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Intracellular Expression of Human Immunodeficiency Virus Type 1 (HIV-1) Protease Variants Inhibits Replication of Wild-Type and Protease Inhibitor-Resistant HIV-1 Strains in Human T-Cell Lines

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The enzymatic activity of the human immunodeficiency type 1 (HIV-1) protease (PR) is crucial to render HIV-1 virions mature and infectious. Hence, genetic intervention strategies based on *trans*-dominant (td) variants of the HIV-1 PR might be an alternative to current pharmacological and gene therapy regimens for AIDS. CD4-positive human CEM-SS T-cell lines were generated which constitutively expressed HIV-1 td PR variants. HIV-1 infection experiments demonstrated severely reduced HIV-1 replication in these td PR CEM-SS cell lines compared with control T cells expressing wild-type PR. Furthermore, replication of an HIV-1 isolate bearing a PR inhibitor-resistant PR was blocked, showing that genetic intervention strategies based on td PRs can be effective against HIV-1 isolates containing PR inhibitor-resistant mutants.

Retroviral proteases (PRs) play an important role in the life cycle of retroviruses (15). In human immunodeficiency virus type 1 (HIV-1), structural and enzymatic components of the virion are expressed as polyprotein precursors, i.e., Gag ($p55^{gag}$) and Gag-Pol ($p160^{gag\text{-}pol}$) (36), respectively, which assemble into immature virions on the cytoplasmic side of the cell membrane (11). The HIV-1 PR belongs to the class of aspartyl PRs (28), but in contrast to eukaryotic aspartyl PRs (e.g., pepsin), proteolytically active HIV-1 PR is a dimer consisting of two monomers in which each subunit contributes the amino acid triplet Asp-Thr-Gly (DTG) to form the active site (37). The HIV-1 PR monomer is part of the $p160^{gag\text{-}pol}$ polyprotein precursor, and therefore, dimerization during the virion assembly process is necessary to allow autoproteolytic release and activation of the PR (8). During and after virion budding from the cell membrane, proteolytic processing of the $p55^{gag}$ and $p160^{gag\text{-}pol}$ polyprotein precursors is initiated by the viral PR, leading to mature Gag (MA, CA, and NC; for nomenclature, see reference 16) and Pol (PR, RT, IN, and RNase H) proteins, condensation of the retroviral core, and hence, infectious virions (11, 14, 29). Inhibition of PR-mediated polyprotein processing, either by site-directed mutagenesis of the PR active site (11, 14, 29) or by application of PR inhibitors (23, 24), has demonstrated the essential role of the HIV-1-encoded PR in the generation of infectious virions. In recent years, several PR inhibitors with in vitro anti-HIV efficacy have been developed and are currently being evaluated in clinical trials (21).

Inhibition of HIV-1 replication by intracellular expression of dominant negative Rev, Gag, or Env variants (3–5, 10, 19, 34, 35) has been demonstrated, leading to the approval of a phase I clinical study inserting the dominant negative RevM10 variant into peripheral blood lymphocytes (25). These approaches are effective because the wild-type (wt) viral proteins multimerize with a dominant negative protein variant, forming inactive complexes. Previously, Babé et al. (2) demonstrated in cotransfection experiments that HIV-1 PR is also a target for dominant negative inhibition of HIV-1 replication when wt and mutant (D25N) PR proteins are present. This inhibition is thought to result from the formation of enzymatically inactive wt-mutant PR heterodimers, which have been observed in in vitro assays (1).

In this report, we show that inhibition of HIV-1 replication by intracellular expression of dominant negative PR variants is an effective anti-HIV strategy. With a previously described cell culture assay (2), we show that mutation of the viral frameshift sequence, combined with an engineered PR variant (Lys-25 Trp-49 Trp-50; herein referred to as KWW) (22), yielded a 12-fold increase in *trans*-dominant (td) inhibition of proteolytic HIV-1 polypeptide processing in comparison with the td PR D25N variant (2). CEM-SS T-cell lines constitutively expressing these td PR inhibitors showed reduced viral replication (up to 4 orders of magnitude) of two laboratory HIV-1 isolates (SF2 and HXB3) as measured by p24 enzyme-linked immunosorbent assay (ELISA). Increasing intracellular expression levels of the td PR inhibitors through manipulation of the viral frameshift yielded a more pronounced inhibition of HIV-1 replication. Combining the td PR inhibitor with a small-molecule PR inhibitor increased overall anti-HIV efficacy. Finally, we demonstrate effective inhibition of viral replication by td PRs of an HIV-1 isolate carrying an inhibitor-resistant PR. These results provide proof of the concept that a clinically relevant anti-HIV gene therapy based on dominant negative HIV-1 PR inhibitors can be developed. Furthermore, genetic intervention strategies can be effective against HIV-1 isolates resistant to small-molecule inhibitors.

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MATERIALS AND METHODS

Plasmid construction. The HIV-gpt plasmid has been described previously (27). Engineered mutations were introduced into the PR gene by site-directed mutagenesis as described previously (32). The frameshift constructions were made with the oligonucleotide 5′-CAGGCTAATTTT**A**TTAGGGAAGATCT -3′ to insert the nucleotide A (in bold) in the phagemid pSPRD25N/KWW at position 1635 of the viral genome. The mutation was confirmed by dideoxy-DNA sequencing (USB) and introduced into the HIV-gpt plasmid, resulting in pFSD25N and pFSKWW.

The HIV-1 ΔEdhfr plasmid was kindly provided by D. Trono (Salk Institute) (35). In this plasmid, HIV-1 HXB2 proviral DNA contains a translational frameshift in the *env* gene and is therefore replication defective (35). In addition, it encodes a dihydrofolate reductase gene in place of *nef* (35). The *Sal*I site in the ΔEdhfr plasmid backbone was destroyed by partial digestion and Klenow treatment, generating ΔEdhfrSalI(−). A *Sal*I-*Xba*I (~5.5 kb) fragment from ΔEdhfrSalI(−) spanning the region between *env* and the 3′ long terminal repeat (LTR) region was used to replace the corresponding region in plasmids HIV-gpt D25N, FS D25N, KWW, and FS KWW, generating ΔEdhfr D25N, FS D25N, KWW, and FS KWW.

Cell culture and transfections. Transient 293T transfections, isolation of viral particles, quantification of p24 CA protein by p24 ELISA, and Western blot (immunoblot) analysis were carried out as previously described (2, 22, 32).

CEM-SS cells (26) were maintained in RPMI 1640 medium (JRH) supplemented with 10% cosmic calf serum (Hyclone). Cells were electroporated (200 V, 960 μF) with 10 μg of linearized plasmid DNA (*Xba*I) and selected in 0.2 μM methotrexate (Sigma) in RPMI 1640 medium with 10% dialyzed fetal calf serum (Hyclone) until control cells were dead. The established cell lines were stable for more than 6 months as determined by intracellular Rev fluorescence-activated cell sorter (FACS) analysis (31). After thawing, cell lines were passaged two or three times before HIV-1 infection.

Northern (RNA) blot and immunoblot analyses. Total cellular RNA was isolated from stably selected CEM-SS cells by using RNAzol B (Biotecx) according to the manufacturer's protocol and analyzed by Northern blot hybridization with ^{32}P-radiolabeled oligonucleotides (4). The oligonucleotide sequences used were TAR (5′-TCGAACCAGAGAGCTCCCAGGCTCAGATCTGGCCA-3′; only the underlined sequence hybridizes to the transactivation response region) and GAPDH (9) (5′-CCATGGTGGTGAAGACGCCAGTGGACTCC-3′).

For Western blot analyses, 2×10^6 cells were harvested, washed once in phosphate-buffered saline, and lysed in phosphate-buffered saline–5 mM EDTA–1% Triton X-100. Following trichloroacetic acid precipitation, cellular proteins were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10 to 20% polyacrylamide gradient gels and transferred to nitrocellulose. Immunoblot analysis was performed with polyclonal HIV-1-seropositive patient serum (kind gift of J. M. McCune), biotinylated goat anti-human immunoglobulin G antibodies, and streptavidin-alkaline phosphatase. Color development was obtained by incubation of the nitrocellulose membrane in a 5-bromo-4-chloro-3-indolylphosphate (BCIP)–nitroblue tetrazolium (NBT) solution.

HIV-1 challenge assays. Polyclonal CEM-SS cells (1×10^6/ml) were inoculated with various doses of HIV-1 SF2 (18), HXB3 (30), or NF6 virus (kind gift of D. Winslow) (20) and incubated at room temperature for 2 h. The HIV-1 NF6 variant contains a PR inhibitor-resistant PR with the mutations V82F and I84V (20). The number of 50% tissue culture-infective doses ($TCID_{50}$) per milliliter of cell-free HIV-1 stock was determined by endpoint titration as previously described (17). Following virus inoculation, cells were diluted in fresh RPMI 1640 medium–10% cosmic calf serum and incubated at 37°C. At different time points thereafter, cell counts were determined and aliquots of the culture supernatants were collected, cleared by centrifugation, and stored at −70°C. HIV-1 replication was determined by analyzing p24 Gag protein synthesis with a p24 ELISA kit (DuPont-NEN). Each p24 value determined represents the mean of at least three independent measurements. Cell counts and HIV-1 p24 antigen release from non-HIV-infected CEM-SS cell lines were determined in parallel. This HIV-1 antigen release per 10^6 cells (ΔEdhfr, ~200 ng of p24; D25N, ~175 pg of p24; FS D25N, ~125 pg of p24; KWW, ~1,700 pg of p24; FS KWW, ~300 pg of p24) was defined as the background and subtracted from the p24 values obtained during HIV-1 infection experiments.

HIV-1 challenge assays in combination with a PR inhibitor. CEM-SS ΔEdhfr and FS KWW cells were inoculated in duplicate with 400 $TCID_{50}$ of HIV-1 HXB3 or NF6 virus per ml as described above. Three days after virus inoculation, cell cultures were split and resuspended in fresh RPMI 1640 medium and one culture was grown in the presence of PR inhibitor A-77003 (kindly provided by D. J. Kempf) (13) at a final concentration of 1 μM. At each passage, cells were resuspended in fresh medium containing the PR inhibitor. Non-inhibitor-treated control cultures and non-HIV-infected control cultures were treated in an identical manner, and HIV-1 antigen levels were determined as described above.

RESULTS

Description of HIV-1 td PR mutants and td PR expression plasmids. Previously, two PR mutants with an active-site mutation (D25N) or a triple mutation (Lys-25 Trp-49 Trp-50 [KWW]) were identified by cotransfection experiments to have a dominant negative phenotype with regard to wt PR activity (2, 22). At the beginning of our studies, it was unclear whether sufficient intracellular td PR could be expressed to effectively block HIV-1 replication in a tissue culture system. To improve td PR expression, a mutation was introduced in the *gag-pol* frameshift region (12) which translocated the *pol* gene into the *gag* reading frame. In transient cotransfection experiments, the frameshift construct pFSD25N inhibited Gag-Pol processing more than the pD25N construct did (Fig. 1A). The frameshift construct inhibited Gag-Pol processing by 50%, as measured by p24 ELISA, relative to the 10% observed with the parental D25N construct (Fig. 1B). The frameshift construct encoding the more potent td PR KWW variant (22) increased PR inhibition relative to pFSD25N, with significant levels of p24 being visible on Western blots only with the pFSD25N

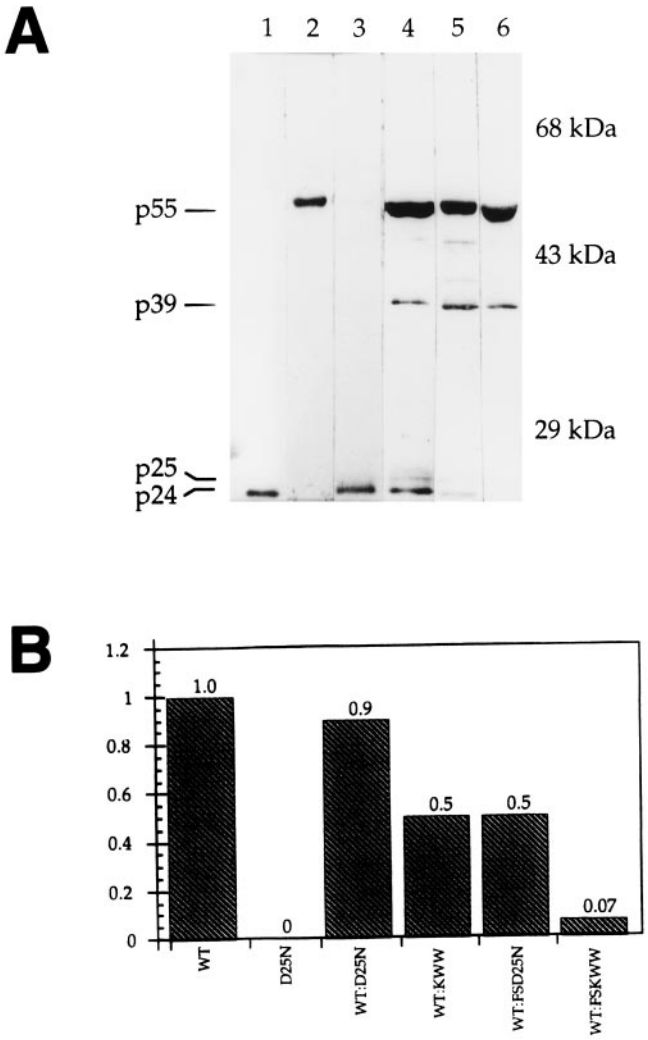

FIG. 1. Inhibition of polyprotein processing in cotransfection experiments. 293T cells were transfected with 10 μg of wt HIV-gpt DNA (lane 1), D25N DNA (lane 2), or a 1:1 ratio of wt DNA and D25N (lane 3), KWW (lane 4), FS D25N (lane 5), or FS KWW (lane 6) DNA. Viral particles were purified by ultracentrifugation and analyzed by anti-p24 immunoblotting following separation by SDS–10% PAGE (A) or by p24 ELISA (B). The data in panel B are ratios of processed to total Gag, determined as previously described (32). The data presented show one representative of five different transfection experiments.

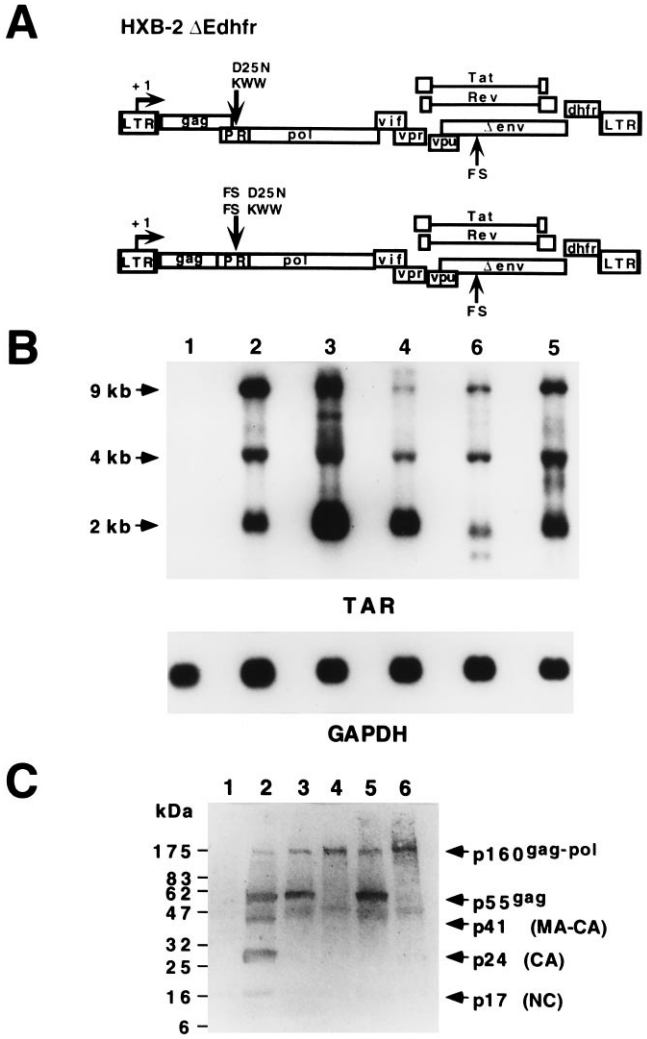

FIG. 2. Schematic representation of HIV-based expression plasmids and molecular characterization of CEM-SS ΔE td PR cell lines. (A) Genomic organization of td PR HIV-based expression plasmids. The names of the expression plasmids are shown above the PR gene. The upper scheme represents the native *gag-pol* arrangement. The lower scheme represents reading frame positions after mutation of the *gag-pol* frameshift. The transcription initiation site in the 5′ LTR is indicated by an arrow. The translational frameshift disrupting translation of the *env* gene is indicated by an arrow below the Δ*env* gene. The *dhfr* gene replacing the *nef* gene is also indicated. (B) Northern blot analysis. Ten micrograms of total RNA was loaded in each lane and hybridized with a ^{32}P-labeled transactivation response region (TAR)-specific oligonucleotide or a glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific oligonucleotide. Locations and sizes of the various mRNA species are indicated on the left. (C) Western blot analysis. Cellular extracts were separated by SDS–10 to 20% gradient PAGE, and Western blots were probed with polyclonal HIV-1 seropositive patient serum. Molecular mass standards are indicated on the left. HIV-specific precursor, intermediate, and mature protein products are indicated on the right. Lanes: 1, CEM-SS; 2, CEM-SS ΔEdhfr; 3, CEM-SS D25N; 4, CEM-SS FS D25N; 5, CEM-SS KWW; 6, CEM-SS FS KWW.

construct (Fig. 1A). Inhibition of polyprotein processing by pFSKWW was sevenfold greater than inhibition by pFSD25N (Fig. 1B).

Both td PR inhibitors were inserted into a modified HXB2 ΔEdhfr expression plasmid (35), generating D25N and KWW (Fig. 2A). The td PR inhibitors were also inserted into a ΔE dhfr plasmid in which the HIV-1 *gag-pol* frameshift (12) was mutated, leading to in-frame expression of Gag-Pol precursors and generating FS D25N and FS KWW (Fig. 2A).

Generation and characterization of CEM-SS td PR cell lines. The ΔEdhfr HIV-1 proviral plasmids encoding the td PR inhibitors were electroporated into the CD4-positive human lymphoid CEM-SS T-cell line, and cells were selected for methotrexate resistance. As a control, a CEM-SS ΔEdhfr cell line was established expressing wt PR from an HIV-1 proviral genome carrying the same modifications as described above but leaving the *gag-pol* frameshift intact. Total cellular RNA was prepared from stably selected CEM-SS cell lines and analyzed by Northern blot analysis with an oligonucleotide probe specific for the TAR element located at the 5′ end of all HIV-1 transcripts (33). Figure 2B shows that all of the CEM-SS cell lines expressed unspliced (~9 kb), singly spliced (~4 kb), and multiply spliced (~2 kb) HIV-1 mRNA transcripts, which is reminiscent of the transcript pattern in the late structural phase of HIV-1 gene expression (7). Rehybridization with a glyceraldehyde-3-phosphate dehydrogenase-specific oligonucleotide demonstrated equal RNA loading in all lanes (Fig. 2B). In addition, intracellular Rev-specific FACS analyses (31) showed comparable intracellular steady-state levels of the splicing-dependent but PR-independent Rev antigen in all cell lines and demonstrated that the cell lines were stable for more than 6 months (data not shown). Western blot analysis of cellular proteins with HIV-1-seropositive patient serum showed that CEM-SS D25N and KWW cell lines expressed high levels of the p55^{gag} precursor and, to a lesser degree, the p160$^{gag\text{-}pol}$ precursor (Fig. 2C, lanes 3 and 5). This Western blot analysis also demonstrated that CEM-SS FS D25N and FS KWW cell lines expressed only the p160$^{gag\text{-}pol}$ precursor, as was expected from the mutation of the *gag-pol* frameshift and the presence of td PRs (Fig. 2C, lanes 4 and 6). In the CEM-SS ΔEdhfr control cell line, both precursors were detected but the expression of wt PR also led to intracellular precursor processing, yielding detectable levels of intermediate (p41 [MA-CA]) and mature (p24 [CA] and p17 [NC]) processing products (Fig. 2C, lane 2), which were absent in all td PR-expressing cell lines. FACS analysis showed that the frequency of surface expression of CD4 (the major receptor for HIV-1) was very high (>95%) and comparable in all established CEM-SS cell lines (data not shown). In summary, these different analyses demonstrated that the established cell lines expressed the transfected constructs correctly and cellular characteristics such as CD4 expression and cellular proliferation were not adversely affected.

Inhibition of HIV-1 SF2 and HXB3 replication in CEM-SS td PR cell lines. CEM-SS ΔEdhfr and CEM-SS D25N and FS D25N cells were inoculated with the HIV-1 SF2 isolate (800 $TCID_{50}$/ml) (18). In the CEM-SS ΔEdhfr control cell line, the amount of released HIV-1 p24 protein increased over time until it reached a plateau between days 14 and 19 postinoculation. In contrast, HIV-1 antigen release remained at low levels in CEM-SS cells expressing td PR variants (Fig. 3A). However, HIV-1 replication was not completely blocked in CEM-SS cells expressing td PR variant D25N or FS D25N, as demonstrated by a logarithmic representation of the data collected (Fig. 3B). The td PR inhibitors did appear to act as dominant negative inhibitors of HIV-1 replication, since HIV-1 p24 antigen levels were 2 to 4 orders of magnitude lower between days 5 and 19 than those in control CEM-SS ΔEdhfr cells (Fig. 3B). Similar results were obtained with a second HIV-1 laboratory strain (HXB3) (30). CEM-SS control cells (ΔEdhfr) and CEM-SS cell lines expressing td PR inhibitors (D25N, FS D25N, KWW, and FS KWW) were inoculated with HIV-1 HXB3 (400 $TCID_{50}$/ml). HIV-1 HXB3 replication in CEM-SS ΔEdhfr control cells was faster than in the HIV-1 SF2 challenge experiments. At day 7, HIV-1 p24 antigen re-

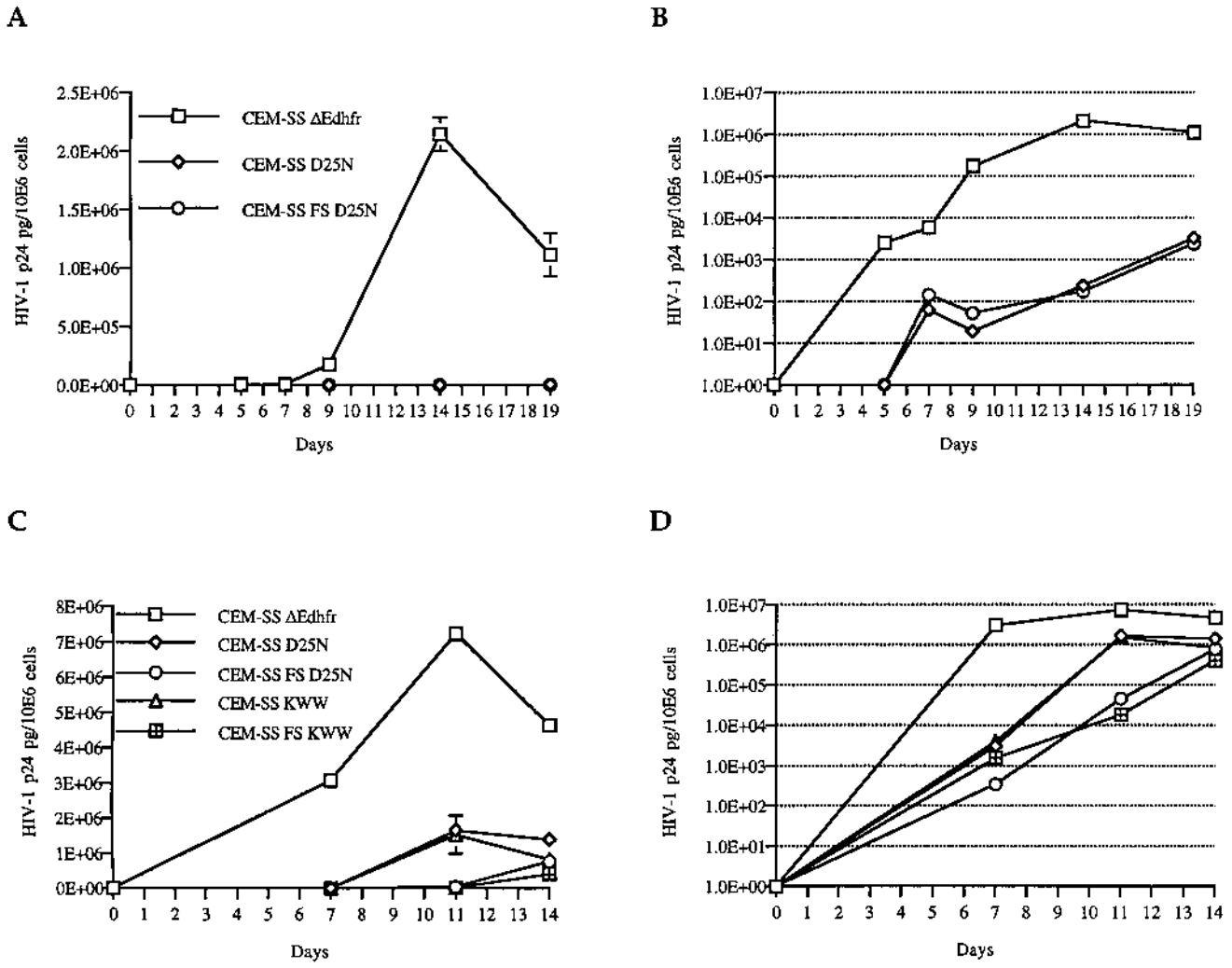

FIG. 3. HIV-1 SF2 and HXB3 challenge experiments. CEM-SS ΔEdhfr and CEM-SS ΔE td PR cell lines were inoculated with 800 $TCID_{50}$ of HIV-1 strain SF2 per ml (A and B) or 400 $TCID_{50}$ of HIV-1 strain HXB3 per ml (C and D), and the culture supernatants were assayed for HIV-1 p24 release at the indicated time points by p24 ELISA. Duplicate HIV-1 challenges were analyzed in A and B and in C and D for CEM-SS KWW and FS KWW. At some time points, error bars are not visible because of minimal variations in the data. Note that graphs B and D are semilogarithmic representations of the data in graphs A and C.

lease in CEM-SS ΔEdhfr control cells (~3 μg/10^6 cells) exceeded the highest HIV-1 p24 levels in CEM-SS ΔEdhfr cells in the HIV-1 SF2 infection experiment at day 14 (~2 μg/10^6 cells) (Fig. 3A and C). In the HIV-1 HXB3 challenge, CEM-SS cell lines expressing the D25N and KWW td PR inhibitors showed low levels of HIV-1 antigen release at days 11 and 14 (Fig. 3C), and CEM-SS cells expressing the FS D25N and FS KWW td PR inhibitors showed even lower HIV-1 antigen release at these time points (Fig. 3C). In a logarithmic representation, the HIV-1 p24 levels in CEM-SS cells expressing the D25N and KWW PR inhibitors were reduced by 3 orders of magnitude at day 7 and nearly 1 order of magnitude at day 11 (Fig. 3D). Furthermore, overexpression of td PR inhibitors in the context of Gag-Pol polyprotein precursors had an additional antiviral effect in this HIV-1 challenge experiment. In CEM-SS FS D25N and FS KWW cells, HIV-1 p24 antigen levels were reduced by 3 to 4 orders of magnitude at day 7 and 2 orders of magnitude at day 11 (Fig. 3D). The CEM-SS cell line expressing the FS KWW td PR inhibitor showed slightly improved anti-HIV activity compared with the CEM-SS FS D25N cell line at later time points (days 11 and 14) (Fig. 3D). In addition, infectivity of the culture supernatants was determined at day 11 after HIV-1 inoculation as previously described (10). Infectivity of the CEM-SS FS D25N supernatants was reduced 32-fold compared with the CEM-SS D25N supernatant, and CEM-SS FS KWW supernatant infectivity was reduced 256-fold compared with the CEM-SS KWW supernatant (data not shown).

Inhibition of HIV-1 NF6 replication in CEM-SS td PR cell lines. Small-molecule PR inhibitors are being tested as antiretroviral agents in clinical trials. The therapeutic benefit has been limited because of the selection of preexisting inhibitor-resistant strains (6). We were interested in the ability of td PR variants to suppress the replication of a PR inhibitor-resistant HIV-1 strain. For these HIV-1 challenges, we used the HIV-1 NF6 strain (kind gift of D. Winslow) (20), which contains two mutations in the PR: valine to phenylalanine at position 82 (V82F) and isoleucine to valine at position 84 (I84V). These mutations confer resistance to the PR inhibitor ABT-538 (20). We performed HIV-1 NF6 infection experiments comparable to the HIV-1 HXB3 challenge (~400 $TCID_{50}$/ml; Fig. 3C and D). The HIV-1 antigen release remained at low levels in

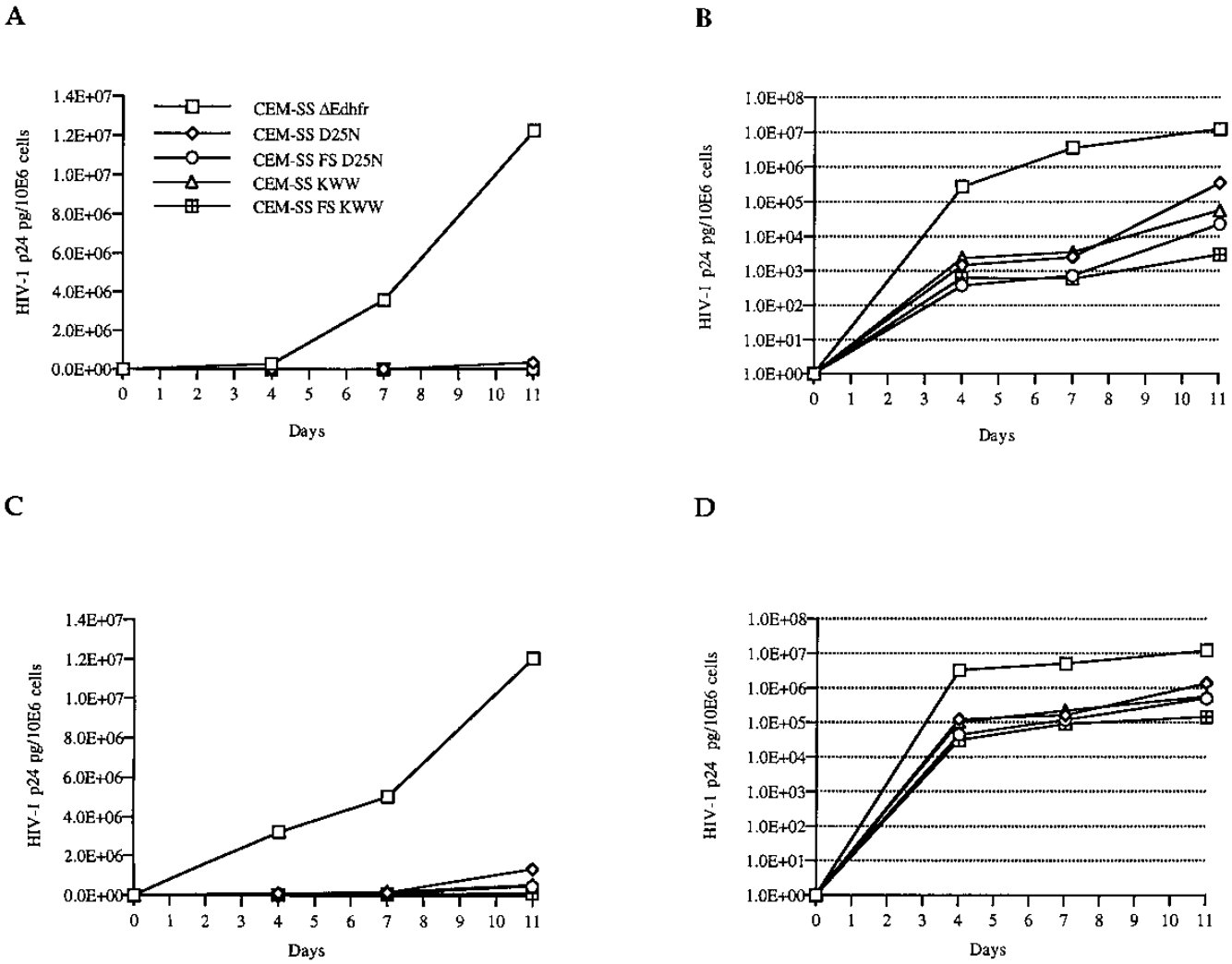

FIG. 4. HIV-1 NF6 challenge experiments. CEM-SS ΔEdhfr and CEM-SS ΔE td PR cell lines were inoculated with ~400 (A and B) or ~40,000 (C and D) $TCID_{50}$ of PR inhibitor-resistant HIV-1 strain NF6 per ml, and the culture supernatants were assayed for HIV-1 p24 release at the indicated time points by p24 ELISA. Note that graphs B and D are semilogarithmic representations of the data in graphs A and C.

CEM-SS cell lines expressing td PR inhibitors, while CEM-SS ΔEdhfr control cells showed slower replication kinetics than in a high-dose HIV-1 NF6 challenge (Fig. 4A and C). On a logarithmic plot, HIV-1 p24 antigen levels were reduced by nearly 2 orders of magnitude at day 4 and by 3 orders of magnitude at day 7 in CEM-SS cell lines expressing td PR inhibitors D25N and KWW (Fig. 4B). Again, higher expression levels of the td PR inhibitors in the CEM-SS FS D25N and FS KWW cell lines seemed to provide an additional antiviral effect, as HIV-1 antigen levels were reduced by 2.5 orders of magnitude at day 4 and 3.5 orders of magnitude at day 7 in these cell lines (Fig. 4B). Figure 4C shows that CEM-SS cells expressing td PR variants suppressed HIV-1 NF6 replication even at high inoculation doses ($\geq 4 \times 10^4$ $TCID_{50}$/ml), while control CEM-SS ΔEdhfr cells released high levels of HIV-1 antigen (~12 μg/10^6 cells), indicating intensive HIV-1 replication. A logarithmic plot showed that at day 4, HIV-1 p24 antigen levels were reduced by 1.5 orders of magnitude in CEM-SS D25N and KWW cell lines and by nearly 2 orders of magnitude in CEM-SS FS D25N and FS KWW cell lines (Fig. 4D). In all CEM-SS td PR cell lines, HIV-1 p24 antigen levels were still reduced by approximately 1.5 orders of magnitude at day 7 (Fig. 4D).

Inhibition of HIV-1 replication in CEM-SS FS KWW cells in combination with a small-molecule PR inhibitor. We were interested in assessing the anti-HIV efficacy of a combination between a small-molecule PR inhibitor and a td PR inhibitor. CEM-SS ΔEdhfr and FS KWW cells were inoculated with the HXB3 or NF6 HIV-1 isolate (400 $TCID_{50}$/ml). Three days after inoculation, cell cultures were split and one culture was propagated in the presence of 1 μM PR inhibitor A-77003 (13). HIV-1 replication kinetics in the CEM-SS FS KWW and ΔEdhfr cell cultures were comparable to those in previous HIV-1 infection experiments without small-molecule PR inhibitors (Fig. 5A and B). Addition of the small-molecule inhibitor completely suppressed HIV-1 HXB3 replication in CEM-SS ΔEdhfr control cells (p24 antigen reduction of >6 orders of magnitude), whereas CEM-SS ΔEdhfr cells in the absence of the PR inhibitor showed active viral replication (Fig. 5A). Interestingly, CEM-SS cells expressing the FS KWW td PR showed a more pronounced antiviral effect in the HXB3 infection experiment in the presence of the PR inhibitor, sug-

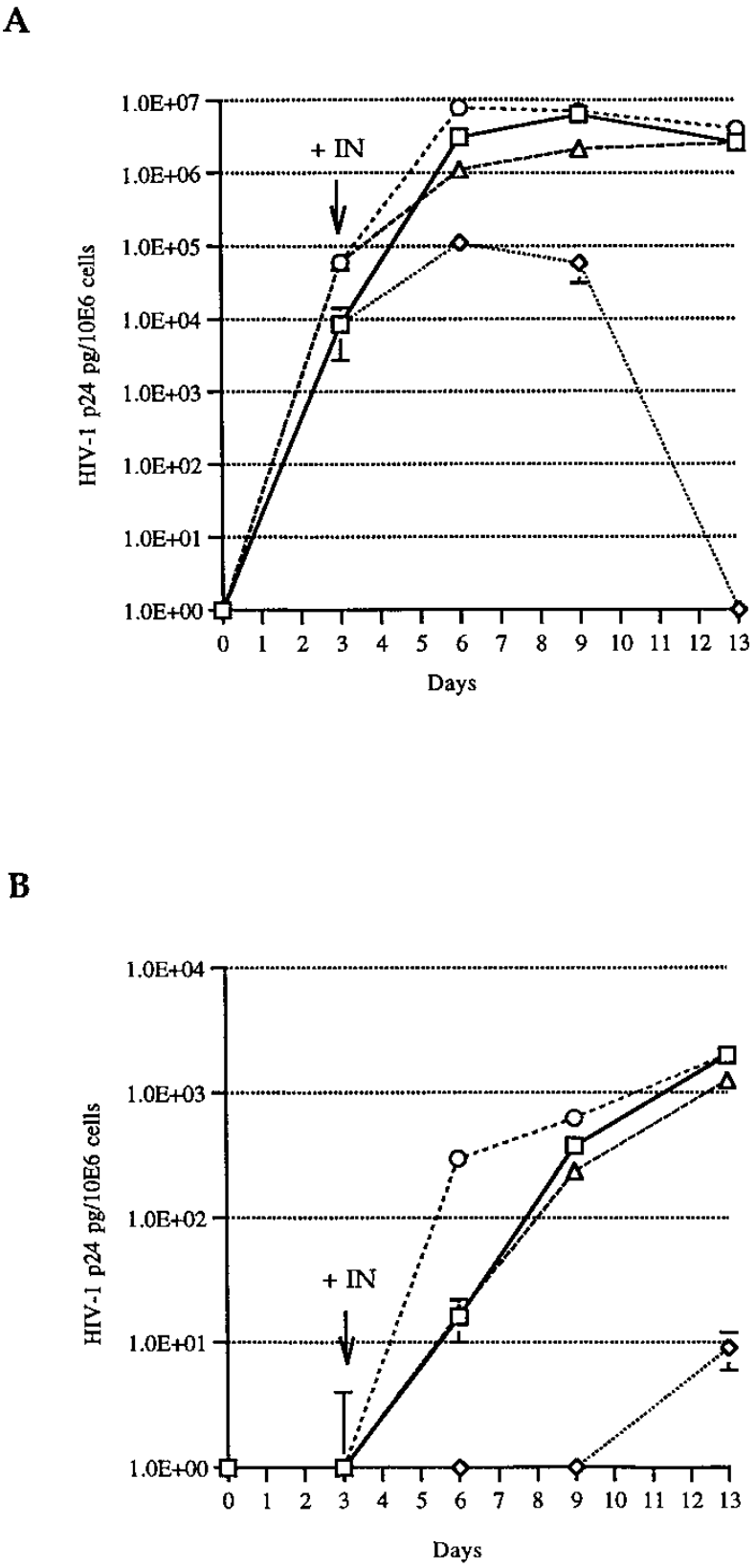

FIG. 5. HIV-1 HXB3 and NF6 challenge experiments in the presence or absence of protease inhibitor A-77003 (IN). The CEM-SS ΔEdhfr (A) and FS KWW (B) cell lines were inoculated in duplicate with 400 $TCID_{50}$ of HIV-1 strain HXB3 or NF6 per ml. Symbols: □, HXB3; ◇, HXB3 plus inhibitor; ○, NF6; △, NF6 plus inhibitor. Three days after HIV-1 inoculation, cell cultures were split and propagated in the presence or absence of PR inhibitor A-77003. Culture supernatants were assayed for HIV-1 p24 release at the indicated time points by p24 ELISA. Duplicate HIV-1 challenges were analyzed. At some time points, error bars are not visible because of minimal variations in the data.

gesting an additive antiviral effect of the combination of an HIV-1 PR inhibitor and the td PR gene therapy strategy. In this case, HIV-1 p24 release was delayed by 6 days, followed by low HIV-1 antigen levels, while in the control HIV-1 infection experiment HIV-1 antigen levels were 2 to 3 orders of magnitude higher (Fig. 5B). In the CEM-SS ΔEdhfr and FS KWW NF6 infection experiment, addition of the PR inhibitor led to a minor (<1 order of magnitude) and transient reduction of viral replication at days 6 and 9, followed by HIV-1 p24 levels comparable to those in the NF6 infection experiment without the PR inhibitor (Fig. 5A and B).

DISCUSSION

In continuation of transient transfection experiments (2, 22), we have generated human CEM-SS T-cell lines constitutively expressing td variants of the HIV-1 PR. In this report, we provide proof of the principle that constitutive expression of td PR inhibitors can efficiently suppress HIV-1 replication. This suppression was observed with laboratory-adapted HIV-1 strains and, more importantly, with HIV-1 isolates bearing an inhibitor-resistant PR.

Our transient studies showed that increasing td PR gene expression also led to improved inhibition of Gag-Pol precursor processing. More importantly, engineering efforts to improve the td PR phenotype (as in KWW) further enhanced antiviral efficacy over the td PR D25N mutant, presumably as a consequence of preferential association of PR heterodimers (22). In our HIV-1 challenge assays, these differences were not readily detectable. In intermediate-dose HIV-1 HXB3 and NF6 infection experiments, both td PR variants D25N and KWW showed comparable anti-HIV efficacies (Fig. 3D and 4B). However, in these infection experiments, increased td PR expression levels led to a moderate increase in antiretroviral activity as measured by the reduction of HIV-1 p24 antigen and infectious virion release (Fig. 3D and 4B). This is also reflected in the observation that HIV-1 antigen release in control, D25N, and KWW cell lines plateaued at the end of the experiments, indicating viral cytopathic effects (Fig. 3D). In contrast, cell lines expressing higher levels of td PR inhibitors (FS D25N and FS KWW) did not show these effects but did display continuously increasing levels of HIV-1 p24 antigen release. On average, the td PR reduced HIV-1 HXB3 (400 $TCID_{50}$/ml) replication by 2 to 3 orders of magnitude as measured 7 to 10 days postinoculation. As a reference, HIV-1 replication was reduced by 1.5 to 2 orders of magnitude in transduced CEM-SS cells expressing the td RevM10 gene from the CMV promoter under similar infection conditions (3, 4, 19). However, the data generated by these two different experimental techniques (retroviral transduction versus transfection and the CMV promoter versus the HIV LTR for transgene expression) do not allow direct comparison of the therapeutic potencies of these two strategies. In high-dose HIV-1 NF6 challenges, all td PR inhibitors revealed comparable antiviral potencies, regardless of the identity of the td PR variant or expression level (Fig. 4D). This suggests that an intracellular threshold td PR inhibitor level must be achieved to inhibit HIV-1 replication, which is dependent on the initial viral load. It is also interesting that the combination of a PR inhibitor and a td PR variant has greater antiviral efficacy than the td PR strategy alone (Fig. 5B). This is most likely due to the inhibition of existing wt PR homodimers.

By using a small-molecule PR inhibitor and a PR inhibitor-resistant HIV-1 isolate, we provide the first experimental evidence that a genetic intervention strategy could remain effective when inhibitor-resistant HIV-1 PR variants arise during pharmacologic therapy. Hence, in the treatment of AIDS, it would allow the sequential or concomitant application of conventional drug-based antiviral therapies and td-PR-based gene therapy to treat emerging drug-resistant viral strains in vivo. As expected, small-molecule PR inhibitors are more effective because effective systemic drug concentrations can be rapidly achieved. However, this effect is transient and selects for preexisting PR inhibitor-resistant mutants (6). In contrast, the intracellular immunization strategies are effective at the cellular level, when intracellular td PR expression levels sufficient to prevent HIV-1 replication in individual cells are obtained. With the present gene transfer and gene expression efficacies, HIV-1 replication occurs only in cells which do not express effective td PR levels. Therefore, selection pressure on HIV-1 is minimal or nonexistent because the virus can replicate in transduced cells with ineffective td PR expression levels, supporting the concept of combination therapy. HIV-1 virions produced in an HIV-1 NF6 infection experiment from cells expressing a td PR inhibitor were still unaffected by the small-molecule PR inhibitor (Fig. 5B), suggesting that these HIV-1 virions contain the original inhibitor-resistant PR. We cannot exclude the possibility that our results are specific to HIV-1 strain NF6, but in the PR of this virus, two critical amino acid residues are mutated, conferring broad cross-resistance to several PR inhibitors in vitro and in vivo (6, 20). Additional studies with primary clinical HIV-1 isolates obtained from patients after long-term PR inhibitor therapy should conclusively answer this question. In addition, as retroviral PRs are well conserved (28), it might also be of interest to analyze the antiviral efficacy of the td PR gene therapy strategy against other clinically relevant HIV-1 and HIV-2 isolates.

Gene delivery into the appropriate cell population (e.g., peripheral blood lymphocytes or hematopoietic stem progenitor cells), targeting of the td PR to the budding HIV-1 virion, and evaluation of antiretroviral activity in these cells are important next steps which must be taken towards the development of a clinically relevant gene therapy protocol. Following infection of cell lines expressing td PR inhibitors with HIV-1, immature virions would contain Gag-Pol precursors carrying heterodimers of wt and td PRs. These heterodimers are unable to support virion maturation, thus blocking viral infectivity and replication. For these reasons, the use of td Gag variants (34, 35) is an obvious strategy which could be combined with td PR variants. Expression of the td PR in fusion with the td *gag* gene from a retroviral vector imitating the genomic organization of HIV-1 would target the td Gag-td PR to the budding virion and result in an additive antiviral effect. Alternatively, the td PR strategy might also be combined with other gene therapy approaches (e.g., td RevM10, ribozymes, RNA decoys) which exert their antiviral effects during different stages of the retroviral life cycle and with different modes of action. Anti-HIV efficacy can be addressed in primary human cells, which allows simultaneous evaluation of transduction efficiency, levels and maintenance of transgene expression, safety, and toxicity. The cellular toxicity of the td PR approach is apparently minimal because of the use of proteolytically inactive td PR variants.

In summary, we have shown that human CEM-SS T-cell lines stably expressing td PR inhibitors are able to significantly inhibit HIV-1 replication. Our findings may be useful for the development of an antiviral gene therapy strategy based on the expression of td PR inhibitors for the clinical treatment of AIDS.

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