance. *Viruses* 6:4095–4139. <http://dx.doi.org/10.3390/v6104095>.
24. Stadel KM, Richman DD. 2013. Rates of emergence of HIV drug resistance in resource-limited settings: a systematic review. *Antivir Ther* 18:115–123. <http://dx.doi.org/10.3851/IMP2437>.
25. Bertagnolio S, Perno CF, Vella S, Pillay D. 2013. The impact of HIV drug resistance on the selection of first- and second-line ART in resource-limited settings. *J Infect Dis* 207(Suppl 2):S45–S48. <http://dx.doi.org/10.1093/infdis/jit121>.
26. Hosseinipour MC, Gupta RK, Van Zyl G, Eron JJ, Nachega JB. 2013. Emergence of HIV drug resistance during first- and second-line antiretroviral therapy in resource-limited settings. *J Infect Dis* 207(Suppl 2):S49–S56. <http://dx.doi.org/10.1093/infdis/jit107>.
27. Dam E, Quercia R, Glass B, Descamps D, Launay O, Duval X, Krausslich HG, Hance AJ, Clavel F. 2009. Gag mutations strongly contribute to HIV-1 resistance to protease inhibitors in highly drug-experienced patients besides compensating for fitness loss. *PLoS Pathog* 5:e1000345. <http://dx.doi.org/10.1371/journal.ppat.1000345>.
28. Doyon L, Croteau G, Thibeault D, Poulin F, Pilote L, Lamarre D. 1996. Second locus involved in human immunodeficiency virus type 1 resistance to protease inhibitors. *J Virol* 70:3763–3769.
29. Gatanaga H, Suzuki Y, Tsang H, Yoshimura K, Kavlick MF, Nagashima K, Gorelick RJ, Mardy S, Tang C, Summers MF, Mitsuya H. 2002. Amino acid substitutions in Gag protein at non-cleavage sites are indispensable for the development of a high multiplicity of HIV-1 resistance against protease inhibitors. *J Biol Chem* 277:5952–5961. <http://dx.doi.org/10.1074/jbc.M108005200>.
30. Mammano F, Petit C, Clavel F. 1998. Resistance-associated loss of viral fitness in human immunodeficiency virus type 1: phenotypic analysis of protease and gag coevolution in protease inhibitor-treated patients. *J Virol* 72:7632–7637.
31. Myint L, Matsuda M, Matsuda Z, Yokomaku Y, Chiba T, Okano A, Yamada K, Sugiura W. 2004. Gag non-cleavage site mutations contribute to full recovery of viral fitness in protease inhibitor-resistant human im-

- munodeficiency virus type 1. *Antimicrob Agents Chemother* 48:444–452. <http://dx.doi.org/10.1128/AAC.48.2.444-452.2004>.
32. Pettit SC, Henderson GJ, Schiffer CA, Swanstrom R. 2002. Replacement of the P1 amino acid of human immunodeficiency virus type 1 Gag processing sites can inhibit or enhance the rate of cleavage by the viral protease. *J Virol* 76:10226–10233. <http://dx.doi.org/10.1128/JVI.76.20.10226-10233.2002>.
 33. Robinson LH, Myers RE, Snowden BW, Tisdale M, Blair ED. 2000. HIV type 1 protease cleavage site mutations and viral fitness: implications for drug susceptibility phenotyping assays. *AIDS Res Hum Retroviruses* 16:1149–1156. <http://dx.doi.org/10.1089/088922200414992>.
 34. Zhang YM, Imamichi H, Imamichi T, Lane HC, Falloon J, Vasudevachari MB, Salzman NP. 1997. Drug resistance during indinavir therapy is caused by mutations in the protease gene and in its Gag substrate cleavage sites. *J Virol* 71:6662–6670.
 35. Bally F, Martinez R, Peters S, Sudre P, Telenti A. 2000. Polymorphism of HIV type 1 gag p7/p1 and p1/p6 cleavage sites: clinical significance and implications for resistance to protease inhibitors. *AIDS Res Hum Retroviruses* 16:1209–1213. <http://dx.doi.org/10.1089/08892220050116970>.
 36. Maguire MF, Guinea R, Griffin P, Macmanus S, Elston RC, Wolfram J, Richards N, Hanlon MH, Porter DJ, Wrin T, Parkin N, Tisdale M, Furfine E, Petropoulos C, Snowden BW, Kleim JP. 2002. Changes in human immunodeficiency virus type 1 Gag at positions L449 and P453 are linked to I50V protease mutants in vivo and cause reduction of sensitivity to amprenavir and improved viral fitness in vitro. *J Virol* 76:7398–7406. <http://dx.doi.org/10.1128/JVI.76.15.7398-7406.2002>.
 37. Nijhuis M, van Maarseveen NM, Laster S, Schipper P, Coakley E, Glass B, Rovenska M, de Jong D, Chappey C, Goedegebuure IW, Heilek-Snyder G, Dulude D, Cammack N, Brakier-Gingras L, Konvalinka J, Parkin N, Krausslich HG, Brun-Vezinet F, Boucher CA. 2007. A novel substrate-based HIV-1 protease inhibitor drug resistance mechanism. *PLoS Med* 4:e36. <http://dx.doi.org/10.1371/journal.pmed.0040036>.
 38. Verheyen J, Litau E, Sing T, Daumer M, Balduin M, Oette M, Fatkenheuer G, Rockstroh JK, Schuldenzucker U, Hoffmann D, Pfister H, Kaiser R. 2006. Compensatory mutations at the HIV cleavage sites p7/p1 and p1/p6-gag in therapy-naïve and therapy-experienced patients. *Antivir Ther* 11:879–887.
 39. Prabu-Jeyabalan M, Nalivaika EA, King NM, Schiffer CA. 2004. Structural basis for coevolution of a human immunodeficiency virus type 1 nucleocapsid-p1 cleavage site with a V82A drug-resistant mutation in viral protease. *J Virol* 78:12446–12454. <http://dx.doi.org/10.1128/JVI.78.22.12446-12454.2004>.
 40. Rabi SA, Laird GM, Durand CM, Laskey S, Shan L, Bailey JR, Chioma S, Moore RD, Siliciano RF. 2013. Multi-step inhibition explains HIV-1 protease inhibitor pharmacodynamics and resistance. *J Clin Invest* 123:3848–3860. <http://dx.doi.org/10.1172/JCI67399>.
 41. Murakami T, Ablan S, Freed EO, Tanaka Y. 2004. Regulation of human immunodeficiency virus type 1 Env-mediated membrane fusion by viral protease activity. *J Virol* 78:1026–1031. <http://dx.doi.org/10.1128/JVI.78.2.1026-1031.2004>.
 42. Wyma DJ, Jiang J, Shi J, Zhou J, Lineberger JE, Miller MD, Aiken C. 2004. Coupling of human immunodeficiency virus type 1 fusion to viron maturation: a novel role of the gp41 cytoplasmic tail. *J Virol* 78:3429–3435. <http://dx.doi.org/10.1128/JVI.78.7.3429-3435.2004>.
 43. Gallego O, de Mendoza C, Corral A, Soriano V. 2003. Changes in the human immunodeficiency virus p7-p1-p6 gag gene in drug-naïve and pretreated patients. *J Clin Microbiol* 41:1245–1247. <http://dx.doi.org/10.1128/JCM.41.3.1245-1247.2003>.
 44. Holguin A, Alvarez A, Soriano V. 2006. Variability in the P6gag domains of HIV-1 involved in viral budding. *AIDS* 20:624–627. <http://dx.doi.org/10.1097/01.aids.0000210619.75707.21>.
 45. Ibe S, Shibata N, Utsumi M, Kaneda T. 2003. Selection of human immunodeficiency virus type 1 variants with an insertion mutation in the p6(gag) and p6(pol) genes under highly active antiretroviral therapy. *Microbiol Immunol* 47:71–79. <http://dx.doi.org/10.1111/j.1348-0421.2003.tb02788.x>.
 46. Martins AN, Arruda MB, Pires AF, Tanuri A, Brindeiro RM. 2011. Accumulation of P(T/S)AP late domain duplications in HIV type 1 subtypes B, C, and F derived from individuals failing ARV therapy and ARV drug-naïve patients. *AIDS Res Hum Retroviruses* 27:687–692. <http://dx.doi.org/10.1089/aid.2010.0282>.
 47. Peters S, Munoz M, Yerly S, Sanchez-Merino V, Lopez-Galindez C, Perrin L, Larder B, Cmarko D, Fakan S, Meylan P, Telenti A. 2001. Resistance to nucleoside analog reverse transcriptase inhibitors mediated by human immunodeficiency virus type 1 p6 protein. *J Virol* 75:9644–9653. <http://dx.doi.org/10.1128/JVI.75.20.9644-9653.2001>.
 48. Tamiya S, Mardy S, Kavlick MF, Yoshimura K, Mistuya H. 2004. Amino acid insertions near Gag cleavage sites restore the otherwise compromised replication of human immunodeficiency virus type 1 variants resistant to protease inhibitors. *J Virol* 78:12030–12040. <http://dx.doi.org/10.1128/JVI.78.21.12030-12040.2004>.
 49. Cao J, McNeven J, McSweyn M, Liu Y, Mullins JI, McElrath MJ. 2008. Novel cytotoxic T-lymphocyte escape mutation by a three-amino-acid insertion in the human immunodeficiency virus type 1 p6Pol and p6Gag late domain associated with drug resistance. *J Virol* 82:495–502. <http://dx.doi.org/10.1128/JVI.01096-07>.
 50. Page KA, Liegler T, Feinberg MB. 1997. Use of a green fluorescent protein as a marker for human immunodeficiency virus type 1 infection. *AIDS Res Hum Retroviruses* 13:1077–1081. <http://dx.doi.org/10.1089/aid.1997.13.1077>.
 51. Brindeiro PA, Brindeiro RM, Mortensen C, Hertogs K, De Vroey V, Rubini NP, Sion FS, De Sa CA, Machado DM, Succi RC, Tanuri A. 2002. Testing genotypic and phenotypic resistance in human immunodeficiency virus type 1 isolates of clade B and other clades from children failing antiretroviral therapy. *J Clin Microbiol* 40:4512–4519. <http://dx.doi.org/10.1128/JCM.40.12.4512-4519.2002>.
 52. Lee J, Lee HJ, Shin MK, Ryu WS. 2004. Versatile PCR-mediated insertion or deletion mutagenesis. *Biotechniques* 36:398–400.
 53. Wei X, Decker JM, Liu H, Zhang Z, Arani RB, Kilby JM, Saag MS, Wu X, Shaw GM, Kappes JC. 2002. Emergence of resistant human immunodeficiency virus type 1 in patients receiving fusion inhibitor (T-20) monotherapy. *Antimicrob Agents Chemother* 46:1896–1905. <http://dx.doi.org/10.1128/AAC.46.6.1896-1905.2002>.
 54. Freed EO, Martin MA. 1994. Evidence for a functional interaction between the V1/V2 and C4 domains of human immunodeficiency virus type 1 envelope glycoprotein gp120. *J Virol* 68:2503–2512.
 55. Waheed AA, Ono A, Freed EO. 2009. Methods for the study of HIV-1 assembly. *Methods Mol Biol* 485:163–184. http://dx.doi.org/10.1007/978-1-59745-170-3_12.
 56. Petropoulos CJ, Parkin NT, Limoli KL, Lie YS, Wrin T, Huang W, Tian H, Smith D, Winslow GA, Capon DJ, Whitcomb JM. 2000. A novel phenotypic drug susceptibility assay for human immunodeficiency virus type 1. *Antimicrob Agents Chemother* 44:920–928. <http://dx.doi.org/10.1128/AAC.44.4.920-928.2000>.
 57. Marzolini C, Buclin T, Decosterd LA, Biollaz J, Telenti A. 2001. Nelfinavir plasma levels under twice-daily and three-times-daily regimens: high interpatient and low inpatient variability. *Ther Drug Monit* 23:394–398. <http://dx.doi.org/10.1097/00007691-200108000-00012>.
 58. van der Lugt J, Lange J, Avihingsanon A, Ananworanich J, Sealoo S, Burger D, Gorowara M, Phanuphak P, Ruxrungtham K. 2009. Plasma concentrations of generic lopinavir/ritonavir in HIV type-1-infected individuals. *Antivir Ther* 14:1001–1004. <http://dx.doi.org/10.3851/IMP1410>.
 59. Zhang KE, Wu E, Patick AK, Kerr B, Zorbas M, Lankford A, Kobayashi T, Maeda Y, Shetty B, Webber S. 2001. Circulating metabolites of the human immunodeficiency virus protease inhibitor nelfinavir in humans: structural identification, levels in plasma, and antiviral activities. *Antimicrob Agents Chemother* 45:1086–1093. <http://dx.doi.org/10.1128/AAC.45.4.1086-1093.2001>.
 60. Martinez-Picado J, Savara AV, Sutton L, D'Aquila RT. 1999. Replicative fitness of protease inhibitor-resistant mutants of human immunodeficiency virus type 1. *J Virol* 73:3744–3752.
 61. Zennou V, Mammano F, Paulous S, Mathez D, Clavel F. 1998. Loss of viral fitness associated with multiple Gag and Gag-Pol processing defects in human immunodeficiency virus type 1 variants selected for resistance to protease inhibitors in vivo. *J Virol* 72:3300–3306.
 62. Yu XF, Dawson L, Tian CJ, Flexner C, Dettnerhofer M. 1998. Mutations of the human immunodeficiency virus type 1 p6Gag domain result in reduced retention of Pol proteins during virus assembly. *J Virol* 72:3412–3417.
 63. Shehu-Xhilaga M, Crowe SM, Mak J. 2001. Maintenance of the Gag/Gag-Pol ratio is important for human immunodeficiency virus type 1 RNA dimerization and viral infectivity. *J Virol* 75:1834–1841. <http://dx.doi.org/10.1128/JVI.75.4.1834-1841.2001>.
 64. Clavel F, Mammano F. 2010. Role of Gag in HIV resistance to protease inhibitors. *Viruses* 2:1411–1426. <http://dx.doi.org/10.3390/v2071411>.
 65. Banke S, Lillemark MR, Gerstoft J, Obel N, Jorgensen LB. 2009. Positive

- selection pressure introduces secondary mutations at Gag cleavage sites in human immunodeficiency virus type 1 harboring major protease resistance mutations. *J Virol* 83:8916–8924. <http://dx.doi.org/10.1128/JVI.00003-09>.
66. Brann TW, Dewar RL, Jiang MK, Shah A, Nagashima K, Metcalf JA, Falloon J, Lane HC, Imamichi T. 2006. Functional correlation between a novel amino acid insertion at codon 19 in the protease of human immunodeficiency virus type 1 and polymorphism in the p1/p6 Gag cleavage site in drug resistance and replication fitness. *J Virol* 80:6136–6145. <http://dx.doi.org/10.1128/JVI.02212-05>.
 67. Cote HC, Brumme ZL, Harrigan PR. 2001. Human immunodeficiency virus type 1 protease cleavage site mutations associated with protease inhibitor cross-resistance selected by indinavir, ritonavir, and/or saquinavir. *J Virol* 75:589–594. <http://dx.doi.org/10.1128/JVI.75.2.589-594.2001>.
 68. Malet I, Roquebert B, Dalban C, Wirden M, Amellal B, Agher R, Simon A, Katlama C, Costagliola D, Calvez V, Marcelin AG. 2007. Association of Gag cleavage sites to protease mutations and to virological response in HIV-1 treated patients. *J Infect* 54:367–374. <http://dx.doi.org/10.1016/j.jinf.2006.06.012>.
 69. Feher A, Weber IT, Bagossi P, Boross P, Mahalingam B, Louis JM, Copeland TD, Torshin IY, Harrison RW, Tozser J. 2002. Effect of sequence polymorphism and drug resistance on two HIV-1 Gag processing sites. *Eur J Biochem* 269:4114–4120. <http://dx.doi.org/10.1046/j.1432-1033.2002.03105.x>.
 70. Coren LV, Thomas JA, Chertova E, Sowder RC, II, Gagliardi TD, Gorelick RJ, Ott DE. 2007. Mutational analysis of the C-terminal gag cleavage sites in human immunodeficiency virus type 1. *J Virol* 81:10047–10054. <http://dx.doi.org/10.1128/JVI.02496-06>.
 71. de Marco A, Heuser AM, Glass B, Krausslich HG, Muller B, Briggs JA. 2012. Role of the SP2 domain and its proteolytic cleavage in HIV-1 structural maturation and infectivity. *J Virol* 86:13708–13716. <http://dx.doi.org/10.1128/JVI.01704-12>.
 72. Muller B, Anders M, Akiyama H, Welsch S, Glass B, Nikovics K, Clavel F, Tervo HM, Keppler OT, Krausslich HG. 2009. HIV-1 Gag processing intermediates trans-dominantly interfere with HIV-1 infectivity. *J Biol Chem* 284:29692–29703. <http://dx.doi.org/10.1074/jbc.M109.027144>.
 73. Wang W, Naiyer N, Mitra M, Li J, Williams MC, Rouzina I, Gorelick RJ, Wu Z, Musier-Forsyth K. 2014. Distinct nucleic acid interaction properties of HIV-1 nucleocapsid protein precursor NCp15 explain reduced viral infectivity. *Nucleic Acids Res* 42:7145–7159. <http://dx.doi.org/10.1093/nar/gku335>.
 74. Wu T, Gorelick RJ, Levin JG. 2014. Selection of fully processed HIV-1 nucleocapsid protein is required for optimal nucleic acid chaperone activity in reverse transcription. *Virus Res* 193:52–64. <http://dx.doi.org/10.1016/j.virusres.2014.06.004>.
 75. Checkley MA, Luttge BG, Soheilian F, Nagashima K, Freed EO. 2010. The capsid-spacer peptide 1 Gag processing intermediate is a dominant-negative inhibitor of HIV-1 maturation. *Virology* 400:137–144. <http://dx.doi.org/10.1016/j.virol.2010.01.028>.
 76. Lee SK, Harris J, Swanstrom R. 2009. A strongly transdominant mutation in the human immunodeficiency virus type 1 gag gene defines an Achilles heel in the virus life cycle. *J Virol* 83:8536–8543. <http://dx.doi.org/10.1128/JVI.00317-09>.
 77. Nalam MN, Schiffer CA. 2008. New approaches to HIV protease inhibitor drug design. II. Testing the substrate envelope hypothesis to avoid drug resistance and discover robust inhibitors. *Curr Opin HIV AIDS* 3:642–646. <http://dx.doi.org/10.1097/COH.0b013e3283136cee>.