JOURNAL OF VIROLOGY, Oct. 1994, p. 6782–6786
0022-538X/94/$04.00+0

Vol. 68, No. 10

The Activity of the Protease of Human Immunodeficiency Virus Type 1 Is Initiated at the Membrane of Infected Cells before the Release of Viral Proteins and Is Required for Release To Occur with Maximum Efficiency

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Received 22 April 1994/Accepted 19 July 1994

The final steps in the production of the type C retroviruses include assembly of the viral core particle and release of virions from the surface of the infected cell. The core proteins are translated as part of one of two precursors, Gag and Gag/Pol, which are cleaved by a virally encoded protease. We examined the interaction between the processing of the human immunodeficiency virus type 1 Gag precursor and the membrane-based assembly and budding of virions. Our results indicate that cleavage by the viral protease is initiated at the membrane of the infected cell during virus release and that protease activity is required for virion release to occur with maximum efficiency.

The proteins of the retroviral capsid are contained in two precursors, Gag and Gag/Pol, which are cleaved to their mature forms by the viral protease. The final stages in the production of retroviruses include association of the Gag and Gag/Pol precursors, both with the membrane of the infected cell and with each other; activation of the virally encoded protease; processing of the precursors; and the final assembly and budding of virus particles (6, 26). Although some of these events can occur independently of the others (e.g., protease-mediated cleavage of the precursors may occur without membrane association [3, 8, 15] and virus particles containing unprocessed precursors may bud from the surface of infected cells [5, 8, 6–18, 22, 27, 30, 31]), production of infectious virus requires that these events occur in a coordinated fashion. We examined the terminal phases in the budding of human immunodeficiency virus type 1 (HIV-1) to define the interaction between the assembly of virus particles and precursor processing.

The exact timing of Gag and Gag/Pol processing during the retrovirus life cycle is uncertain. Although cell-associated precursor cleavage has been demonstrated for all type C retroviruses studied (1, 4, 7, 9, 10, 12, 19, 21, 23–25, 32, 35), the question of whether processing as part of virion formation occurs at the membrane or solely within the virion remains unanswered. We have previously reported that the processing of the Gag precursor of HIV-1 which gives rise to virions occurs either at the membrane of the infected cell or in the virion immediately after budding (15). Studies with Moloney murine leukemia virus have revealed both cell-associated processing and the presence of precursors outside the cell surface (1, 7, 21). In this report, we demonstrate that processing of the Gag precursor of HIV-1 is initiated before the assembled virus buds from the surface of the infected cell and that protease activity is required for budding to proceed with maximum efficiency.

For these experiments, we used CEM cells, a human T-cell lymphoma cell line (a gift of M. Cloyd, University of Texas, Galveston), which were maintained in RPMI 1640 containing 10% (vol/vol) fetal calf serum. CEM cells were infected with virus recovered after transfection of HeLa cells with the full-length HXB2 infectious clone (28). Chronically infected cell lines were produced as described previously (15).

Our previous studies indicated that the Gag precursor is processed either at the membrane or within the virion, immediately after the virus is released from the surface of the infected cell (15). To determine the site of action of the protease, we looked for unprocessed precursors in virus collected at very short intervals after release from infected cells. An aliquot containing 10^7 CEM cells chronically infected with an HIV isolate (HIV-1G) was resuspended in iced phosphate-buffered saline (PBS) and vortexed vigorously to remove extracellular, mature virions associated with the cell membrane. The cells were collected by centrifugation in a microcentrifuge and resuspended in 1 ml of fresh medium at 37°C. The samples were then filtered immediately through a 0.45-μm-pore-size syringe filter which retained the infected cells but allowed the newly released virus to pass through. One-third of the filtered supernatant was added to an equal amount of radioimmunoprecipitation assay buffer with 4% sodium dodecyl sulfate (SDS), blocking any further protease activity. By using this technique, we estimated that we were able to recover virus which had left the cell for no more than 10 s. Another one-third of the filtered aliquot was incubated at 37°C for 1 min before the addition of radioimmunoprecipitation assay buffer with SDS, and the final one-third was incubated for 30 min before disruption. In this way, extracellular processing, if required, could occur.

These samples were then subjected to Western (immunoblot) analysis with monoclonal antibodies directed against the mature Gag proteins (Fig. 1). The samples were stained with monoclonal antibodies to either the amino-terminal matrix

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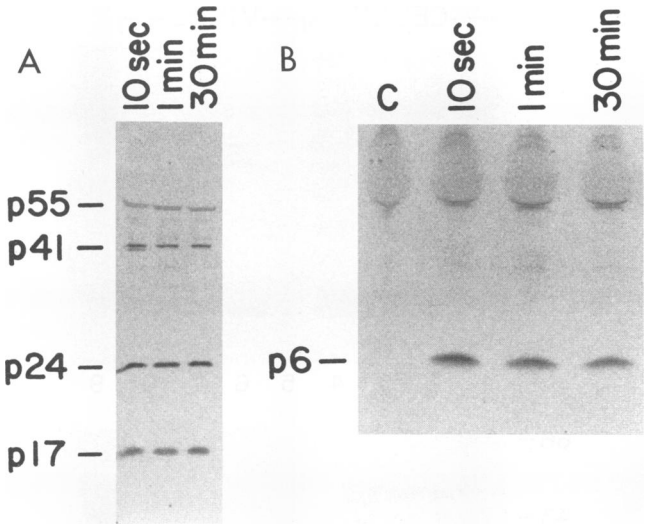

FIG. 1. Processing of the Gag precursor of HIV-1 immediately after release from infected cells. Virus was recovered from infected cells as described in the text. The virion-associated protease was either inactivated by addition of a radioimmunoprecipitation assay buffer with 4% SDS immediately (10 s) after recovery or allowed to incubate at 37°C for 1 or 30 min. The samples were subjected to Western analysis, and the blots were stained with monoclonal antibodies directed against p17 and p24 (A) or p6 (B).

protein (p17), the adjacent capsid protein (p24), (Fig. 1A), or the carboxy-terminal p6 protein (28, 33, 34) (Fig. 1B). In each case, processing appeared to be largely complete by the initial time point (Fig. 1A and B). The aliquots removed before the addition of SDS and allowed to incubate at 37°C for either 1 or 30 min appeared to be unchanged. Although a small amount of unprocessed protein was present at the initial time point, this persisted when the virus was incubated at 37°C, suggesting that it represents membrane-associated protein which has passed through the filter.

The virtual absence of unprocessed Gag protein in virions harvested at 10 s after washing of the cells suggests that precursor processing occurs either in the virion within this time period or at the membrane, just before or during release. We reasoned that proteolytic cleavage of the precursors at the time of capsid formation could play a role in maximizing the efficiency of budding, perhaps by inducing a conformational change in the immature capsid, which is composed of unprocessed precursors. Alternatively, if processing is delayed until after the newly formed virus leaves the surface of the infected cell, the presence or absence of protease activity would have no effect on the efficiency of virion release. We decided, therefore, to examine the interaction between release of newly assembled virions and precursor processing by interfering with protease activity and observing the rate at which viral proteins leave the cell. CEM cells chronically infected with HIV-1 were pulse-labeled with [^{35}S]methionine in the presence of various concentrations of an HIV-1 protease inhibitor (Fig. 2). The cells were then resuspended in complete medium which also contained the inhibitor. Aliquots of cells and medium were removed at the end of the pulse period and at 1, 2, and 3 h during the chase period. Viral proteins were recovered from the infected cells and supernatant by immunoprecipitation with serum obtained from an HIV-1-seropositive person. Previously, we have demonstrated that this antiserum is capable of efficiently identifying the unprocessed Gag precursor (16). At

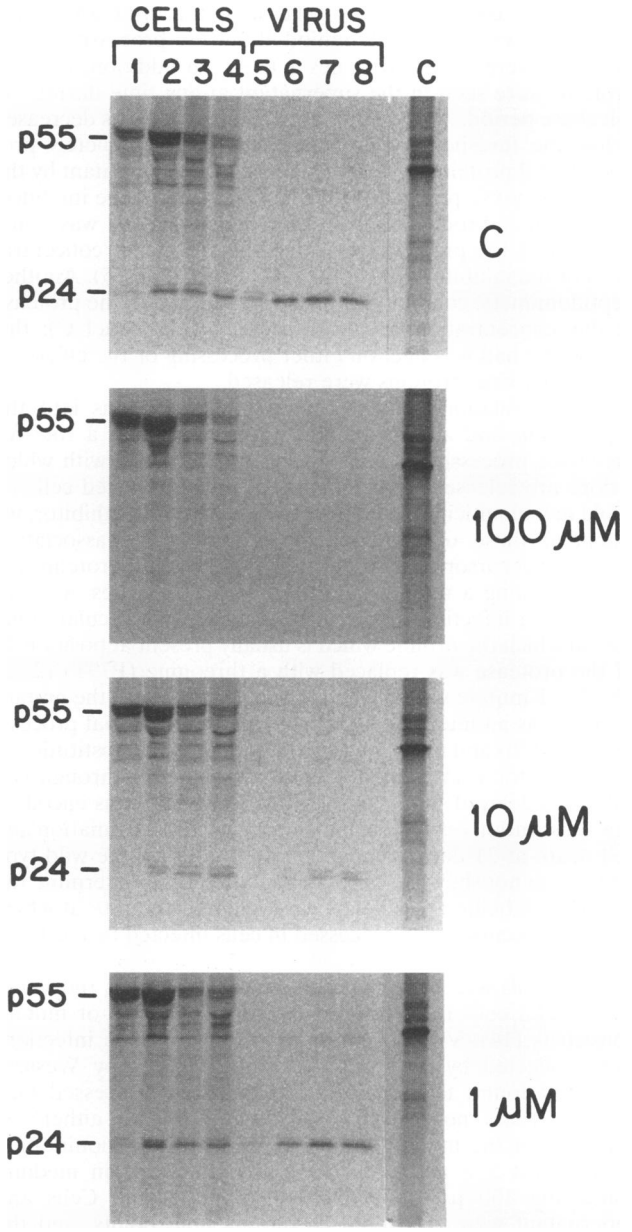

FIG. 2. Pulse-chase analysis of viral proteins from HIV-1-infected cells treated with an inhibitor of the viral protease. CEM cells chronically infected with HIV-1 were grown in methionine-free medium for 3 h and then labeled with 150 μCi of [^{35}S]methionine per ml (1,000 Ci/mmol; New England Nuclear, Boston, Mass.) for 30 min. The radiolabeled cells were washed twice with iced PBS and suspended in complete, unlabeled medium. The cells were grown either in a 100 μM of concentration of a control peptide (C) or in a 100, 10, or 1 μM concentration of the inhibitor beginning 3 h before labeling. The cells were maintained at that concentration throughout the experiment. The inhibitor of the HIV-1 protease used in these studies was SKF 107461 (17). The control peptide, EC15547-71-2, is a peptidomimetic inhibitor which has structural and physical properties similar to those of SKF 107461 but is 100-fold less active in enzyme inhibition assays (data not shown). Aliquots containing 4×10^6 cells and supernatant were recovered at the end of the pulse period (lanes 1 and 5) and at 1 (lanes 2 and 6), 2 (lanes 3 and 7), and 3 (lanes 4 and 8) h after the cells were transferred to complete medium. The cell fraction (lanes 1 to 4) was separated from the virus fraction (lanes 5 to 8) by centrifugation, and the radiolabeled viral proteins were isolated by immunoprecipitation as described elsewhere (11).

the highest concentration of the inhibitor (100 μM), Gag processing was completely inhibited and no processing intermediates were observed (lanes 1 to 4). In addition, no viral proteins were seen in the supernatant at any time during the 3-h chase period. As the inhibitor concentration was decreased below the threshold of protease inhibition, completely processed viral proteins began to appear in the supernatant by the end of the pulse period. At all concentrations of the inhibitor, viral protein production was unimpaired. There was some turnover of the precursor over time at the higher concentrations of the inhibitor (100 and 10 μM; lanes 4 and 5). Another peptidomimetic compound which does not inhibit the protease at this concentration was used as a control (panel C); this compound had no effect on either processing or the efficiency with which viral proteins were released.

This correlation between the release of virions into the supernatant and activity of the protease suggests a role for precursor processing in maximizing the efficiency with which virions are released from the surface of the infected cell. To avoid any unanticipated pleiotropic effects of the inhibitor, we extended these observations by examining the association between precursor processing and release of viral proteins in a virus encoding a mutant enzyme. For these studies, we constructed an infectious clone from the HXB2 molecular clone (28) in which the proline which is usually present at position 79 of the protease was replaced with a threonine (P79T) (21a). The P79T mutant was selected for analysis because the mutant enzyme has an intermediate phenotype in a bacterial processing assay (20) and although virus containing this substitution is infectious for transformed T cells, virus spread through cell culture is delayed. Infection of CEM cells with virus encoding this substitution resulted in maximum syncytium formation and cell death at 21 days, compared with 7 days for the wild-type virus (data not shown). As part of an attempt to determine the mechanism behind this delay, we evaluated the rate at which the Gag precursor was processed in cells infected by the P79T mutant.

CEM cells were infected with supernatant virus recovered from HeLa cells transfected with either wild-type or mutant constructs (14). Virus particles produced by these infections were collected by ultracentrifugation, analyzed by Western blot, and found to contain only completely processed Gag proteins (data not shown). Cells infected with either the wild-type or the mutant virus were grown in methionine-free medium for 3 h and then labeled continuously in medium containing 150 μCi of [^{35}S]methionine per ml. Cells and supernatant were recovered at various time points, and the viral proteins were immunoprecipitated with antiserum from an HIV-1-seropositive person (Fig. 3). For the wild-type virus (upper panel, lanes 1 to 9), both processed and unprocessed cell-associated, labeled viral proteins were seen after 30 min of labeling (lane 1). At this time, a small amount of the p24 capsid protein was seen in the supernatant (lane 5). Labeled Gag proteins, processing intermediates, and completely processed p24 protein were seen in the infected cells (lanes 2 to 4) and accumulating in the supernatant (lanes 6 to 8) over the rest of the 3-h labeling period. In the cells infected with the P79T mutant, however, both processing and the release of viral proteins were delayed (lower panel, lanes a to h). Only the unprocessed Gag precursor was seen in the infected cells during the first hour of labeling (lanes a and b), and some processing intermediates were apparent after 3 h (lane d). There was no evidence of viral proteins in the supernatant during the labeling period (lanes e to h).

To confirm these results, wild-type virus and the P79T mutant were transiently expressed in HeLa cells by using the calcium phosphate transfection method (14). Release of viral proteins into the supernatant was measured by a p24 antigen enzyme-linked immunosorbent assay (Abbott Laboratories, Abbott Park, Ill.). Twenty-four hours after transfection, the amount of p24 antigen was significantly less in the supernatant from the cells transfected with the P79T mutant than in that from cells transfected with the wild-type virus (data not shown).

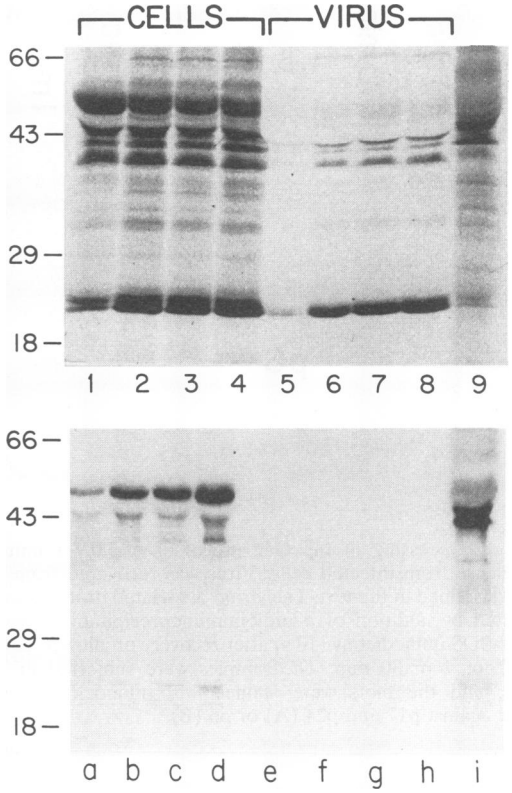

FIG. 3. Continuous labeling of CEM cells infected with wild-type HIV or the P79T protease mutant. CEM cells were infected with either wild-type HIV-1 derived from the HXB2 infectious clone (upper panel, lanes 1 to 8) or the P79T mutant virus (lower panel, lanes a to h). At the peak of viral infection, as measured by cell killing and syncytium formation, the infected cells were washed in PBS and transferred to methionine-free medium for 3 h. The viral proteins were then continuously labeled with [^{35}S]methionine. Aliquots of cells and virus were recovered after 30 min (wild-type virus, lanes 1 and 5; P79T, lanes a and e), 1 h (wild type, lanes 2 and 6; P79T, lanes b and f), 2 h (wild type, lanes 3 and 7; P79T, lanes c and g), and 3 h (wild type, lanes 4 and 8; P79T, lanes d and h). Uninfected CEM cells were used as controls (lanes 9 and i). The cell fraction of each aliquot (wild type, lanes 1 to 4; P79T, lanes a to d) was separated from the virus fraction (wild type, lanes 5 to 8; P79T, lanes e to h) by centrifugation, and the radiolabeled viral proteins were recovered by immunoprecipitation.

To determine more accurately the rate at which viral proteins are released from cells infected with the P79T mutant, we next performed pulse-chase experiments. Cells infected with either the wild type or the P79T mutant were grown in methionine-free medium for 1 h and pulse-labeled with 150 μCi of [^{35}S]methionine per ml. The cells were then washed twice with PBS and transferred to unlabeled, complete medium. Aliquots of cells and medium were removed at several time points, and the viral proteins were recovered by immunoprecipitation. The samples were evaluated by SDS-polyacrylamide gel electrophoresis, and the rate at which the

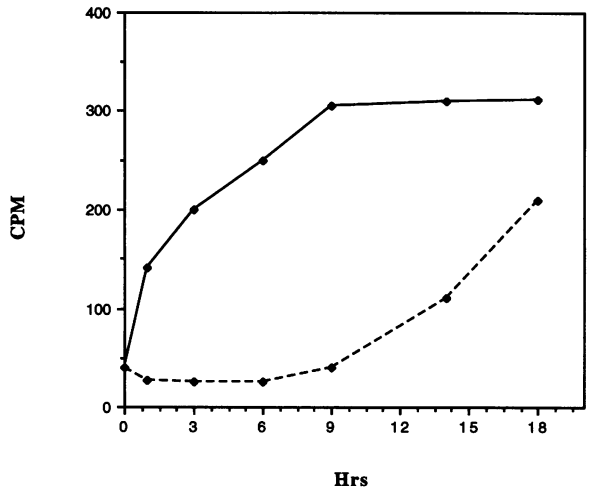

FIG. 4. Comparison between the rates at which viral protein is released from cells infected with wild-type virus or P79T protease mutant virus. CEM cells infected with either wild-type HIV-1 or the P79T mutant were pulse-labeled with [^{35}S]methionine and chased with complete medium. Aliquots of supernatant were recovered at the end of the pulse-labeling period (0 h) and at 1, 3, 6, 9, 14, and 18 h after transfer to complete medium. Viral proteins were immunoprecipitated with serum from an HIV-1-seropositive person and subjected to SDS-polyacrylamide gel electrophoresis. The radioactivity in the dried gels was quantitated with a radioanalytical analyzing system (Ambis Systems). Shown are the summed counts per minute of the radiolabeled viral proteins in the cell-free supernatant of cells infected with wild-type virus (solid line) and the P79T mutant (dashed line).

labeled viral proteins were released into the supernatant was quantitated by a radioanalytical analyzing system (Ambis Systems, San Diego, Calif.) (Fig. 4). Viral proteins produced by infection with the wild-type virus were released into the supernatant with a half-life of approximately 90 min. In contrast, the release of viral proteins from the cells infected with the P79T mutant was slower; radiolabeled viral proteins were first identified outside the cells after 9 h in complete medium. It is of note that only completely processed Gag proteins were found in the supernatant of the P79T-infected cells. Therefore, a delay in processing is associated with a delay in the release of viral proteins.

In summary, we examined the interaction between the processing of the HIV-1 Gag precursor and the membrane-based assembly and budding of virions. Although it has been clearly demonstrated that mutations which block protease activity do not prevent particle formation, it is also clear that complete processing of the Gag precursor is required for normal virus assembly and infectivity (5, 8, 16–18, 22, 27, 30, 31). Even partial inhibition of Gag processing results in aberrantly assembled viruses (16), and mutations which block cleavage at each of the processing sites in Gag disrupt normal assembly (8).

A prerequisite for any linkage between protease activity and release of viral proteins is that processing is initiated before virion release. We examined such an association by trying to identify the exact site of action of the viral protease. Specifically, we wanted to determine whether processing is initiated at the membrane before virion release or whether protease activity is delayed until after the virus has left the surface of the infected cell. Our attempts to examine newly formed virus particles revealed only completely processed precursors. In addition, whether protease activity was inhibited chemically or by a mutation in the protease, there was a close correlation between a delay in processing and a delay in release of the maturing virus particles. A recent report by Shin and coworkers indicates that an integrase mutation which impedes processing also delays release of viral proteins into the supernatant (29).

The data presented here indicate that processing of the Gag precursor is initiated at the membrane of infected cells and that the final steps of virus assembly are delayed when processing is slowed. If any additional processing occurs after virion release, it must happen over a very short period of time. Cell-associated processing is a common feature of retrovirus-infected cells (1, 4, 7, 9, 10, 12, 19, 21, 23–25, 32, 35), although a link between processing and virion release has not been made previously. In several systems, unprocessed viral proteins have been seen in virions (1, 7, 21). Several reports indicate that the efficiency with which protease-deficient Rous sarcoma virus and Moloney murine leukemia virus Gag-containing particles are released from the surface of infected cells is not markedly diminished (2, 13, 36–38). However, these studies were performed either with cell lines which produce very high levels of viral proteins and/or with chimeric subviral constructs. Since assembly of the viral core is likely dependent on the local concentration of Gag and Gag/Pol precursors, moderate differences in the efficiency of viral release could easily be obscured in these systems. These differences may also be difficult to identify in systems using chimeric constructs or those expressing Gag in the absence of other viral proteins. Therefore, we do not know if this represents experimental variation or a fundamental difference in the assembly of these viruses.

We thank T. Meek and G. Dreyer for providing the protease inhibitors and for helpful advice.

A.H.K. is a Scholar of the American Foundation for AIDS Research and was supported by Physician-Scientist Award 1956006143 AI from the National Institute of Allergy and Infectious Diseases. This study was also supported by award RO1-AI125321 from the National Institute of Allergy and Infectious Diseases.

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