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Specific Inhibitor of Human Immunodeficiency Virus Proteinase Prevents the Cytotoxic Effects of a Single-Chain Proteinase Dimer and Restores Particle Formation

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The active form of the retroviral proteinase (PR) is a homodimer of monomeric subunits expressed as integral parts of the viral *gag-pol* precursor polyproteins, and dimerization of polyproteins is presumed to be important for regulation of PR activity. Expression of a single-chain dimer of the human immunodeficiency virus (HIV) type 1 PR as a component of the viral polyprotein has been shown to prevent particle assembly and viral infectivity (H.-G. Kräusslich, Proc. Natl. Acad. Sci. USA 88:3213–3217, 1991). Ro31-8959, a specific inhibitor of HIV PR, blocked proteolysis of polyproteins containing either wild-type or single-chain dimer PR at the same inhibitor concentration. Different inhibitor concentrations gave three phenotypic effects for the linked PR: at a concentration of 10 nM, cytotoxicity was prevented yet viral polyproteins were almost completely processed and no particles were released. The majority of HIV capsid proteins was found in the soluble cytoplasmic fraction, whereas at a concentration of 1 μM inhibitor most HIV *gag* proteins were associated with an insoluble fraction. Release of particles consisting of partially processed polyproteins was observed at 100 nM Ro31-8959, and polyprotein processing was blocked at 10 μM. Particles derived from the dimer-containing provirus were noninfectious independently of the inhibitor concentration. Production of infectious HIV after transfection of wild-type provirus was abolished at 100 nM and markedly reduced at 10 nM Ro31-8959.

Production of infectious retroviral particles involves the ordered association of two structural polyproteins (pr55^{gag} and pr160$^{gag\text{-}pol}$ in the case of human immunodeficiency virus [HIV] type 1). Proteolytic processing of the polyproteins by the viral proteinase (PR; for the nomenclature of retroviral proteins, see reference 18), which is expressed as a monomeric subunit on the *gag-pol* polyprotein, is presumed to initiate during budding of the viral particle, and PR activity is not required for particle release (reviewed in reference 16). However, inactivation of PR, either by genetic alteration of its active site (12) or by specific PR inhibitors (2, 6, 19, 21, 26), completely abolishes viral infectivity, making these inhibitors attractive candidates for the treatment of AIDS (14). Retroviral PRs belong to the aspartic proteinases (reviewed in references 9 and 11), and the active enzyme has been shown to be a dimer of identical polypeptide chains (17, 20, 23, 33). The requirement for dimerization to initiate the processing cascade may prevent premature cleavage of viral polyproteins, which would dissociate the components of the virion from the site of assembly. Concentration of *gag-pol* polyproteins during virus assembly would kinetically favor dimerization and may therefore be an important factor in the initiation of processing.

Recombinant DNA technology has been employed to express HIV-1 and Rous sarcoma virus PRs as active, genetically linked dimer molecules in both bacterial and mammalian cells (3–5, 13). Expression of proviral (pNL43-2PR [13]) and subviral (pK-R-gp2PR [13]) constructs containing two copies of the PR-coding region linked by seven codons specifying a flexible hinge region in mammalian cells yielded complete processing of viral polyproteins and prevented particle formation and viral infectivity (13). Furthermore, transfected cells expressing the single-chain PR dimer were progressively lost over time, suggesting that in these cells rapid autocatalytic processing of polyproteins containing the linked dimer occurred and either the PR dimer or any of the polyprotein cleavage products may be cytotoxic, leading to rapid cell death.

Specific inhibitors of HIV PR that prevent viral spread in tissue culture have recently been reported (2, 6, 19, 21, 26). These inhibitors should also block all effects directly caused by the enzymatic activity of the single-chain PR dimer, and transfection of proviral plasmid pNL43-2PR (13) was therefore performed in the presence of specific HIV PR inhibitor Ro31-8959 (this compound was synthesized at Roche Welwyn by S. Redshaw of J. A. Martin's group; it was originally named compound XVII; 26). Figure 1A and B shows the same representative panel of cells 60 h after transfection with pNL43-2PR and stained either for DNA (4′,6-diamidino-2-phenylindole dihydrochloride; Fig. 1A) to visualize cell nuclei or with serum from a patient with AIDS (Fig. 1B). Virtually no fluorescence-positive cells were found in the absence of inhibitor. Transfection in the presence of Ro31-8959, on the other hand, completely restored expression of cell-associated HIV antigen (Fig. 1C and D; note that samples from the same transfection were analyzed in panels B, C, and D). At 10 μM inhibitor, no difference was seen between cells transfected with wild-type plasmid pNL4-3 (data not shown) and those transfected with pNL43-2PR (Fig. 1C).

HIV-specific proteins were analyzed 48 h after cotransfection of subviral expression vector pK-R-gp2PR, which contains *gag* and the single-chain PR dimer under control of the cytomegalovirus immediate-early promoter-enhancer (13), and *rev* expression plasmid pMTcrev (8) into COS-7 cells in the presence of different concentrations of Ro31-8959. Western immunoblots of cell lysates using an antiserum against the capsid (CA) protein revealed a dramatic increase of

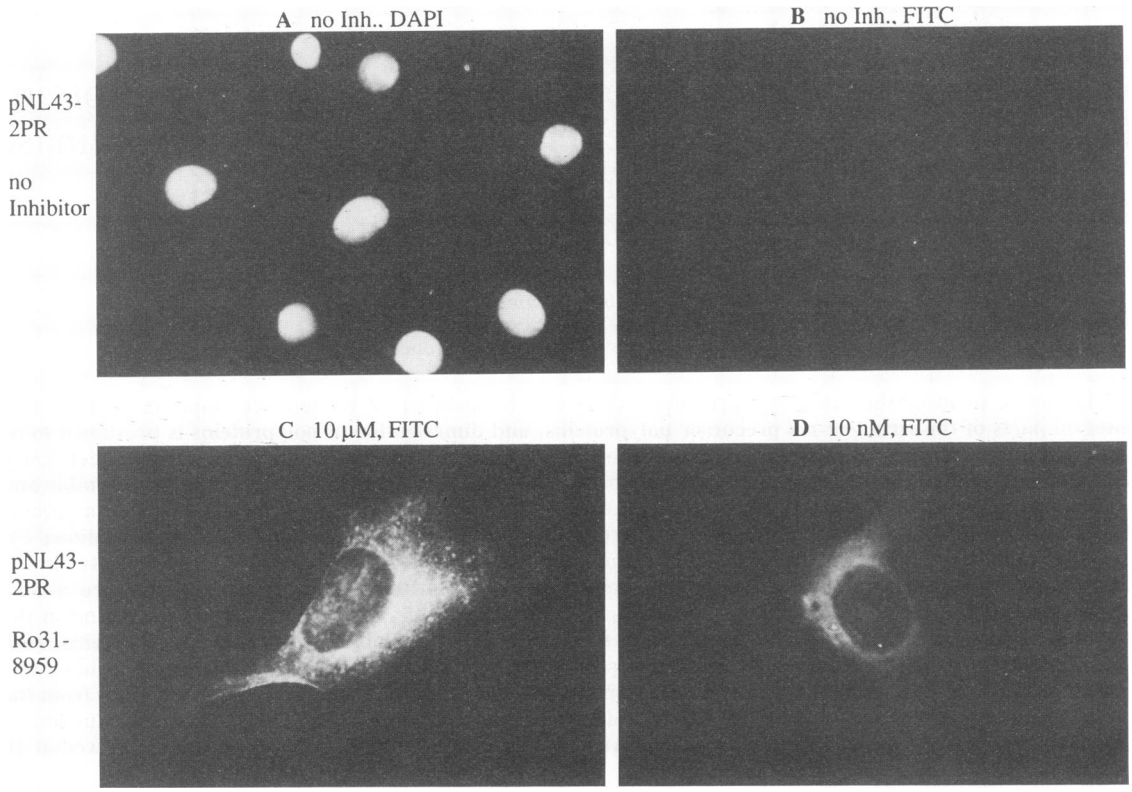

FIG. 1. Indirect immunofluorescence analysis of COS-7 cells 60 h after transfection with pNL43-2PR in the absence (A and B) or presence of 10 μM (C) or 10 nM (D) Ro31-8959. Plasmid pNL43-2PR (13) is derived from HIV-1 proviral plasmid pNL4-3 (1) and contains two copies of PR linked by a seven-codon hinge region. Approximately 5×10^6 subconfluent COS-7 cells were electrotransfected with 10 μg of DNA as previously described (22). Subsequently, cells were diluted into fresh medium and samples of this suspension were added to culture dishes containing medium with or without inhibitor (Inh.). Ro31-8959 (26) was dissolved in dimethyl sulfoxide to a concentration of 25 mM and diluted in dimethyl sulfoxide to 200 times the desired final concentration before addition to the medium. All control experiments received the same concentration of dimethyl sulfoxide (0.5%) without inhibitor. Cells were fixed with methanol and acetone as previously described (22) and were probed with serum from a patient with AIDS and then a fluorescein isothiocyanate (FITC)-labeled second antiserum (B to D) or stained for DNA with 0.1 μg of 4′,6-diamidino-2-phenylindole dihydrochloride per ml (A).

cell-associated antigens (Fig. 2A; compare lanes 1 and 2; note that cell samples from the same transfection were analyzed in lanes 1 to 5) and almost complete inhibition of processing at an inhibitor concentration of 10 μM (Fig. 2A, lane 2). Partial inhibition of processing with almost no processed CA protein was observed at a concentration of 1 μM Ro31-8959 (Fig. 2A, lane 3), and at 100 nM there was primarily a matrix (MA)-CA intermediate (containing the MA and CA domains) and processed CA with little residual $pr55^{gag}$ (lane 4). Little inhibition of polyprotein cleavage occurred at 10 nM Ro31-8959, and the processed CA protein was the main product. Comparable levels of PR inhibition were seen after transfection of wild-type plasmid pK-R-gpII containing only one copy of the PR-coding region (22) in the presence of Ro31-8959. The same relative amounts of precursor polyprotein, intermediate processing products, and mature CA protein were observed (data not shown), suggesting that similar concentrations of inhibitor are present in the cytoplasm, where the single-chain dimer is active, and at the plasma membrane, where retroviral PRs are normally activated.

Transfection of constructs expressing the single-chain PR dimer in the presence of Ro31-8959 showed that cytotoxicity was prevented at a concentration of 10 nM Ro31-8959, at which little or no inhibition of HIV polyprotein processing occurred (Fig. 1D and 2A, lane 5). These results indicate that cell toxicity is directly caused by the proteolytic activity of the single-chain PR dimer, since no toxic effects were observed despite the presence of all polyprotein cleavage products. Several cellular proteins have been identified as substrates of HIV PR in vitro, including components of the cytoskeleton (27, 28, 30, 32), calmodulin (29), and a precursor of transcription factor NFκB (25). Moreover, injection of purified PR directly into mammalian cells resulted in abnormal distribution of vimentin intermediate filaments (27). It appears likely, therefore, that cell toxicity of the single-chain PR dimer is caused by proteolytic cleavage of cellular proteins. Additional evidence implicating active HIV PR as a cytotoxic agent is provided by experiments showing that transient transfection of constructs expressing only the single-chain PR dimer or wild-type HIV PR at high levels induced rapid cell death (7). Considerable intracellular polyprotein processing has also been found in cells lytically infected with HIV-1 (10). Intracellular PR activity may be caused by high-level expression of HIV structural proteins, leading to cytoplasmic dimerization, and may directly contribute to HIV-induced cell killing, similar to what is observed with the single-chain dimer. Conceivably, cytoplasmic activation of PR may be incompatible with survival of infected cells and chronic infection with HIV may therefore

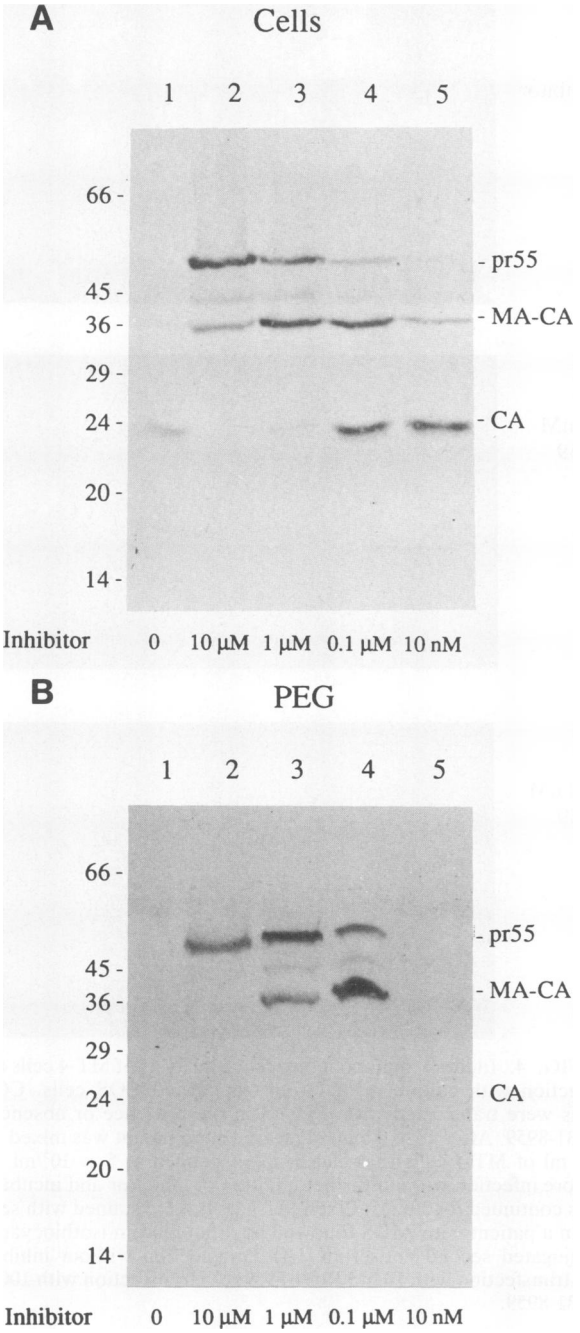

FIG. 2. Western blot analysis of *gag* gene products after transient transfection. COS-7 cells were cotransfected with pK-R-gp2PR (13) and *rev* expression vector pMTcrev (8) in the presence of various concentrations of Ro31-8959, as indicated. (A) Cells were harvested 48 h after transfection by being scraped off of the plate, and they were then resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. (B) Media were cleared for 10 min at 500 × *g*, and particles were precipitated with polyethylene glycol (PEG) as previously described (22) and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Western blots were stained with rabbit polyclonal antiserum against CA followed by an alkaline phosphatase-conjugated second antibody (Jackson Immunochemicals, Inc.). Molecular mass standards are indicated (in kilodaltons) on the left, HIV-specific proteins are identified on the right, and inhibitor concentrations are given at the bottom.

be dependent on low or absent expression of *gag-pol* proteins.

Culture media from transfected cells were analyzed for release of particles by polyethylene glycol precipitation followed by immunoblotting. In agreement with previous experiments (13), no HIV-specific antigen could be precipitated from the medium of pK-R-gp2PR transfected cells (Fig. 2B, lane 1) although significant amounts of nonparticulate antigen were detected by enzyme-linked immunosorbent assay (ELISA). Particle release was not restored at 10 nM Ro31-8959, and no HIV-specific antigen was precipitated at this inhibitor concentration (Fig. 2B, lane 5) despite the presence of large amounts of cell-associated antigen (Fig. 2A, lane 5). Release of extracellular particles was restored at inhibitor concentrations of 100 nM (Fig. 2B, lane 4) and higher (lanes 2 and 3), indicating that lack of particle production was indeed due to the activity of the single-chain PR dimer. These particles consisted of different relative amounts of $pr55^{gag}$ and a processing intermediate containing MA and CA (Fig. 2B) but did not contain the completely processed CA protein. Only uncleaved $pr55^{gag}$ was found in particles released at a concentration of 10 μM Ro31-8959 (Fig. 2B, lane 2). These results were confirmed by using a commercial HIV-1 antigen capture ELISA (Organon Teknika) that detects the cleaved CA protein but not $pr55^{gag}$ or intermediate processing products. No ELISA antigen was detected when transfected cells were kept in 1 or 10 μM Ro31-8959, and significantly reduced levels were obtained at a concentration of 100 nM.

The subcellular distribution of HIV-specific antigens expressed in the presence of various concentrations of Ro31-8959 was analyzed by fractionation of cells that had been cotransfected with pK-R-gp2PR and pMTcrev. At an inhibitor concentration of 1 μM, almost the entire immunoreactive protein was associated with an insoluble P100 fraction with less than 5% remaining in the soluble S100 fraction (Fig. 3). Conversely, fractionation of cells that had been kept at 10 nM Ro31-8959 yielded most of the immunoreactive protein in the soluble fraction (Fig. 3). Moreover, completely processed CA was found almost exclusively in the S100 fraction and only MA-CA and minute quantities of intact $pr55^{gag}$ remained in the P100 fraction (Fig. 3). No particles were observed at this inhibitor concentration, despite the presence of processed cell-associated *gag* proteins located in a soluble cytoplasmic compartment. These results support the hypothesis that HIV morphogenesis requires targeting of unprocessed polyproteins to the site of assembly and PR needs to remain inactive until all virion components are confined in the budding particle. Uncoupling of processing and assembly, as in the case of the insufficiently inhibited single-chain PR dimer, leads to release of processed structural proteins from their targeting domain, and only products containing MA but lacking the C-terminal domains of *gag* are localized to the membrane. This may also be the case at higher inhibitor concentrations, since processed CA protein was found intracellularly but not in the extracellular particle at 100 nM Ro31-8959. Similarly, overexpression of wild-type monomeric PR as part of a *gag-pol* fusion protein resulted in complete intracellular processing of the polyprotein and abolished particle production, which was restored by mutation of the PR active site (15, 22). Moreover, high-level expression of HIV structural polyproteins in lytically infected cells also appears to cause premature intracellular processing, giving rise to completely processed capsid proteins in the cytoplasm which are not incorporated into virus (10). Taken together, these results suggest that inefficient

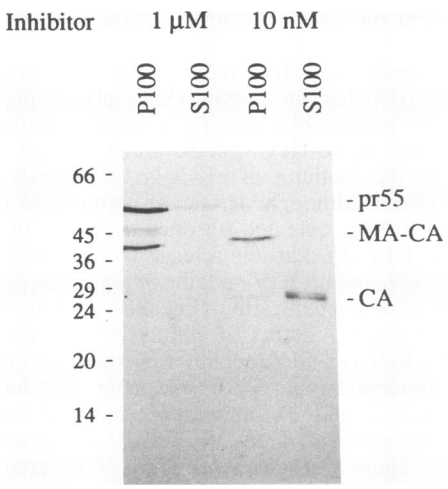

FIG. 3. Western blot analysis of subcellular fractions from transfected cells. COS-7 cells were cotransfected with pK-R-gp2PR and pMTcrev in the presence of either 1 μM or 10 nM Ro31-8959, as indicated at the top. After 48 h, subcellular fractionation was performed by hypotonic lysis of transfected cells, followed by homogenization and centrifugation to give soluble (S100) and insoluble (P100) fractions (24). S100 was precipitated with 12.5% trichloroacetic acid for 60 min at 0°C, and the precipitate was collected by centrifugation. P100 and trichloroacetic acid-precipitated S100 were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed by Western blotting with antiserum against CA. Molecular mass standards are shown (in kilodaltons) on the left, and HIV-specific proteins are identified on the right.

dimerization due to a low concentration of polyproteins may in fact be an essential element of PR regulation. They do not, however, rule out the possibility of additional regulatory factors which may inactivate or activate the viral enzyme.

Supernatants from COS-7 cells that had been transfected with proviral constructs pNL4-3 and pNL43-2PR were incubated with $CD4^+$ lymphocytic cell line MT-4 to assay for production of infectious virus. Replicated HIV was readily detected for wild-type construct pNL4-3, and after 3 days the MT-4 culture was greater than 50% infected (Fig. 4A). No infectious virus could be recovered when pNL4-3-transfected cells were kept at 100 nM Ro31-8959 (Fig. 4C; inhibitor was added at the time of transfection only). No fluorescent cells and no HIV antigen, as measured by ELISA, was detected, even after prolonged incubation with reporter cell line MT-4 or when culture medium from these MT-4 cells was used for a second round of infection of fresh MT-4 cells (data not shown). Less than 5% immunofluorescence-positive MT-4 cells were detected after 3 days of incubation with medium from pNL4-3-transfected COS cells kept in 10 nM Ro31-8959 (Fig. 4B). Similarly, extracellular CA antigen, as measured by ELISA, was three to five times reduced compared with uninhibited controls. Particles released at these inhibitor concentrations consisted mainly of partially or completely processed *gag* proteins, and a concentration of 10 μM Ro31-8959 was required to prevent processing of $pr55^{gag}$. Antiviral activity of PR inhibitors therefore does not require complete blockage of PR activity. These results suggest that partially processed *gag* and *gag-pol* polyproteins may interact to form nonfunctional multimers and may thereby *trans*-dominantly interfere with the replication of infectious virus, as previously observed for HIV-1 *gag* mutants (31).

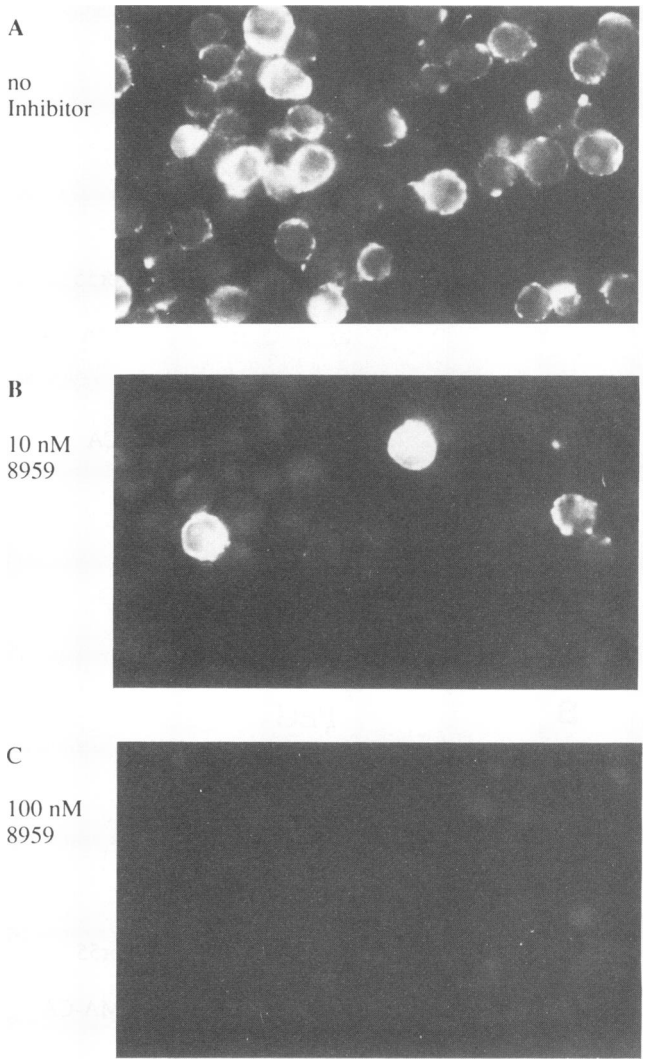

FIG. 4. Indirect immunofluorescence analysis of MT-4 cells after infection with culture media from transfected COS cells. COS-7 cells were transfected with pNL4-3 in the presence or absence of Ro31-8959. After 48 h, 0.5 ml of cleared supernatant was mixed with 0.5 ml of MT-4 cells that had been suspended at 5×10^5/ml 24 h before infection without further addition of inhibitor and incubation was continued for 3 days. Fixed MT-4 cells were stained with serum from a patient with AIDS followed by a fluorescein isothiocyanate-conjugated second antiserum. (A) Transfection without inhibitor; (B) transfection with 10 nM Ro31-8959; (C) transfection with 100 nM Ro31-8959.

Transfection of pNL43-2PR, on the other hand, yielded no infectious virus, independently of the inhibitor concentration used. No fluorescence-positive cells and no increase in extracellular ELISA antigen were detected when MT-4 cells were incubated with media from pNL43-2PR-transfected cells that had been kept at 10 nM to 10 μM Ro31-8959 (data not shown). The same result was obtained when MT-4 cells were treated with 10 nM Ro31-8959 prior to addition of medium from COS cells that had been transfected with pNL43-2PR in the presence of 100 nM Ro31-8959 (data not shown). This result could be explained by lack of particle formation, by inhibition of uncoating similar to what is presumed to occur in the case of the wild-type construct, or

by induction of rapid death of infected cells by PR toxicity. Toxic effects appear unlikely, however, since incubation of $CD4^+$ cells with 10 nM Ro31-8959 (which does not abolish HIV infection) prior to addition of culture medium from transfected cells yielded no fluorescence-positive cells. Release of particles after transfection of pNL43-2PR was restored only at a concentration of 100 nM Ro31-8959 or higher, which also blocked production of infectious HIV in the case of wild-type provirus, and it was therefore not possible to analyze any specific defect of the dimer-containing provirus.

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