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Potent Inhibition of Human Immunodeficiency Virus Type 1 (HIV-1) Replication by Inducible Expression of HIV-1 PR Multimers

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Constructs were generated in which expression of human immunodeficiency virus type 1 (HIV-1) protease (PR) was placed under control of the HIV-1 long terminal repeat, thus requiring the HIV-1 Tat protein for expression of PR. The activity of PR was assessed by cotransfection with a construct producing a Gag substrate. Expression of PR as an intramolecular multimer resulted in a large increase in PR activity in comparison with the level obtained with the expression of PR as a monomer. A cytotoxic effect of PR expression was also exhibited by the constructs expressing PR multimers. $CD4^+$ T-cell lines were generated with a construct producing PR as a linked tetramer and screened for PR activity and inducibility. The replication of HIV-1 in these cell lines was several orders of magnitude reduced in comparison with that in cell lines not expressing PR. Infection in these cell lines could be detected early after infection but disappeared over time. Infection of the PR-expressing cell lines could be increased several orders of magnitude by the addition of a specific inhibitor of PR, U75875 (Upjohn), after infection of the cells, demonstrating that the potent inhibition of HIV-1 replication in these cells was directly due to the expression of PR.

Human immunodeficiency virus type 1 (HIV-1) gene expression is tightly controlled through the effects of viral regulatory proteins. High levels of expression from the viral long terminal repeat (LTR) require expression of the HIV-1 Tat protein (9, 27, 31). Without the Tat protein, viral replication is abolished. The HIV-1 Gag and Pol proteins are translated as polyprotein precursor molecules which interact with the genomic viral RNA and other viral proteins to form new viral particles. These polyprotein precursors are cleaved, subsequent to viral assembly, by a virally encoded protease (PR) (17). Cleavage of the Gag and Gag/Pol precursors is necessary for the formation of infectious virions (16). Cleavage of the precursor molecules can be inhibited through the use of various specific inhibitors of PR (for a review, see reference 7). Some cleavage of precursors by PR can be detected in the cytoplasm of infected cells; however, most cleavage appears to be delayed until viral assembly and budding occur (12, 13). This may be a result of the fact that PR must dimerize to be active (15, 24, 34). Several methods have been used to examine the effects of overexpression of HIV-1 PR on viral particle formation. PR has been expressed as a single-chain PR dimer within the context of a proviral construct (18). In these experiments, the clone containing the PR dimer was defective in both viral particle formation and infectivity. The dimer PR in this construct was shown to be more active than the monomer PR in its ability to cleave the Gag/Pol precursor molecule both in vitro and in vivo. Additionally, transient transfection of this construct into COS-7 cells led to an increase in cytotoxicity of the transfected cells; 48 h after transfection, no viable cells expressing HIV-1 antigens were found. The effect of overexpression of the HIV-1 PR has also been investigated by elimination of the requirement for a ribosomal frameshifting event to express PR (10, 14, 23, 25). This was accomplished by mutating HIV-1 sequences to place Gag and Pol proteins in the same reading frame. Different groups used these Gag/Pol fusions within the context of an HIV-1 provirus, as well as within other vectors. In all of these cases, a defect in particle formation was demonstrated for those vectors which overexpress PR. Overprocessing of the precursor molecule was observed in these experiments. PR appears to be toxic to mammalian cells when produced at high levels (12, 13, 18, 19, 21, 23). Cleavage of cellular proteins by the PR may be responsible for the cytotoxic effects of PR overexpression (1, 26, 30, 32, 33). There appears to be adequate precedent that overexpression of HIV-1 PR in *cis*, within the context of a Gag/Pol precursor molecule, inhibits HIV-1 replication by interfering with particle formation. In this study, we investigated the ability of HIV-1 PR to function independently as an antiviral gene when expressed in *trans*.

MATERIALS AND METHODS

Expression vectors. The construct LTRPR was derived from the construct LTRNef by PCR mutagenesis (4). This resulted in alteration of sequences (to DNA sequence **ATG**GAA<u>ACTAGT</u>; amino acid sequence Met-Glu-Thr-Ser) surrounding the Nef initiation codon (bold) to create an *Spe*I site (underlined) just downstream of this site. This allowed insertion of an *Spe*I-to-*Kpn*I (PR-containing) fragment (3), resulting in fusion of PR in frame to the Nef initiation codon and to Nef sequences downstream of the *Kpn*I site (3). Full details on plasmid construction are available from us. The construct LTR2XPR was generated by isolation of the *Spe*I-*Xba*I (PR-containing) fragment from LTRPR (see Fig. 1) and reinsertion of this fragment into the *Spe*I site of LTRPR. This generated an in-frame fusion of two identical copies of PR. The construct LTR2XPR was generated by isolation of the *Spe*I-*Xba*I fragment (containing two copies of PR) from LTR2XPR and reinsertion of this fragment into the *Spe*I site of LTR2XPR. This generated an in-frame fusion of four identical copies of PR. LTRPR was digested with *Spe*I and *Xba*I and religated to generate LTRΔNef, in which the PR-containing *Spe*I-*Xba*I fragment was deleted. CSF-A was generated by treatment of pYKJRCSF/EBV (2) with *Avr*II and religation. This construct contains a large in-frame deletion between two *Avr*II sites at positions 2012 and 5672 in the HIV genome (indicated by an open box in Fig. 1) that removes sequences from Gag-NC to Vpr. A 2.2-kb *Eco*RI fragment containing the Epstein-Barr virus (EBV) ori P (2) was inserted in place of the simian virus 40

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neo-containing *Eco*RI fragment in the LTR4XPR vector by standard molecular techniques.

Tissue culture and transfection. JM (29) and 729 cells were maintained in Iscove's medium supplemented with 1% Pen/Strep, 0.5% glutamine, and 10% fetal calf serum (all products were from BRL/Gibco). Cells were electroporated as previously described (2). Cells were incubated for 24 h in the presence or absence of the HIV-1 protease inhibitor (U75875; Upjohn) before being harvested for protein extraction. U75875 (5) was maintained as a stock solution at 5 mM in dimethyl sulfoxide at −20°C. Stable cell lines were selected and maintained in 1.5 mg of G418 (BRL/Gibco) per ml.

Western blotting (immunoblotting). Cells were harvested by centrifugation at 1,000 × *g* for 5 min. Cells were washed twice in phosphate-buffered saline (PBS) (137 mM NaCl, 3 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4) and lysed in 200 to 400 μl of NP40 lysis buffer (0.65% Nonidet P-40, 0.15 M NaCl, 10 mM Tris [pH 7.8], 1.5 mM $MgCl_2$). Nuclei were removed by low-speed (1,000 × *g*/2 min) and high-speed (16,000 × *g*/5 min) centrifugations. A 10- to 20-μl volume of the cytoplasmic extract was mixed with an equal volume of sample buffer (8 mM Tris [pH 8.0], 2.0% sodium dodecyl sulfate [SDS], 0.1 M dithiothreitol, 10% glycerol, 0.1% bromophenol blue), loaded onto a lane of an SDS-polyacrylamide gel (between 6 and 15% polyacrylamide), and run in the Laemmli buffer system (22). The proteins were transferred to nitrocellulose by using a Transblot apparatus (Bio-Rad) with 25 mM Tris–192 mM glycine–20% (wt/vol) methanol as the blotting buffer. The membrane was blocked for 1 to 16 h with 5% bovine serum albumin in TBST (20 mM Tris [pH 7.6], 150 mM NaCl, 0.05% Tween 20). The membranes were then subjected to incubations with human anti-HIV-1 (dilution, 1:4,000; NIH AIDS Reagent Program) for 1 h, biotinylated goat anti-human immunoglobulin (1:4,000; Amersham) for 1 h, streptavidin (1:2,000; Amersham) for 30 min, and biotinylated horseradish peroxidase (1:2,000; Amersham) for 30 min. All incubations were performed in TBST, and the membranes were rinsed twice and washed (5 min) twice with TBST between incubations. A final 10-min wash was also performed. The membrane was then developed by using the ECL system (Amersham) and exposed to film (X-Omat-AR; Kodak).

Quantitative RNA PCR. RNA was prepared from cytoplasmic extracts as previously described (2). The samples were treated with RNase-free DNase twice to remove DNA (Boehringer Mannheim). The samples were resuspended in 100 μl of H_2O, and 8 μl was assayed by a quantitative RNA PCR protocol as previously described (2). All reverse transcription reactions included a mock (without reverse transcriptase) reaction done in parallel to demonstrate the RNA nature of the resultant PCR signals. The sequences and nucleotide positions for the oligonucleotide primer pairs used for the amplification of HIV sequences contained within the LTR4XPR construct are as follows: U3Sal, 5′-CTAGTACCAGTcGAcCCAGAGAAGG-3′ (nucleotides 9245 to 9269 of HIV-1JR-CSF); NefSpe 5′-atccaaactagtGCAGTCCTTGTAGTACTCCG-3′ (capital letters indicate homology to nucleotides 9430 to 9411 of HIV-1_{JR-CSF}). PCRs were performed by using a 9600 Gene Amp PCR system (Perkin-Elmer Cetus) for 25 cycles of amplification. The conditions used were denaturation for 30 s at 94°C and annealing and extension for 2 min at 65°C.

Viral infections and immunofluorescence. Infection of cell lines with HIV-1_{SF-2} (NIH AIDS Reagent Program) was performed in the presence of Polybrene (10 μg/ml) for 1 to 2 h as previously described (35). Generation of virus stocks for infections was accomplished by propagation of virus in JM cells and harvesting of cell-free supernatants at the peak of viral infection was determined by immunofluorescence. Immunofluorescence was performed essentially as described elsewhere (11). Briefly, pelleted cells were washed twice in PBS (see above) and resuspended in 100 to 300 μl of PBS. A 10-μl volume of cells was spotted onto a glass microscope slide and allowed to dry. Dried slides were fixed for 10 min in ice-cold acetone. A 40-μl volume of human anti-HIV-1 antisera (dilution, 1:200; NIH AIDS Reagent Program) was distributed over each fixed spot of cells. Slides were incubated in a 37°C incubator (87% humidity, 6% CO_2) for 20 to 30 min. Slides were washed twice for 5 min in PBS and once for 5 min in H_2O. Slides were air dried, and 40-μl of fluoresceinated goat anti-human immunoglobulin (dilution, 1:40; Cappel) was distributed over each fixed spot of cells. Slides were incubated in a 37°C incubator (87% humidity, 6% CO_2) for 20 to 30 min and washed as described above. Slides were air dried and covered with 70% glycerol in PBS, and a coverslip was applied. Infected (fluorescing) cells were examined and counted with a microscope equipped for epifluorescence (Zeiss).

RESULTS

Activity and cytotoxicity of PR expression vectors. We had previously shown that HIV-1 PR, when expressed as a heterologous fusion protein, appeared to express high levels of proteolytic activity and cleaved the precursor fusion protein to completion (3). In contrast to results with PR expressed as intramolecular dimers within the context of the Gag/Pol precursor (18), no cytotoxic effect was seen with these constructs. We wished to generate constructs that would inducibly express maximal levels of proteolytically active PR as well as induce a cytotoxic effect in the infected cells. To assure inducibility of the constructs, we expressed PR under the control of the HIV-1 LTR. This necessitated the expression of the HIV-1 Tat protein to achieve high levels of expression from the constructs. Since the constructs expressing PR as a monomer did not demonstrate any cytotoxic effect (3), we generated additional constructs expressing PR as intramolecular multimers in the absence of any Gag or Pol sequences. The constructs expressing monomer, dimer, or tetramer intramolecular configurations of PR are shown in Fig. 1. The PR cleavage sites flanking each of the PR monomers were retained. Additional amino acids were also added during construction of the multimers such that a total of 16 amino acids were present between adjoining PR cleavage sites. As a substrate to assess the activity of the various PR-expressing constructs, we used a construct, CSF-A, that has deletions of all of *pol* (PR) and *vif* and part of *gag* and *vpr*. This construct should generate an approximately 53-kDa Gag/Vpr precursor molecule as a substrate for PR and should also produce Tat. The production of Tat by this construct should allow induction of PR expression from the LTR-driven PR constructs. The CSF-A construct was cotransfected into lymphoid cells together with the PR-expressing constructs. Aliquots of the transfected cells were incubated in the presence or absence of a specific HIV-1 PR inhibitor (U75875; Upjohn) (5). This allowed the direct assessment of effects attributable to PR. As a control, a construct expressing an inactive PR, LTRNefPRΔ3 (3), was cotransfected with CSF-A. After 24 h of incubation, HIV-1 protein production was analyzed by Western blotting with anti-HIV-1 antisera (Fig. 2A). In all transfections, the expected $gp160^{Env}$ and an approximately 53-kDa protein, consistent in size with the predicted Gag/Vpr fusion protein, were seen. A protein doublet at approximately 24/25 kDa was seen only in the transfections with PR-expressing constructs. These bands are consistent with the expected appearance of processed p24/p25 CA Gag proteins in the presence of PR. The level of p24/p25 CA was vastly increased in the transfections with the constructs expressing intramolecular multimers of PR in comparison with the level in transfections with the monomer PR construct. A 41-kDa protein was also detected in those transfections with the constructs expressing intramolecular multimers of PR. This product is consistent with the expected partially processed Gag precursor protein consisting of p17 and p24. No processed products were observed in the transfections done with the PR-negative construct or in the transfection mixtures incubated in the presence of the PR inhibitor. These results indicated that the expression of PR as a multimer vastly increased the intracellular level of PR activity. This did not seem to be due merely to increased expression of PR, as the effect appeared to be much greater than two- or fourfold. In other experiments, adjusting for the two- or fourfold difference in PR copy number did not compensate for the difference in PR activity seen between constructs expressing PR as monomers and multimers (data not shown). Interestingly, the level of $gp160^{Env}$ appeared to be affected by the presence of the PR inhibitor; in the absence of PR inhibitor, a reduction in the level of $gp160^{Env}$ was demonstrated in the transfections done with the constructs expressing multimers of PR. Since Env is not a substrate for PR, the disappearance of Env indicated that the expression of PR multimers was causing a cytotoxic effect similar to that which was previously observed (18).

In the experiments detailed above, the expression of the substrate construct had been optimized by insertion of a fragment of DNA containing the EBV origin of replication into the plasmid. This allows for increased expression from the construct in EBV-transformed cells (2). The PR-expressing con-

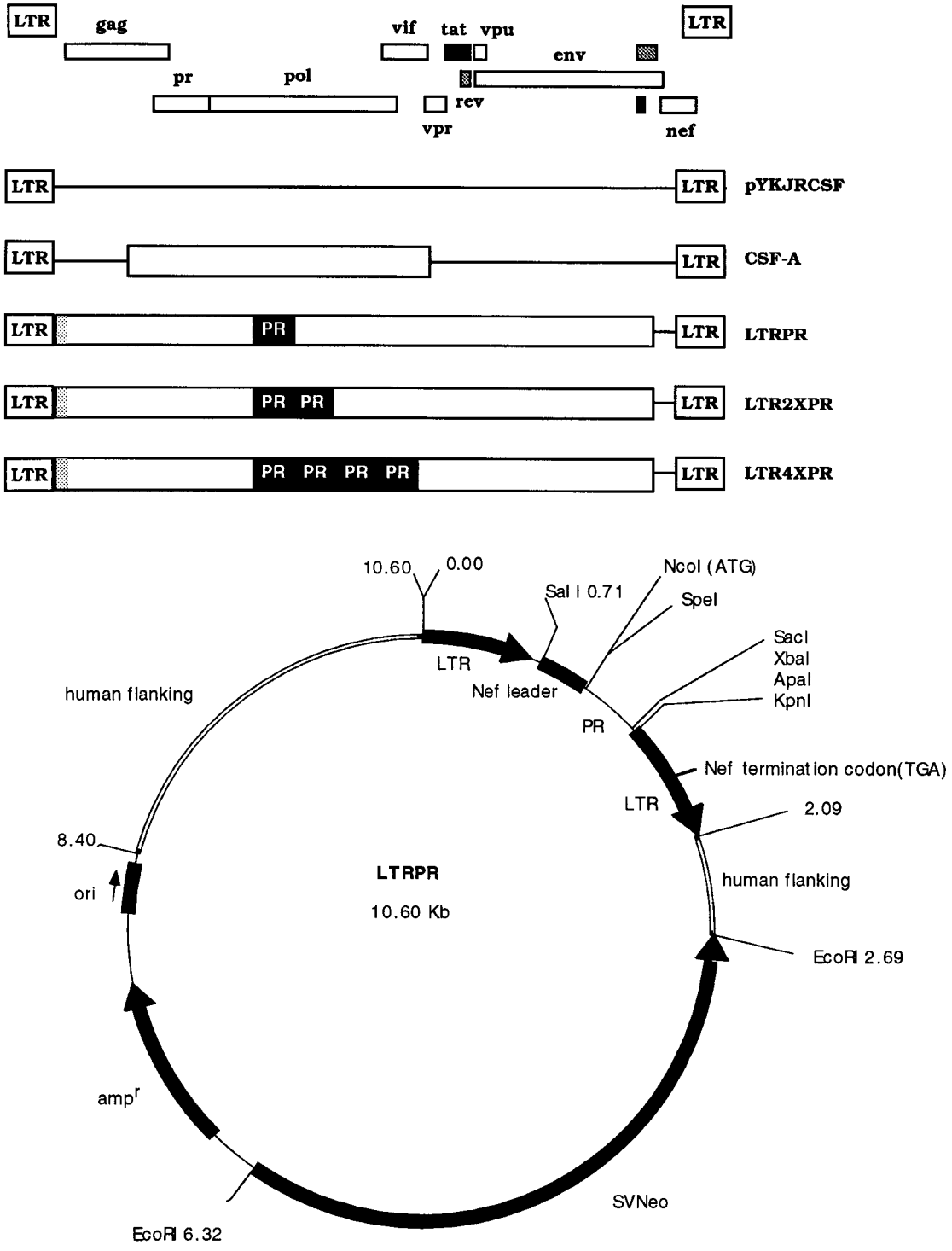

FIG. 1. Schematic representation of the constructs generated to examine HIV-1 PR activity and cytotoxicity. The complete LTRPR construct and the coding region changes in each of the fusion constructs are illustrated. The locations of deleted sequences are indicated by the unfilled boxes. The locations of the reinserted PR-containing fragments are indicated by black boxes. The Nef leader sequence is indicated by the stippled boxes. The Nef translational initiation (ATG) and termination (TGA) sites are shown.

struct did not contain this DNA fragment. To further define the limits to which we could extend the activity and cytotoxicity of the PR-expressing constructs, we assessed the impact of increasing the ratio of the transfected LTR4XPR construct to the substrate CSF-A construct. The results are shown in Fig. 2B. As shown in Fig. 2A, the appearances of p41 and p24/p25 are dependent upon PR expression. Increasing the ratio of PR to substrate resulted in the nearly complete disappearance of both p53ΔGag/Vpr and $gp160^{Env}$. These results indicated that sufficient overexpression of PR multimers could result in

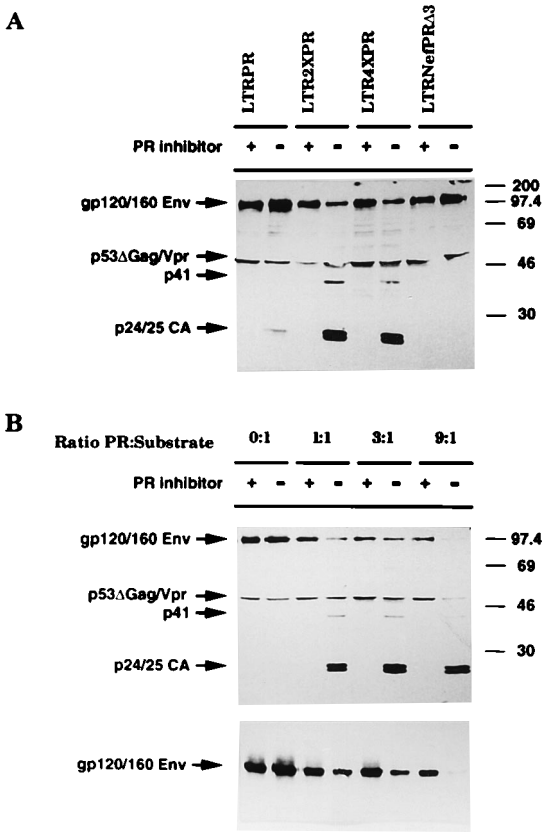

FIG. 2. Proteolytic activity and cytotoxicity of PR expression vectors. (A) 729 B-lymphoid cells were transfected with 25 μg each of the indicated constructs and incubated in the presence (+) or absence (−) or 3 μM U75875. Cytoplasmic extracts were prepared at 24 h posttransfection. Protein expression was examined by Western blotting with anti-HIV-1 antisera. (B) 729 B-lymphoid cells were transfected with 5 μg of CSF-A and LTR4XPR at the indicated ratio. A total of 50 μg of transfected DNA was achieved by the addition of an appropriate amount of PR-negative construct DNA (LTRΔNef). Cytoplasmic extracts were prepared at 24 h posttransfection. Protein expression was examined by Western blotting with anti-HIV-1 antisera. The locations of HIV-1-specific protein products are indicated by arrows. The locations of molecular mass markers (in kilodaltons) are shown. The lowermost panel shows the same samples analyzed on a lower-percentage gel to better visualize Env.

nearly complete elimination of the Gag substrate and Env. One would expect that both of these effects would be extremely detrimental to viral replication if they could be reproduced in infected cells.

To further assess the impact of the level of PR expression relative to the level of substrate, the DNA fragment containing the EBV origin of replication was inserted into the LTR4XPR construct. This should serve to better equalize expression from the PR and substrate constructs. These constructs were cotransfected at a substrate/PR ratio of 1:5 and assessed by Western blotting (Fig. 3). A range of PR concentrations was used to provide additional information as to the procession of cleavage. In the absence of PR inhibitor, little or no p53ΔGag/Vpr precursor and processing intermediate p41 were seen. Only low levels of $gp160^{Env}$ were detected in the absence of PR inhibitor, and these levels increased concomitantly with increasing concentrations of PR inhibitor. The ordered appearance of the various proteins with increasing concentrations of PR inhibitor was consistent with ordered inhibition of cleavage events; the cleavage of p41 to p24/p25 appeared to be more sensitive to inhibition than was the cleavage of p53 to p41. The ordered inhibition of cleavage events correlates well with previous results concerning the ordered cleavage of the HIV-1 Gag precursor (6, 8, 20). Additionally, the cleavage of the precursor protein appeared to be more difficult to inhibit than the cytotoxic effect was. This ordered effect of cleavage at specific sites preceding the cytotoxic effect has also been demonstrated with other PR-expressing constructs (data not shown). These results supported the idea that a high level of proteolytic activity against an exogenous Gag substrate and a cytotoxic effect could be achieved with an inducible construct expressing PR multimers.

PR-expressing T-cell lines. In cotransfection experiments, inducible expression of multimers of PR could be optimized to result in complete cleavage of precursor molecules, as well as cytotoxic effects. Therefore, we wished to assess the ability of inducibly expressed multimers of PR to inhibit HIV-1 replication in stable T-cell lines. The inducible nature of LTR4XPR should allow the establishment stable T-cell lines in the absence of cytotoxic effects, as coexpression of Tat was necessary for expression of PR from this construct. We expected that a stable T-cell line containing this vector would express only low constitutive levels of PR. We also expected that high levels of PR would be produced in the presence of the HIV-1 Tat protein. Tat would be provided upon transfection or infection of the cell line. Theoretically, the effects of this expression would be twofold. First, the Gag and Gag/Pol precursors of the virus would be cleaved, inhibiting virus assembly. Second, the cytotoxic effects of PR expression would make this construct a suicide vector (for a review, see reference 28) and result in specific killing of infected cells. The presence of a simian virus 40-driven neomycin phosphotransferase cassette in the construct allowed selection of G418 resistance clones in the parental $CD4^+$ T-cell line, JM (29). Resistant cells were cloned, propagated, and assayed for PR activity by transfection with the Gag substrate-producing construct, CSF-A. Production of Tat by this construct should activate expression of PR from this construct. Western analysis was performed to determine the processing of the Gag substrate (Fig. 4). Two clones were identified as producing active PR by disappearance of the 53-kDa precursor and appearance of a 24-kDa product. These two PR-producing clones (JM4XP3 and JM4XP6), as well as a PR-negative clone (JM4XP1), were chosen for further analysis. Fluorescence-activated cell sorting (FACS) analysis, at the Medical University of South Carolina FACS facility, demonstrated all three of these clones to be >98% CD4 positive (data not shown). The presence of the PR construct in the cell lines

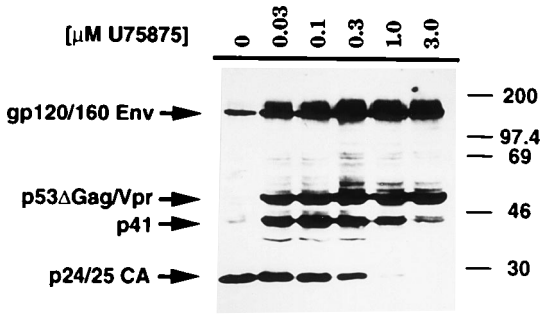

FIG. 3. Proteolytic activity and cytotoxicity of intramolecular PR tetramers. 729 B-lymphoid cells were transfected with 40 μg of LTR4XPR and 10 μg of CSF-A. Aliquots of cells were incubated in the indicated concentrations of U75875. Cytoplasmic proteins were analyzed by Western blotting with anti-HIV-1 antisera. The locations of HIV-1-specific protein products are indicated by arrows. The locations of molecular mass markers (in kilodaltons) are shown.

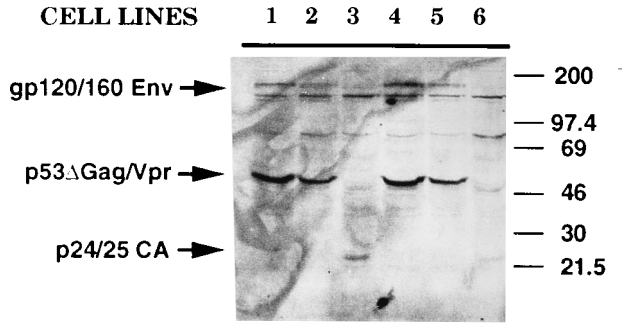

FIG. 4. PR expression in selected cell lines. Six G418-resistant clones from JM cells transfected with LTR4XPR were transfected with CSF-A. Cytoplasmic proteins were analyzed by Western blotting with anti-HIV-1 antisera. The locations of HIV-1-specific protein products are indicated by arrows. The locations of molecular mass markers (in kilodaltons) are shown.

resulted in little or no difference between either the growth rates or gross morphologies of the cell lines (data not shown).

We had previously demonstrated that the LTR-driven gene expression from similar constructs was inducible by the HIV-1 Tat protein (3). In order to determine whether the stable cell lines were still inducible by Tat, we transfected the cell lines with a Tat-producing vector and assessed LTR-specific gene expression by quantitative RNA PCR (Fig. 5). Both the JM4XP3 and JM4XP6 cell lines demonstrated low basal levels of RNA which could be substantially induced in the presence of the Tat protein. No HIV-1 RNA expression in the JM4XP1 cell line was seen (data not shown). These results indicated that the PR-expressing cell lines retained the Tat inducibility specified by the LTR subsequent to integration in these clones. This is not surprising, since we expected that lack of induction would be selected against in our screen for PR-producing clones and high-level constitutive expression would be cytotoxic and selected against accordingly.

Inhibition of HIV-1 replication by PR overexpression. We wished to determine whether the inducible expression of PR in T-cell lines would result in any inhibitory effect on the replication of HIV-1. Therefore, we infected the PR-negative JM4XP1 and the PR-expressing JM4XP3 and JM4XP6 cell lines with HIV-1_{SF-2}. We also included a nonselected $CD4^+$ JM cell clone (JM#4) as a control in some experiments. We examined the levels of viral replication in these cell lines by indirect immunofluorescence with anti-HIV-1 antisera at various time points postinfection. The percentage of cells positive for HIV-1 antigens (infected) was determined. A representative infection profile for each of the cell lines is shown in Fig. 6. The PR-producing cell lines (JM4XP3 and JM4XP6) consistently showed only low levels of infection in many different experiments. Any detectable infection was detected at early time points and decreased with time, such that no infected cells were detected by 21 days postinfection. The PR-negative cell lines (JM4XP1 and JM#4) showed high levels of infection, more than 50% of cells being infected by day 7 postinfection and demonstrating a major cytopathic effect by day 11 postinfection. These results demonstrated that the PR-expressing cell lines were extremely resistant to infection by HIV-1.

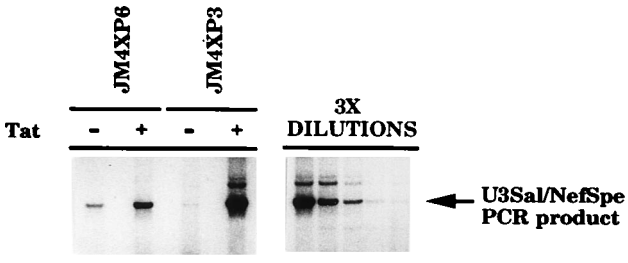

FIG. 5. Inducibility of PR-expressing cell lines. The indicated cell lines were mock transfected (−) or transfected with a Tat-producing construct (+). Cytoplasmic RNA was prepared at 24 h posttransfection and treated with RNase-free DNase to remove contaminating plasmid DNA. The RNA was then subjected to quantitative RNA PCR as described elsewhere (2) by using an oligonucleotide primer pair specific for the HIV-1 LTR sequences. Threefold dilutions of RNA were used to demonstrate the quantitative nature of the procedure.

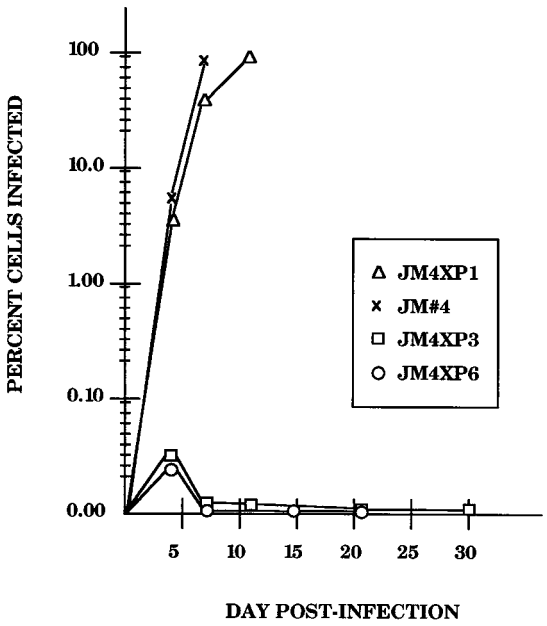

FIG. 6. Resistance of PR-expressing cell lines to productive HIV-1 replication. The indicated cell lines were infected with HIV-1_{SF-2}. The percentage of cells infected at various time points after infection was determined by indirect immunofluorescence with anti-HIV-1 antisera (pooled AIDS patient sera) and fluoresceinated secondary antisera (goat anti-human).

To determine that PR expression was directly responsible for the block to HIV-1 infection in these cells, we infected the PR-producing JM4XP6 cell line with HIV-1 and then split the infected cell population into two flasks. One flask received 1 μM the PR inhibitor U75875, and the other did not. We expected that if the block to infection were directly due to PR production, then the JM4XP6 cell line should be capable of higher levels of infection in the presence of PR inhibitor. As a control, the PR-negative JM4XP1 cell line was infected similarly. In this case, we would expect that the presence of PR inhibitor should not result in higher levels of infection but might result in lowered levels of infection (due to inhibition of viral spread). We examined the cells by indirect immunofluorescence at day 4 postinfection (Fig. 7). In the absence of PR inhibitor, <0.02% of the JM4XP6 cells were shown to be infected. In contrast, >8% of the JM4XP6 cells in the culture that received PR inhibitor were infected. This represents a >400-fold increase in infection in the presence of PR inhibitor. Similar results were seen with the PR-expressing JM4XP3 cell line. Removal of the PR inhibitor at day 4 postinfection resulted in a gradual loss of infected cells from the JM4XP6 infection. The PR-negative JM4XP1 cell line demonstrated high levels of infection in both the presence and the absence of PR inhibitor (Fig. 7). These results indicate that the expression of PR was directly responsible for the inhibition of HIV-1

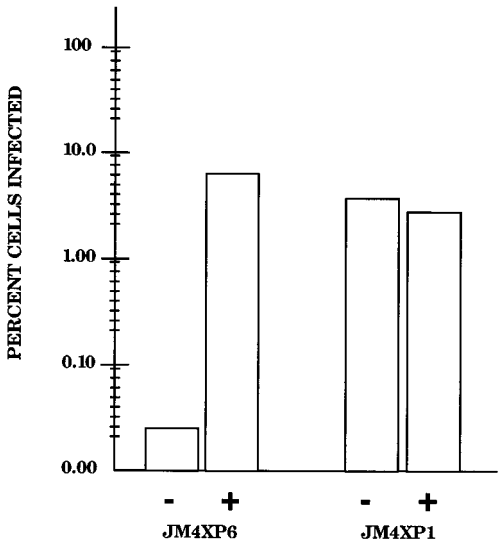

FIG. 7. Infection of resistant cell lines in the presence of PR inhibitor U75875. The indicated cell lines were infected with HIV-1_{SF-2}. Subsequent to infection, cells were incubated in the presence (+) or absence (−) of 1 μM U75875. The percentage of cells infected at 4 days postinfection was determined by indirect immunofluorescence with anti-HIV-1 antisera (pooled AIDS patient sera) and fluoresceinated secondary antisera (goat anti-human).

replication seen with the PR-producing cell lines. The spread of the virus was inhibited by PR, and the reduction in the level of infected cells indicates that infected cells were being eliminated. Therefore, inducible expression of PR multimers appears to exert a potent antiviral effect within cultured T cells, controlling viral spread and killing infected cells.

DISCUSSION

We assessed the ability of various PR-expressing constructs to cleave an exogenous Gag substrate and mediate cytotoxic effects. We find that intramolecular multimers of PR, dimers and tetramers, are capable of dramatically increasing the level of PR activity and cytotoxic effects in comparison with the levels obtained with monomers. It seems likely that this is due to the fact that PR functions as a multimer and is inactive in its monomer state (15, 24, 34). The expression of PR as intramolecular multimers may facilitate the actual formation of the multimeric, active PR. It is interesting that our recent work with PR monomer-expressing constructs (as Nef fusion proteins) indicated that these monomeric constructs were resulting in complete cleavage of the Nef-PR fusion protein precursor molecules in the absence of any cytotoxic effect (3). These constructs were also shown to exhibit a lack of cytotoxicity and low relative cleavage of exogenous Gag substrate by the cotransfection assay described in this paper (data not shown). Therefore, high levels of self cleavage of a PR-expressing precursor molecule do not necessarily serve as a prediction of high levels of *trans* cleavage of an exogenous substrate or a cytotoxic effect.

We assessed whether inducibly expressed multimers of PR might serve as an antiviral gene in inhibiting the replication of HIV-1 in T-cell lines. The inhibition of HIV-1 replication by PR was shown to be specific for PR and highly effective. The use of indirect immunofluorescence to detect infected cells allowed for a very accurate determination of the extent of viral inhibition over a 10,000-fold range. Although HIV-1-infected cells could be seen early after infection of PR-expressing T-cell lines, the levels were very low and diminished with time. This may be due to the initial infection of cells prior to cytotoxicity. The PR-expressing cell lines were able to maintain resistance to HIV-1 infection even after 3 months of continuous culture. Over more prolonged periods of time, one might expect selection against the PR expression capacity of the cell line if the basal level of PR is at all deleterious (or subject to spontaneous induction). Tighter control of PR expression, by adding an additional level of control such as Rev inducibility, would reduce these effects.

We believe that the use of an inducible HIV-1 PR multimer vector offers great potential for a gene therapy approach to HIV-1 infection. This gene contains many attributes which make it ideal as an antiviral gene. In particular, its small size, specificity, enzymatic nature, and cytotoxic effects make it potentially very potent and adaptable to many experimental systems. Generation of a mutated HIV-1 which is resistant to the effects of this gene is highly unlikely. In order for an HIV-1 strain to develop resistance, the amino acid sequence of many PR cleavage sites would need to be altered, with the ability of the virally encoded PR to cleave at these sites being conserved. Therefore, changes in the virally encoded PR would need to accompany changes in the cleavage sites. As PR is a wild-type gene, this scenario seems very doubtful. Alternately, resistance might be achieved by alterations in the inducibility (by Tat) of the PR expression construct. In this case, mutation of Tat would need to be accompanied by mutation of the TAR element. We believe that the ability of PR to serve as an inhibitory gene and a suicide vector makes it an unusually interesting candidate for anti-HIV gene therapy. Indeed, the basic principle is potentially applicable to the inhibition of any retrovirus. It is our hope to develop HIV-1 PR as an antiviral gene, with the goal of application of this technology to the inhibition of HIV-1 replication in a clinical setting. Obviously, many other major hurdles, such as delivery of an antiviral gene to the appropriate cell type, will have to be addressed before gene therapy for HIV-1 infection becomes a reality.

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