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NOTES

Progression of Early Steps of Human Immunodeficiency Virus Type 1 Replication in the Presence of an Inhibitor of Viral Protease

HELMUT JACOBSEN,* LUDWINA AHLBORN-LAAKE, ROLAND GUGEL, AND JAN MOUS
Department PRTB, F. Hoffmann-LaRoche AG, CH-4002 Basel, Switzerland

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We have evaluated a possible role for human immunodeficiency virus type 1 protease during early steps of replication. For these studies, a specific inhibitor of human immunodeficiency virus protease, Ro31-8959, was used. Synthesis of viral cDNA, its integration into cellular DNA, and its transcription were determined during a one-step, acute infection of MT-4 cells. No consistent difference in any of these parameters was noted between control-infected cultures and those treated with protease inhibitor. However, no infectious progeny virus was produced in treated cultures, and thus spread of infection was severely restricted. Our results do not support an essential activity of viral protease in early steps of replication but are in line with its established role in *gag* and *gag-pol* processing and in maturation to infectious progeny virus.

Maturation of retroviruses requires the activity of a virally encoded protease for cleavage of the *gag* and *gag-pol* precursor proteins. Proteolytic cleavage gives rise to a set of polypeptides which are essential for virion structure, i.e., the *gag* products, and reverse transcription and integration of the infecting viral genome, i.e., the *pol* products (5). In the case of human immunodeficiency virus type 1 (HIV-1), the *gag* products include the matrix protein p17, the capsid protein p24/25, and the nucleocapsid p15, which is further cleaved into peptides p7 and p6. The *pol* gene encompasses the protease itself (p10), the reverse transcriptase (RT)/RNase H (p66/p51), the p15 RNase H, and the integrase (p32). It is expressed as a fusion protein with *gag*, generating a p160 protein translated from a reading frame on a large RNA containing both the *gag* and *pol* genes by a ribosomal frameshift mechanism (7). Genetic and biochemical studies have demonstrated that the *pol*-encoded protease of HIV-1 is responsible for the cleavage of the *gag* and *gag-pol* precursor proteins (4, 13, 14, 16, 17, 19, 26). In the absence of functional protease, immature noninfectious virus particles are produced (12). While the essential function of the virus protease in late steps of retroviral replication is firmly established, any additional role in early steps of infection is less clear. Active protease in isolated capsids of equine infectious anemia virus and postmaturation cleavage of its associated nucleocapsid protein have been reported recently, and a function for viral protease in early infection has been proposed (21).

A specific and potent inhibitor of HIV protease, designated compound XVII or Ro31-8959, has been reported recently (24). Its activity in acute and chronic infections of HIV-1 has been described (3). We have used this inhibitor as a tool to study a possible role of protease in early steps of HIV infection up to the point of integration and transcription of the infecting viral genome.

We chose to measure cDNA synthesis and integration in the presence or absence of protease inhibitor in a synchronized one-cycle infection of MT-4 cells. Cells were infected with freshly harvested culture supernatant of HIV-1_{IIIB}-infected MT-4 cells (8, 20). Inhibitors were added before infection and maintained throughout the experiment. Zidovudine (AZT [18, 28]), a proven inhibitor of HIV RT, was assayed in parallel to the protease inhibitor Ro31-8959. Ro31-8959 has been shown to inhibit HIV-1 growth in JM cells with mean 50 and 90% inhibitory concentrations (IC_{50} and IC_{90}) of 2.7 and 16 nM, respectively (24). A similar inhibitory potency has been determined for HIV-1_{IIIB} in MT-4 cells (data not shown). To prevent secondary infection, soluble CD4 was included in the medium after the infecting virus supernatant was washed off (CD4-Hγ3a; 10 μg/ml [31]). To ensure the effectiveness of the inhibitors, we determined the RT activity in the culture supernatants. Whereas RT activity started to accumulate between 16 and 24 h postinfection (p.i.) in control-infected cultures, essentially no RT activity was found in inhibitor-treated cultures. Thus, both Ro31-8959 and AZT were inhibitory under the chosen experimental conditions (Fig. 1).

Infected cells were harvested at various time points, and DNA was isolated and separated into its low- and high-molecular-weight components by the Hirt procedure (10). Approximately 4×10^7 cells were lysed in 4 ml of Tris-EDTA (TE) buffer (pH 8) containing 0.75% sodium dodecyl sulfate (SDS) (5 min at 4°C) and adjusted to 1.2 M NaCl. After overnight incubation at 4°C, the lysate was centrifuged (1 h at 200,000 × *g*). The supernatant containing low-molecular-weight DNA was treated with proteinase K, phenol-chloroform extracted, and precipitated in ethanol. The pellet containing high-molecular-weight DNA was dissolved in 4 ml of TE and treated similarly. Subsequently, it was analyzed for viral DNA by the Southern blot technique (Fig. 2 and 3a) (25, 29). Low-molecular-weight cDNA was weakly detected in the Hirt extract at 4 h p.i., reached a maximum at the 8-h time point, and decreased subsequently. The earlier, more pronounced form is probably the linear form,

* Corresponding author.

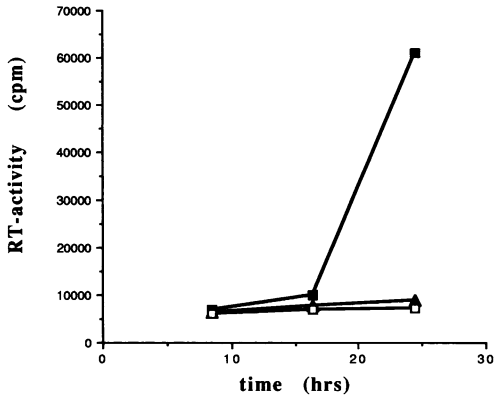

FIG. 1. Kinetics of RT activities in supernatants of HIV-1-infected MT-4 cells. Cells were treated with inhibitors prior to infection (AZT at −24 h; Ro31-8959 at −6 h) or infected without prior treatment (■). The multiplicity of infection was 0.1 to 0.5, as judged by indirect immunofluorescence analysis. AZT (□) and Ro 31-8959 (▲) were added to final concentrations of 100 and 1,000 nM, respectively. At various time points, culture supernatants were harvested, virus was pelleted, and RT activity of the pellet was determined (6). RT activity is expressed as radioactivity incorporated into insoluble material per 500 μl of culture supernatant.

as deduced from its mobility relative to molecular weight markers. The low-mobility form appearing at later time points would consequently correspond to the nicked circular species. A high-mobility supercoiled form was not detected, possibly because of the extraction conditions. The kinetics of cDNA synthesis are comparable to those in previously reported one-step growth experiments of HIV-1 in H9 cells, including the early peak and later disappearance of the cDNA signal (11). As clearly shown in this experiment, there is no significant difference between control infections and Ro31-8959-treated cultures with respect to both kinetics and the strength of the cDNA signal. As expected, no cDNA synthesis occurred in cultures receiving AZT.

Integration of cDNA into the host genome was determined by Southern blot analysis of high-molecular-weight, nuclear DNA (Fig. 3A). A positive signal was observed at 24 h p.i. and increased toward 32 h. Because of the randomness of the integration site in each infected cell, a broad radioactive band was obtained. The analysis proved that the presence of

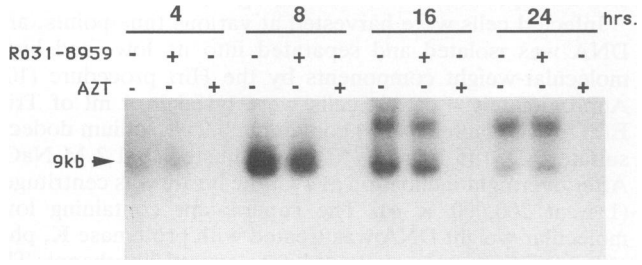

FIG. 2. Kinetics of cDNA synthesis in HIV-1-infected MT-4 cells, indicating effects of Ro31-8959 and AZT. Cells were infected and treated with inhibitor as described for Fig. 1. At the indicated times, culture aliquots were harvested, and DNA was isolated from 4×10^7 cells and separated into its low- and high-molecular-weight components by the Hirt procedure (10). Viral cDNA in the low-molecular-weight fraction was detected by Southern blotting and hybridization to an HIV-1-specific, ^{32}P-labelled probe (25, 27).

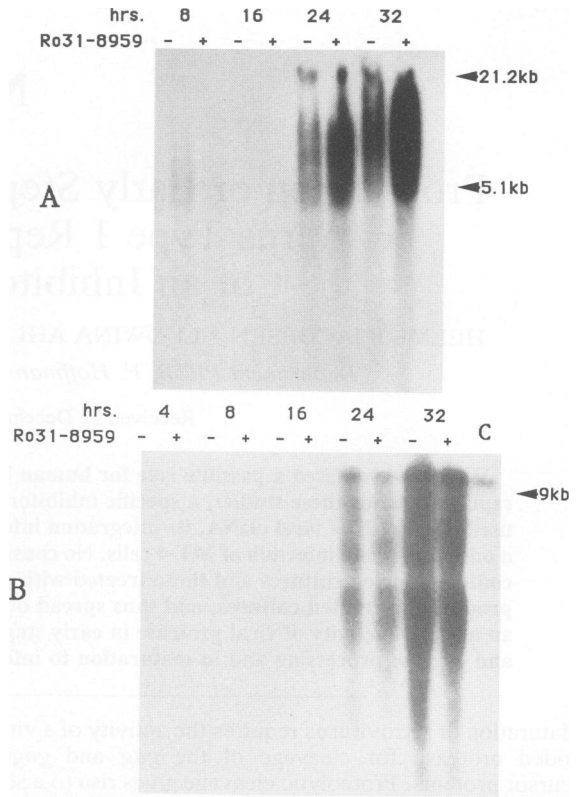

FIG. 3. Integration and transcription of viral DNA. (A) The high-molecular-weight DNA was obtained as described in the text. Integrated viral DNA was demonstrated after digestion with *Xba*I (which does not cleave viral sequences), Southern blotting, and hybridization to the HIV-1-specific probe. (B) Total RNA was isolated from 2×10^7 cells by the procedure of Chirgwin et al. (2) in parallel to DNA extractions, electrophoresed under denaturing conditions, and transferred to nylon membrane. Viral transcripts were detected by hybridization as described above.

protease inhibitor did not prevent integration of proviral DNA. The differences in signal strength between control and Ro31-8959-treated cultures are due to different amounts of DNA applied, as judged from the ethidium bromide-stained gel (data not shown). Thus, it appears that both cDNA synthesis and subsequent integration into the cell genome do not require active viral protease.

Steady-state levels of viral RNA were determined by Northern blot analysis of total cellular RNA (Fig. 3B), which was isolated in parallel to DNA extractions. HIV-specific transcripts became detectable from 24 h p.i. onward and continued to increase toward later time points. No RNA signal was observed at earlier time points. The kinetics of transcription followed closely those for integration of viral DNA, in keeping with previous observations that the integrated rather than the episomal viral DNA is the primary source of transcription in T-cell lines. Again, no differences in kinetics and steady-state levels of viral RNA were noted between RNA from control-infected cells and from cultures receiving the protease inhibitor.

Finally, we measured the release of viral particles into the culture supernatants (Fig. 4). Viral particles were pelleted from culture supernatants and dissolved in SDS sample buffer, and protein was resolved by SDS-polyacrylamide gel electrophoresis (PAGE). The *gag* protein was measured by

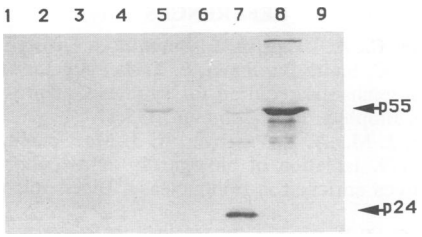

FIG. 4. Immunoblot analysis of viral proteins in supernatants of HIV-1-infected MT-4 cells. Treatment, infection, and harvesting of viral particles from culture supernatants were done as described for Fig. 1. Cultures were untreated (lanes 1, 4, and 7) or treated with Ro31-8959 (lanes 2, 5, and 8) or AZT (lanes 3, 6, and 9). Aliquots were harvested after 8 h (lanes 1 to 3), 16 h (lanes 4 to 6), or 24 h (lanes 7 to 9). Pelleted virus was dissolved in SDS-PAGE sample buffer. After electrophoresis and blotting (9, 30), the viral proteins containing p24 capsid sequences were detected by staining with a p24-specific mouse monoclonal antibody (gag3; gift of M. Brockhaus, Hoffmann-LaRoche) and alkaline phosphatase-coupled goat anti-mouse antibody (Bio-Rad).

immunoblotting with a mouse monoclonal antibody directed against the capsid protein p24 of HIV-1 (15, 30). As shown in Fig. 4, no viral proteins could be detected in supernatants from AZT-treated cells, whereas comparable amounts of viral proteins are found in supernatants from control and Ro31-8959-treated cultures. However, in the latter case, no processing to mature p24 occurred and the prevalent forms are the precursors $p55^{gag}$ and $p160^{gag\text{-}pol}$, indicating a near complete elimination of protease activity by the inhibitor.

To prove that productive infection had occurred, we measured production of infectious virus during and after treatment with the inhibitor. Cultures were treated with Ro31-8959 and infected at a low multiplicity of infection (ca. 0.01 infectious particles per cell). Spread of infection was analyzed by immunofluorescent staining of cells expressing viral antigens (Fig. 5). In the presence of Ro31-8959, only a few cells (2 to 3%) showed a positive signal after 3 days of infection (Fig. 5C), in marked contrast to control-infected cultures, in which extensive infection had occurred; i.e.,

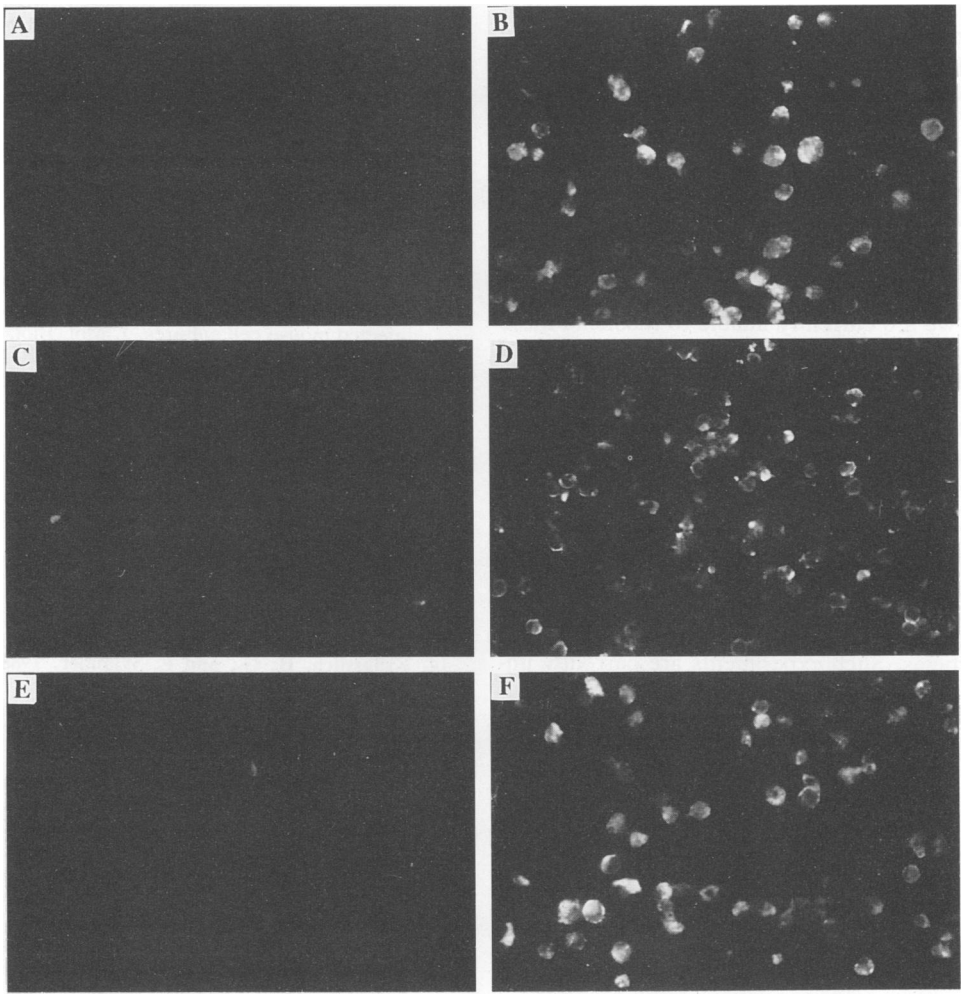

FIG. 5. Immunofluorescence analysis of HIV-1 antigen-expressing MT-4 cells treated with Ro31-8959. Cells were infected at a low multiplicity of infection (ca. 0.01); incubated with inhibitor as indicated below, and analyzed for virus infection by indirect immunofluorescence with a human anti-HIV-1 serum and fluorescein isothiocyanate-coupled goat anti-human immunoglobulin G. (A) Uninfected control; (B) infected cells, analyzed on day 3 p.i.; (C) cells infected and subsequently incubated in the presence of 10 μM Ro31-8959, analyzed on day 3 p.i.; (D) cells treated as for panel C, and on day 3 the inhibitor was washed off and incubation continued to day 6; (E) cells infected and subsequently incubated in the presence of soluble CD4 immunoglobulin (10 μg/ml) (31), analyzed on day 3 p.i.; (F) cells treated as for panel C, and on day 3 the CD4 immunoglobulin was washed off and incubation continued to day 6.

more than 50% of cells stained positive for viral antigen (Fig. 5B). After removal of inhibitor and another 3-day incubation, infection had spread to >50% of cells, indicating the resumption of infectious virus production (Fig. 5D). A similar pattern is observed in cultures treated with soluble CD4, which blocks secondary infection by neutralization of progeny virus (Fig. 5E and F).

Comparable results were obtained when RT activity was determined as a parameter for production of mature virus. From initial infection throughout cultivation, RT activity in the medium was low to undetectable in the presence of inhibitor. However, at 24 h after removal of Ro31-8959, RT activity became detectable and continued to accumulate (data not shown). Thus, viral protease inhibitor did not prevent the establishment of a primary, de novo infection but reversibly blocked the production of infectious virus and thus prevented further spread of infection.

Recently, it has been reported that purified capsids of a lentivirus, equine infectious anemia virus, contain an active aspartic protease, and cleavage of the nucleocapsid protein p11 was observed upon incubation of such subviral particles. Extending these observations, the authors proposed that cleavage of p11 might be necessary to uncoat the genomic RNA for subsequent reverse transcription and suggested that viral protease thus might be essential for early steps of infection (21–23). Furthermore, inhibition of synthesis of proviral DNA by another protease inhibitor (UK-88,947) has been reported in acute infections of H9 cells by HIV-1_{IIIB} (1). These results are clearly different from the results reported here, which failed to show any effect of HIV-1 protease inhibitor Ro31-8959 on cDNA synthesis and integration. In the absence of further information on the characteristics of the other protease inhibitor, e.g., with respect to its specificity, we are currently unable to reconcile these divergent results. We note, however, that in the study of Baboonian et al. (1) on inhibition of proviral DNA synthesis, their protease inhibitor was used at a concentration of 12 μM, while the IC_{50}s against representative aspartic proteases, renin and pepsin, were reported to be 580 and 450 nM, respectively. We used our viral protease inhibitor at 1 μM, while the IC_{50} against known human aspartic proteases exceeds 10 μM (24). We have performed experiments like those shown in Fig. 5 with H9 cells and have obtained results similar to those described in this study for MT-4 cells. It may be of importance to directly compare the state of processing of the NC protein in HIV-1 and EIAV equine infectious anemia virus particles. Such experiments are currently under way.

Inhibitors of HIV protease may soon become new therapeutic modalities which are mechanistically distinct from the currently used inhibitors of RT, e.g., AZT, dideoxycytidine, and dideoxyinosine, which prevent de novo infections without affecting replication in chronically infected cells. This different mode of action should be advantageous since it precludes the appearance of cross-resistance and potentially allows for synergistic interactions. From the observations by us and others on the effect of protease inhibitors in different types of HIV infection in cell culture, it is evident that this class of inhibitors also by itself will not cure an established infection. However, it may be successful in preventing further spread of infection and thus be of significant therapeutic benefit.

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