The C Terminus of Human Immunodeficiency Virus Type 1 Matrix Protein Is Involved in Early Steps of the Virus Life Cycle

XIAOFANG YU,1 QIAN-CHUN YU,2 TUN-HOU LEE,1 AND MAX ESSEX1*

Department of Cancer Biology, Harvard School of Public Health, 665 Huntington Avenue, Boston, Massachusetts 02115,1 and Department of Molecular Genetics & Cell Biology, University of Chicago, Chicago, Illinois 606372

Received 10 April 1992/Accepted 1 June 1992

Deletion mutations at the C terminus of the matrix (MA) protein of human immunodeficiency virus type 1 (HIV-1) were generated by site-directed mutagenesis. The resultant mutant viruses had a severe defect in viral infectivity. This defect did not involve late steps of the virus life cycle, as the synthesis and processing of the Gag polyprotein and the assembly and release of mutant virions were not greatly affected. The incorporation of viral proteins and the viral RNA genome was similar for mutant and wild-type virions. In contrast, the early steps of the virus life cycle were severely affected, as the synthesis of viral DNA postinfection was dramatically reduced in mutant-virus-infected cells. One stretch of amino acids that was deleted in one of the mutants has a significant homology with a region in VP1 of the picornavirus family. This region of VP1 is presumably involved in poliovirus penetration into cells. These results suggest that in addition to its functional role in virus assembly, the MA protein of HIV-1, and possibly of other retroviruses, plays an important role in virus entry.

Assembly of retroviruses requires the correct folding and intracellular transport of the Gag polyprotein, association of the Gag polyprotein with the cellular membrane, and ultimately the release of assembled virions from the plasma membranes of infected cells. The matrix (MA) protein plays an important role in all of these aspects. In most retroviruses, the MA protein is modified by the addition of myristic acid at the N-terminal glycine residue (23). This modification is required for Gag polyprotein transport (19, 26) and association with the cellular membrane (2, 18, 26) and assembly of type C retroviruses and lentiviruses at the plasma membrane (2, 9, 18, 26). In addition to their role in myristylation, sequences within the MA protein are important for Gag polyprotein transport and virus assembly (10, 27).

Deletions or point mutations within the MA protein of Mason-Pfizer monkey virus (MPMV) affected the stability of Gag polyprotein and virus assembly (21, 22). Point mutations also blocked the transport of preassembled MPMV capsids to the plasma membrane and blocked the release of virions from the cell surface (22). More significantly, a single amino acid substitution in the MA protein of MPMV converted a type D retrovirus to a type C retrovirus (20).

Despite our knowledge about the functional role of the MA protein in virus assembly, little is known about the role of the protein in the early steps of the virus life cycle. For this reason, we generated a series of in-frame deletion mutations in the MA protein of human immunodeficiency virus type 1 (HIV-1). Two of these mutations did not affect virus assembly and were analyzed for their potential defects in the early steps of the virus life cycle.

Mutant D9 contains an in-frame deletion of 10 amino acids (105 through 114) and a substitution of isoleucine with leucine at position 104 at the carboxy terminus of p17 (Fig. 1A). Mutant D10 contains a deletion of 13 amino acids (116 through 128) at the carboxy terminus of p17 (Fig. 1A). Wild-type (wt) DNA (HXB2R3 [30]) and mutant DNA (D9 and D10) were transfected into COS-7 cells by the DEAE-dextran method (25). Seventy-two hours posttransfection, comparable virion-associated reverse transcriptase (RT) activities were detected in the supernatants of cells transfected with wt and mutant DNAs (data not shown). This suggests that the assembly and release of virions were not significantly affected by the amino acid deletions introduced in D9 and D10. Cell-free wt and mutant viruses with comparable RT activities were tested on Sup-T1 cells for their infectivity. The infectivity of mutant virions was significantly impaired compared with that of the wt virus. The production of mutant D9 virus was delayed by approximately 10 days, as monitored by RT activity in the supernatants of infected cells (Fig. 1C). Virus production was delayed even more dramatically in cells infected with mutant D10 (Fig. 1C), with no virus production detected in the first 30 days postinfection (Fig. 1C). Cytopathic effects and syncytium formation were also delayed in D9- and D10-infected Sup-T1 and H9 cells (data not shown).

To determine whether mutations in D9 and D10 affect viral Gag protein synthesis or processing or assembly of viral proteins into mature virions, cell and viral lysates from transfected COS-7 cells were subjected to radioimmunoprecipitation assay and immunoblot analysis. COS-7 cells were metabolically labeled with [35S]methionine or [35S]cysteine from 60 to 72 h posttransfection. Cell lysates were prepared and immunoprecipitated with HIV-1-positive human sera as previously described (29). The Gag precursor (p55) and the partially cleaved Gag protein (p41), as well as the cleaved products, p24/p17, were detected in the wt cell lysate (Fig. 2A). Gag-related proteins p55, p41, p24/p25, and p17 were also detected in D9 and D10 cell lysates (Fig. 2A). Mutant p55, p41, and p17 migrated more quickly than the wt Gag proteins, corresponding to the deletions in p17. It appears that the synthesis and processing of mutant Gag proteins were not greatly affected by deletions in D9 and D10.

Viral proteins in mature virions were analyzed by immunoblot using viral pellets from the supernatants of transfected COS-7 cells. Gag proteins p24 and p17, Pol proteins p66, p51, and p34, Env proteins gp120 and gp41 were detected in wt and mutant virions by the HIV-1-positive sera
(Fig. 2B). gp120 was also detected in wt and mutant virions by a sheep anti-gp120 serum (Fig. 2B). There was no significant difference between wt and mutant virions in either the amount or the migration of viral proteins, with the exception that the mutant p17s migrated faster than the wt p17 (Fig. 2B). These results suggest that deletions in D9 and D10 did not affect the cleavage of MA, CA, or Pol proteins in mature virions. The cleavage of NC protein from p15 was also not affected in mutant virions (data not shown).

The HIV-1 Gag precursor, p55, has been implicated in its ability to bind to the viral RNA genome (15). Deletions in p17 may change the conformation of p55 or the structure of the viral RNA genome and therefore affect the incorporation of the viral genome into mature virions. To address this question, viral RNA was analyzed by the dot blot assay. Equal amounts of wt and mutant virions, adjusted by RT activity and immunoblot (Fig. 2B), were blotted onto a nitrocellulose filter as previously described (14). Virus-specific signals were detected by using 2.7-kb Sall-BamHI DNA fragments from HXB2R3 that were labeled with [32P]dCTP. The amounts of viral RNA present did not differ significantly among D9, D10, and wt virions (Fig. 2C).

Deletions in D9 and D10 did not appear to affect the late steps of the virus life cycle, such as viral protein synthesis and processing and virus assembly and release. However, both mutant viruses had severe defects in virus infectivity, suggesting that early steps in the virus life cycle were affected. To test this possibility, we monitored the synthesis of the first viral product (viral DNA) immediately after infection. If the defect of the mutant viruses involved early steps in the virus life cycle, viral DNA synthesis would be blocked. This approach has been successfully used in the past to detect defects in virus entry (4, 13, 16). Equal amounts of wt and mutant viruses from the supernatants of transfected COS-7 cells, as measured by virion-associated RT activity and viral proteins (Fig. 2B), were incubated with Sup-T1 cells at 37°C for 2 h. Cells were then washed twice with phosphate-buffered saline and resuspended in fresh medium. Twelve hours postinfection, viral DNAs were isolated from equal numbers of cells by the Hirt method (11) and analyzed by Southern blot (24). A strong band migrating at the position of linear HIV-1 DNA (approximately 9.7 kb) was detected in the wt-infected cells (Fig. 3). A weaker band, which migrated more slowly than a 12-kb band would and which presumably was the circular form of HIV-1 DNA, was also detected in wt-infected cells (Fig. 3). Although it is possible that virions may carry some input plasmid DNA, the viral DNA isolated from infected cells by the method of Hirt is unlikely to be the input plasmid DNA. The signal of wt HIV-1 DNA was stronger at 72 h postinfection than at 12 h postinfection (Fig. 3), suggesting the presence of newly synthesized viral DNA. Also, under the same condition, the input plasmid DNA migrated differently from the viral DNA isolated by the method of Hirt (data not shown). Both D9 and D10 viruses showed a significantly decreased ability to synthesize viral DNA after infection (Fig. 3). The defect was more dramatic for D10 than for D9, corresponding to the results of the virus infectivity assay (Fig. 1C). Viral DNA synthesis at 72 h postinfection was also impaired in D9- and D10-infected cells compared with that in cells infected with the wt virus (Fig. 3). This suggests that the spread of mutant viruses was also affected.

Our study indicates that the HIV-1 MA protein plays an important role in the early steps of the virus life cycle. Although the MA protein's involvement in the process of reverse transcription cannot be excluded, our preliminary study using transmission electron microscopy suggests that
the entry of the mutant virus might be affected (28). The possibility that the MA protein of HIV-1 is directly involved in virus internalization is worth investigating further.

The possible role of murine leukemia virus Gag proteins in the early stages of virus infection has been previously reported (4). Deletions in murine leukemia virus Gag protein p12, or p12 and p15 (MA) together, have been shown to block viral replication at steps prior to reverse transcription (4). However, it is not clear whether p12 or the MA protein or both were responsible for the observed phenomenon, as the cleavage between the MA protein and p12 was affected in all of the mutants (4). Deletions in D9 and D10 did not affect the synthesis and cleavage of HIV-1 Gag protein or the assembly and release of virions. Therefore, our study suggests that the MA protein itself, at least in HIV-1, plays a role in the early steps of the viral life cycle, such as virus penetration or uncoating.

Mutations at the C terminus of p17 appear to affect viral replication at steps different from those affected by mutations introduced in the first two-thirds of p17 (30). Small deletions in p17 upstream from D9 severely inhibited virus assembly and/or incorporation of viral Env proteins into virions (30). Since deletions in D9 and D10 did not significantly affect virus assembly and incorporation of viral Env proteins, it is possible that the C terminus of p17 forms a distinctive functional domain. A stretch of amino acids at the

FIG. 2. Analysis of viral proteins and virion-associated RNA. (A) Viral proteins from transfected COS-7 cells were analyzed by radiolabeling kit as previously described (30). (B) Viral proteins from purified virions analyzed by immunoblot with a sheep anti-p120 serum (top) and top HIV-1-positive human sera (bottom) as previously described (30). (C) Viral RNA analyzed by dot blot assay as previously described (14). Briefly, equal amounts of wt and mutant virions, as measured by virion-associated RT activity and virion proteins, were blotted onto a nitrocellulose filter. The filter was then hybridized with probes generated by using a random-primed labeling kit (Boehringer Mannheim, Indianapolis, Ind.), with the 2.7-kb SalI-BamHI DNA fragments from HXB2R3 used as templates. Lane 2, virion samples collected from supernatants containing 10^6 cpms of RT activity; lane 3, twofold dilutions of the samples in lane 2; lane 1, samples similar to those in lane 2 but pretreated with DNase-free RNase cocktail (Boehringer Mannheim) at 37°C for 30 min.

FIG. 3. Analysis of viral DNA synthesis after infection. DNA was isolated by the method of Hirt (11) from newly infected Sup-T1 cells at 12 and 72 h postinfection and subjected to Southern blot with [32P]dCTP-labeled probes as described in the legend to Fig. 2.

C terminus of p17, AADTGHSSQV, is highly conserved among different strains of HIV-1 (1). Nearly identical sequences were also found in VP1 of most picornaviruses and, to a lesser extent, in the E protein of flaviviruses (Fig. 1B) (1). Interestingly, the VP1 region of the poliovirus becomes externalized when the virus attaches to infected cells and has been implicated in the process of virus penetration (5). Antibodies raised against this region and surrounding regions neutralized virus infectivity (3, 5, 6). It is possible that the AADTGHSSQV sequence in p17 also plays an important role in virus penetration, as deletion of this sequence in mutant D10 severely impaired virus infectivity (Fig. 1C).

Surrounding sequences may also be involved in virus infectivity, as demonstrated by the mutations in D9 (Fig. 1C), although they may be less critical (Fig. 1C and 3).

Electron microscopic tomography indicates that the narrow end of the HIV-1 core structure is attached to the viral envelope membrane in mature virions (12). It is possible that some of the MA proteins, which are attached to the viral envelope's inner membrane (8, 17), interact with the CA proteins of the core shell and thus are involved in the penetration and/or uncoating of the virus core. The MA protein of retroviruses also interacts with the viral Env protein (7, 30). This interaction may have consequences for postreceptor-binding steps of virus entry. The MA protein mutants described here will be useful for future studies on retroviral penetration and uncoating. With HIVs, such information may eventually be useful for the design of new classes of antiviral inhibitors.

We thank Z. Matsuda, X. Yuan, K. Chow, and M. F. McLean for helpful discussions and technical assistance and E. Conway for editorial assistance.

This work was supported by Public Health Service grants CA-39805 and HL-33774 from the National Institutes of Health and by contract DAMD 17-90-C-0151 from the U.S. Army.

REFERENCES

3. Chow, M., R. Yabrov, J. Bitte, J. Hogle, and D. Baltimore. 1985. Synthetic peptides from four separate regions of the poliovirus type 1 capsid protein VP1 induce neutralizing anti-