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The Matrix Protein of Human Immunodeficiency Virus Type 1 Is Required for Incorporation of Viral Envelope Protein into Mature Virions

XIAOFANG YU, XIN YUAN, ZENE MATSUDA, TUN-HOU LEE, AND MAX ESSEX*

Department of Cancer Biology, Harvard School of Public Health, 665 Huntington Avenue, Boston, Massachusetts 02115

Received 4 March 1992/Accepted 13 May 1992

Accumulating evidence suggests that the matrix (MA) protein of retroviruses plays a key role in virus assembly by directing the intracellular transport and membrane association of the Gag polyprotein. In this report, we show that the MA protein of human immunodeficiency virus type 1 is also critical for the incorporation of viral Env proteins into mature virions. Several deletions introduced in the MA domain (p17) of human immunodeficiency virus type 1 Gag polyprotein did not greatly affect the synthesis and processing of the Gag polyprotein or the formation of virions. Analysis of the viral proteins revealed normal levels of Gag and Pol proteins in these mutant virions, but the Env proteins, gp120 and gp41, were hardly detectable in the mutant virions. Our data suggest that an interaction between the viral Env protein and the MA domain of the Gag polyprotein is required for the selective incorporation of Env proteins during virus assembly. Such an interaction appears to be very sensitive to conformational changes in the MA domain, as five small deletions in two separate regions of p17 equally inhibited viral Env protein incorporation. Mutant viruses were not infectious in T cells. When mutant and wild-type DNAs were cotransfected into T cells, the replication of wild-type virus was also hindered. These results suggest that the incorporation of viral Env protein is a critical step for replication of retroviruses and can be a target for the design of antiviral strategies.

Human immunodeficiency virus type 1 (HIV-1) assembly at the plasma membrane of infected cells is similar to the morphogenesis of type C retroviruses. The Gag precursor polyprotein of HIV-1 appears to be sufficient for virus assembly, since viruslike particles are formed in the absence of all other viral proteins (5, 9, 12, 29, 30). The Gag precursor of HIV-1 is first synthesized as a 55-kDa polyprotein (p55) and is subsequently cleaved by the viral protease to yield four mature Gag proteins: the matrix (MA) protein (p17); the capsid (CA) protein (p24); the nucleocapsid (NC) protein (p9); a proline-rich protein (p6); and two small peptides, p2 and p1 (11). The CA protein, p24, is believed to form the core shell of the virus particle, which is analogous to the function of the CA protein of other retroviruses. Domains within the CA protein have been shown to be critical for Gag polyprotein interaction and the formation of virus particles (10, 28). p9 contains two Cys-His boxes that are conserved among the NC proteins of all retroviruses. Mutations which disrupt the Cys-His motif impair the packaging of the viral RNA genome into mature virions (1, 6, 13, 14). Mutations in the p6 domain do not affect virus assembly, but they are able to block the release of assembled particles from the cell surface (7).

The MA protein of retroviruses plays an important role in virus assembly. In most retroviruses, the MA protein is cotranslationally modified by N-terminal myristoylation (26). Mutations that destroy this modification have completely inhibited HIV-1 and type C retrovirus assembly at the plasma membrane (2, 8, 19, 34, 35). In type D retroviruses, capsid assembly can occur in the absence of myristoylation, but the assembled capsids are not transported to the cell surface (22). It seems that myristoylation is required for high-affinity association of Gag polyprotein with the cellular membrane (2, 19) and is important for intracellular transport of the Gag polyproteins (22, 34). Although cellular receptors for the myristic acid of $p60^{v\text{-}src}$ have been identified (20), it is still not clear whether myristoylation itself is directly involved in Gag polyprotein transport and membrane association. The Gag polyproteins of Rous sarcoma virus (RSV), equine infectious anemia virus, and visna virus are not modified by N-terminal myristoylation (26), but they are competent for virus assembly at the plasma membrane. By analogy to the VP4 of poliovirus, myristoylation could be important for the overall structure of the retrovirus Gag polyprotein (15). In several studies, myristoylation mutant Gag polyproteins lost their ability to interact with wild-type (WT) Gag polyproteins. This was probably due to a change in protein structure, and, as a result, they were excluded from the assembled virions (27, 34).

In addition to myristoylation, sequences within the MA protein are also important for virus assembly and release. Deletions in the MA protein of RSV completely blocked particle formation (36). Particle formation could not be restored with the addition of a myristoylation signal at the N-terminal of mutant Gag polyprotein but was restored by adding a short peptide sequence that contains a targeting signal to the plasma membrane (36). Similarly, amino acid insertions in the MA protein of murine leukemia virus (MuLV) inhibited the transport of mutant Gag polyprotein from the cytoplasm to the plasma membrane and mutant Gag proteins were not incorporated into virions (10). The MA domain's important role in directing the intracellular transport of the Gag polyprotein has also been demonstrated in Mason-Pfizer monkey virus, a type D retrovirus (25). Furthermore, sequences within the MA domain of Mason-Pfizer monkey virus have been found to control the morphogenesis of virus assembly and budding. Single amino acid substitu-

* Corresponding author.

tions in the MA domain of Mason-Pfizer monkey virus converted a type D retrovirus to a type C retrovirus (24). In this study, we addressed the possibility that the MA protein of retroviruses has functional roles in the virus life cycle other than those already identified. The results presented here reveal another important function of the HIV-1 MA protein during virus assembly.

MATERIALS AND METHODS

DNA, cells, and sera. The molecular clone of HIV-1, HXB2R3, was constructed by exchanging the *Xho*I-*Xba*I fragment from HXB2R (39) with the *Xho*I-*Xba*I fragment from HXB3 (18). This fragment includes the 3' long terminal repeat and part of the *nef* coding sequence. In contrast to HXB2 (18), HXB2R3 contains full-length *vpr* and *nef* coding sequences. COS-7 cells were maintained in Dulbecco's modified Eagle's medium plus 10% fetal calf serum. SupT1 cells were maintained in RPMI 1640 medium plus 10% fetal calf serum. HIV-1-positive human sera were kindly provided by K. Meyer of the Fenway Community Health Center, Boston, Mass. The sheep anti-gp120 serum of HIV-1 was obtained from the AIDS Research and Reference Program (catalog no. 192), National Institutes of Health, Bethesda, Md.

Oligonucleotide-directed mutagenesis of p17. The *Sst*I-*Apa*I fragment covering the *gag* region of HXB2R3 was subcloned into pGEM7Z(+) (Promega, Madison, Wis.). Single-stranded uracil-containing DNA was prepared and used for site-directed mutagenesis according to the protocol of the manufacturer (Bio-Rad, Richmond, Calif.). The sequences of primers used for mutagenesis were as follows: D1, 5'-CCC CCT GGC CTT AAC CCG CTT AAT ACT G-3'; D2, 5'-CCA TAC TAT ATG TTT TCG AAT TTT TTC CCA TCG-3'; D3, 5'-AAC TGC GAA TCG TTC TAT TAA TTT ATA TTT TTC-3'; D4, 5'-CAG TAT TTG TCT ACA GAG CTC CCT GCT TGC CC-3'; D5, 5'-TGA TCC TGT CTG AAG GCC TTC TGA TGT TTC-3'; D6, 5'-TAC TGT ATT ATA TAT CGA TGG TTG TAG CTG TCC-3'; D7, 5'-CTT TTA TCT CTA TTC TAG ATC TAA GTT CTT C-3'; D8, 5'-TTG CTC TTC CTC TAT TTG ATG CAC ACA ATA G-3'; Myr$^-$, 5'-TGA CGC TCT CGC GAC CAT CTC TCT CC-3'. The positions of p17 deletion mutants are illustrated in Fig. 1. The myristoylation mutant, Myr$^-$, contains a substitution of valine for glycine at amino acid position 2. Mutants were screened by restriction enzyme digestion and DNA sequencing. The *Bss*HII-*Pst*I fragments which contain the p17 mutations were cloned back into the vector of HXB2R3.

Transfection, infection, and RT assay. COS-7 cells were trypsinized and seeded at about 30% confluence 24 h before transfection. Cells (5×10^6) were then trypsinized, pelleted, and resuspended in 1 ml of TD buffer (25 mM Tris-HCl, pH 7.4; 140 mM NaCl; 5 mM KCl; 0.7 mM K_2HPO_4) containing 400 μg of DEAE-dextran and 2 μg of WT or mutant DNA. Transfection was carried out at 37°C for 30 min. For the transfection of SupT1 cells, the trypsinization step was omitted. SupT1 cells (10^7) were washed once with phosphate-buffered saline and resuspended in 3 ml of TD buffer containing 600 μg of DEAE-dextran and 6 μg of DNA. The DNAs used for each SupT1 transfection were as follows: mock, 6 μg of pUC18; WT, 1 μg of HXB2R3 plus 5 μg of pUC18; WT + D1, 1 μg of HXB2R3 plus 5 μg of D1; WT + D2, 1 μg of HXB2R3 plus 5 μg of D2; WT + Myr$^-$, 1 μg of HXB2R3 plus 5 μg of Myr$^-$. Transfections were carried out at room temperature for 20 min. Virus infectivity was tested with SupT1 cells by using cell-free supernatants of transfected COS-7 cells as previously described (38). Samples used in the reverse transcriptase (RT) assay were prepared from polyethylene glycol-precipitated viral pellets from the supernatant of transfected or infected cells. The assay was performed as previously described (38).

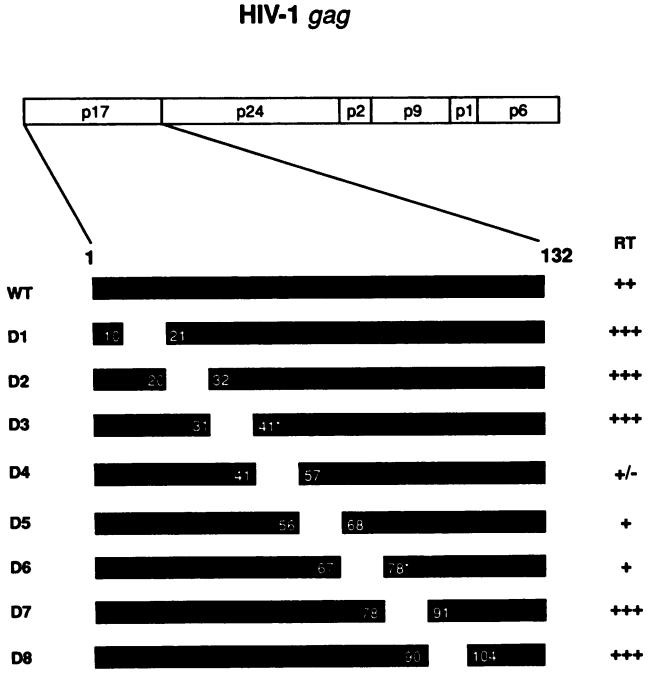

FIG. 1. Construction and virus production of p17 mutants. Mutant plasmids were constructed as described in Materials and Methods. The top diagram shows the mature HIV-1 Gag products. Deletion mutants of HIV-1 p17 are shown as D1, D2, D3, D4, D5, D6, D7, and D8. Numbers within each construct represent the first and last amino acids which were preserved after deletion in each mutant. 41* and 78* indicate amino acid substitutions of leucine to isoleucine at positions 41 and 78, respectively. Virus production was detected by RT activity in the supernatants of transfected COS-7 cells at 72 h posttransfection. Results were representative of three independent experiments. Symbols: +/−, 10,000 to 40,000 cpm/ml; +, 50,000 to 100,000 cpm/ml; ++, 150,000 to 350,000 cpm/ml; +++, 500,000 to 950,000 cpm/ml.

Radioimmunoprecipitation, immunoblot, and pulse-chase analysis. At 60 h posttransfection, COS-7 cells were incubated for 12 h in cysteine-free RPMI 1640 medium containing [^{35}S]cysteine (0.1 mCi/ml; NEN). Supernatants were precleared at 800 × *g* for 30 min and centrifuged through a 20% sucrose cushion at 20,000 rpm (Beckman SW28 rotor) for 2 h to obtain virus pellets. Lysed cells, virus pellets, and supernatants without virus pellets were subjected to radioimmunoprecipitation analysis (RIPA) with HIV-1-positive human sera as previously described (38). For immunoblot analysis of the virion proteins, virus pellets were prepared from the supernatants of COS-7 cells 72 h posttransfection as described above. Virion proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and reacted with HIV-1-positive sera or sheep anti-gp120 serum. For the pulse-chase experiment, COS-7 cells (60 h posttransfection) were labeled in the RPMI 1640 media containing

[^{35}S]cysteine for 30 min and then chased in the complete media for 0.5, 1.5, 3, and 6 h before being analyzed by RIP.

RESULTS

Construction of p17 deletion mutants. The matrix protein of HIV-1 (p17) consists of 132 amino acids. Site-directed mutagenesis was applied to generate in-frame deletion mutants of p17. D1, D2, D4, D5, D7, and D8 contained deletions of 10 to 15 amino acids in p17 (Fig. 1). D3 and D6 contained deletions of 9 and 10 amino acids, respectively, as well as substitutions of leucine to isoleucine at positions 41 and 78, respectively (Fig. 1).

The effect of P17 mutations on Gag polyprotein synthesis, processing, and virus production. To analyze the effect of p17 mutations on virus assembly, mutant and WT proviral DNA were transfected into COS-7 cells by the DEAE-dextran method (33). Seventy-two hours posttransfection, virion-associated RT activity was measured by using supernatants from transfected cells. D4, D5, and D6 had a 2- to 10-fold decrease in virus production compared with production of the WT virus (Fig. 1). Virus production in the other p17 mutants was approximately two- to fivefold greater than production of the WT virus as measured by RT activity in the supernatants of transfected cells (Fig. 1). Since mutations in D4, D5, and D6 significantly affected virus assembly, further studies focused on mutants D1, D2, D3, D7, and D8, unless otherwise indicated.

Sixty hours posttransfection, cells were metabolically labeled with ^{35}[S]cysteine for 12 h and analyzed by RIP. Similar levels of gp120 and gp160 were detected in WT and mutant-transfected cells, suggesting that the efficiencies of transfection were comparable (Fig. 2b). gp120 was also detected in the supernatants of WT and mutant-virus-transfected cells, implying that the transport of viral Env proteins was not affected by the p17 mutations (Fig. 2a). The Gag precursor polyprotein, p55, and the processed products, p24/p25 and p17, were also detected in the WT-transfected cells (Fig. 2b). The level of p55 detected in some of the mutant-transfected cells was lower than that in the WT-transfected cells (Fig. 2b). The processing of the mutant Gag polyproteins was apparently not affected, as p24/p25 and mutant p17 were detected in mutant-transfected cells (Fig. 2b) and/or virus pellets (Fig. 2c). Mutant p17 were less detectable than WT p17 in transfected cells (Fig. 2b), suggesting that they were either very unstable or less immunogenic than the WT p17.

The supernatants from the labeled COS-7 cells were used to purify released virions and subjected to RIPA. In WT virions, p24 and p17 were detected (Fig. 2c). p17 mutants D1, D2, D3, D7, and D8 produced more virions that the WT virus as determined by the increased level of p24, p34, and in some cases p17 (D1 and D2) (Fig. 2c). Slightly smaller p17 proteins, corresponding to deletions in the p17 mutants, were detected in all of the mutant virions (Fig. 2c). The level of p17 detected in the D3 and D7 virions was much lower than that of the WT p17 (Fig. 2c). This may be due to the decreased immunogenicity of mutant p17. When labeled virions were analyzed prior to immunoreaction, the ratios of p17 to p24 in WT and D3 mutant virions were found to be the same (data not shown).

Viral protein synthesis and processing were also analyzed by pulse-chase experiments. The synthesis of mutant Gag p55 was not greatly affected as indicated by the pulse-labeling, whereas mutant p55 disappeared more quickly than WT p55 (Fig. 2d). This was observed both for mutants that had defects in virus assembly (D4 and D6) and for mutants that had slightly increased virus production (D1, D2, D3, D7, and D8) (Fig. 2d and data not shown).

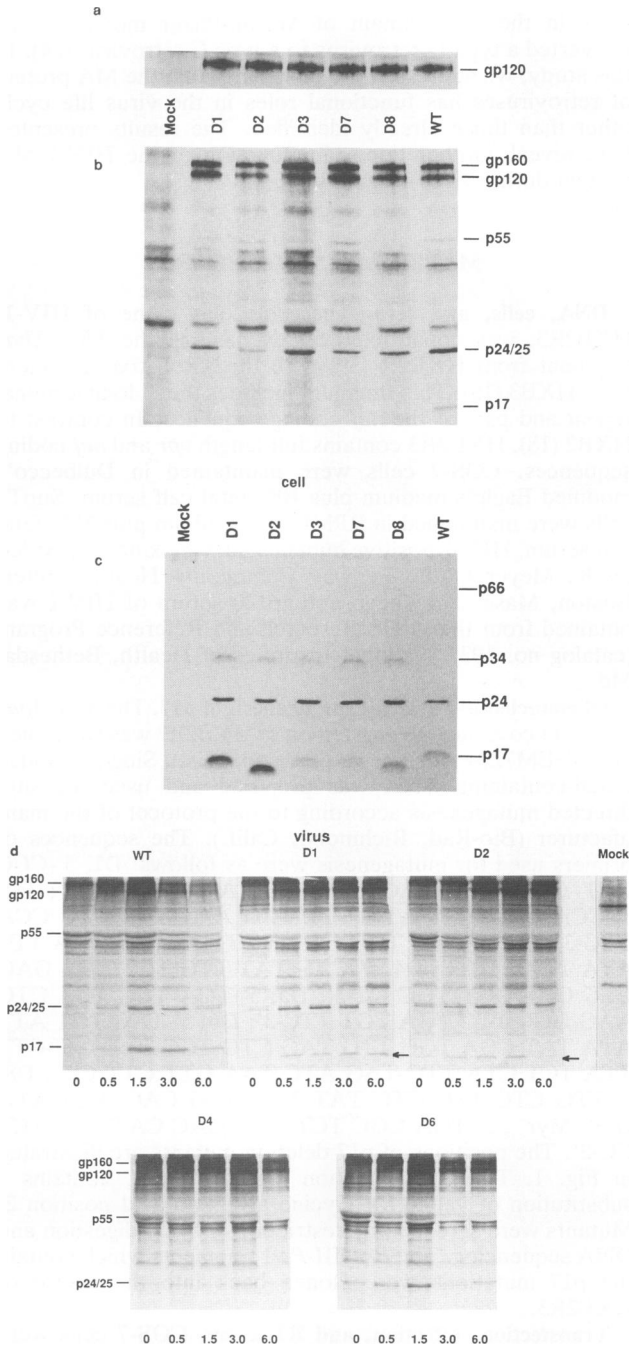

FIG. 2. Detection of viral proteins in transfected COS-7 cells, supernatants, and virus pellets by RIPA (a, b, and c) and pulse-chase labeling (d). Transfected COS-7 cells were metabolically labeled with [^{35}S]cysteine 60 h posttransfection. Samples were separated into cell fractions (cell), viral pellets (virus), and virus-free supernatants (sup) before they were immunoprecipitated with HIV-1-positive sera. (a) sup; (b) cell; (c) virus. (d) Cells were pulse-labeled and chased as described in Materials and Methods. Numbers indicate times after pulse-labeling. Arrows indicate the position of mutant p17.

Mutation in p17 affects viral Env protein incorporation into

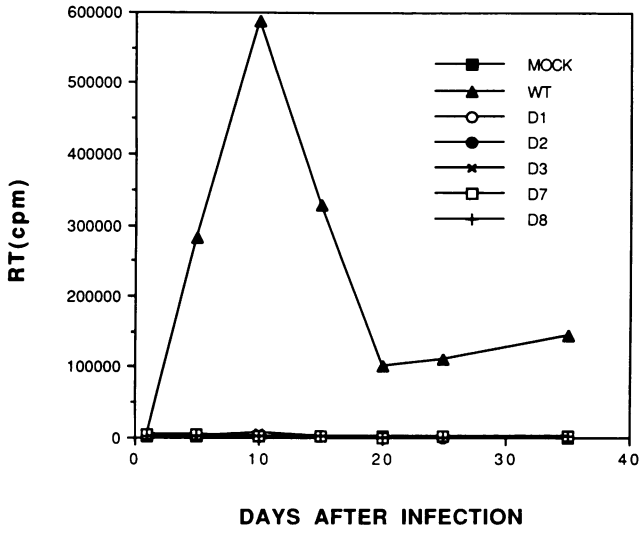

FIG. 3. Virus infectivity assay. Cell-free WT and mutant viruses were prepared from the supernatants of transfected COS-7 cells. Virus infectivity in SupT1 cells was tested as previously described (38). RT values represent samples from 0.1-ml culture supernatants. cpm, counts per minute.

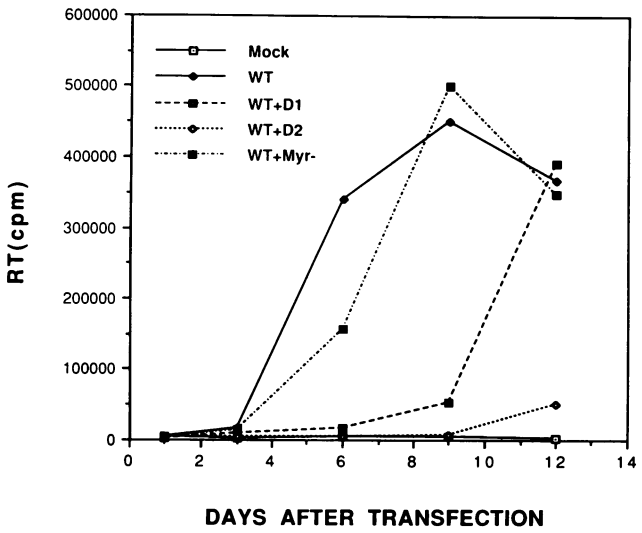

FIG. 5. Dominant negative mutant assay. WT or WT plus mutant DNAs were transfected into SupT1 cells. Virus replication was monitored by the RT activity in the supernatant of transfected cells. RT values represent samples from 0.1-ml culture supernatants. cpm, counts per minute.

mature virions. Although p17 mutants D1, D2, D3, D7, and D8 could efficiently produce virions, mutant viruses were completely noninfectious in SupT1 cells (Fig. 3). To further analyze the defect of these p17 mutants, virions were collected from the culture supernatants 72 h posttransfection. Comparable amounts of WT and mutant virions as adjusted by RT activity were used for an immunoblot assay. In the WT virions, Env proteins (gp41 and gp120), Gag proteins (p24 and p17), and Pol proteins (p66, p51, and p34) were detected (Fig. 4). The amounts of p24 and *pol*-encoded proteins p66, p51, and p34 detected in D1, D2, D3, D7, and D8 virions were comparable to those detected in the WT virions. In D1, D2, and D8, mutant p17 was also detected (Fig. 4). Mutant p17 was not detected in D3 and D7 virions, probably because of the loss of immunogenicity as described above. The most dramatic defect of the p17 mutant virions was that the *env*-encoded proteins gp41 and gp120 were barely detectable in the virions of the p17 mutants by using HIV-1-positive human sera (Fig. 4). Also, no gp120 was detected in p17 mutant virions by using the sheep anti-gp120 serum (Fig. 4). Since the synthesis, processing, and transport of viral Env proteins seem unaffected in cells transfected by p17 mutants (Fig. 2a and b), these observations suggest that the viral Env proteins were not efficiently incorporated into the p17 mutant virions.

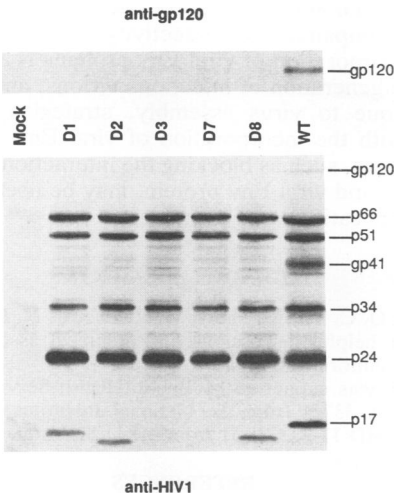

FIG. 4. Analysis of viral proteins by immunoblot. Virions were purified and analyzed as previously described (38). Samples were transferred onto two nitrocellulose filters. One was blotted with HIV-1-positive sera (anti-HIV1). Another was blotted with sheep anti-gp120 of HIV-1 (anti-gp120).

p17 deletion mutants interfere the replication of WT virus. The deletion mutants described in this report did not block virus assembly and release, suggesting that mutant Gag polyproteins can retain their ability to interact with each other and form particles. If the p17 mutant Gag polyproteins can still interact with the WT Gag polyproteins and assemble into virions, the incorporation of Env proteins and infectivity of these resulting virions might be impaired. To test this possibility, WT DNA was cotransfected with either D1 or D2 DNA into SupT1 cells. Virus production, as monitored by RT activity in the supernatants of transfected cells, was quicker and higher in cells transfected with WT DNA alone than in cells cotransfected with WT and D1 or D2 DNAs (Fig. 5). Syncytium formation and cytopathic effects were also delayed in cells cotransfected with WT and D1 or D2 DNAs (data not shown). As a control, WT DNA was cotransfected with the myristoylation mutant, Myr^-, into SupT1 cells. Myristoylation mutant Gag polyproteins of MuLV and spleen necrosis virus were unable to interact with the WT Gag polyproteins and were excluded from virus particles (27, 34). If the myristoylation mutant Gag polyprotein of HIV-1 cannot interact with the WT Gag polyprotein, cotransfection of WT and Myr^- DNAs should not significantly interfere with the replication of WT virus. As expected, virus production (Fig. 5) and the cytopathic effect (data not shown) were not significantly affected in cells

cotransfected with WT and Myr$^-$ compared with cells transfected with WT alone.

DISCUSSION

The MA protein of retroviruses plays an important role in the correct folding and intracellular transport of the Gag polyprotein. In type D retroviruses, small deletions of 7 to 21 amino acids in the MA protein rendered the Gag polyprotein highly unstable and blocked virus assembly (23). Small deletions in the MA protein of RSV or amino acid insertions in the MA protein of MuLV also blocked particle formation (10, 36). Mutant Gag polyproteins of MuLV were unable to be transported to the plasma membrane (36). Therefore, it is somewhat surprising that deletions in some of the HIV-1 MA protein did not greatly affect virus assembly (Fig. 1 and 2c). Although mutant Gag polyproteins appeared to disappear more quickly than the WT Gag polyprotein in transfected cells (Fig. 2d), mutants D1, D2, D3, D7, and D8 all maintained their ability to form virus particles. One explanation may be that these small deletions in p17 did not greatly alter the overall structure and stability of the Gag polyprotein as much as the deletions in D4, D5, and D6. Deletions in D4, D5, and D6 significantly decreased virus assembly (Fig. 1).

The most interesting observation in our study is that although mutations in the p17 region of HIV-1 did not block virus assembly and release, they significantly impaired the incorporation of viral Env proteins into mature virions. Incorporation of viral Env proteins is a unique step in virus assembly. The cellular surface proteins are largely excluded during assembly, while viral Env proteins are selectively incorporated (31, 37). The molecular nature of this process is poorly understood. However, we do know that the MA protein is in close proximity with the virus envelope membrane in mature virions, as it can be cross-linked to the lipid component in avian leukemia virus and in MuLV (17). Immunoelectron microscopic studies have also revealed that the MA protein of HIV-1 is located at the inner leaflet of the virus lipid membrane (4). The localization of the MA protein makes it a likely candidate to interact with the viral Env proteins, such as the transmembrane (TM) protein. Indeed, such interactions in RSV have been suggested by chemical cross-linking studies (3). However, whether such an interaction also takes place during virus assembly and whether it is directly responsible for the incorporation of viral Env proteins into virions have not been demonstrated. Our mutational study provides evidence that the MA protein of HIV-1 is required for the incorporation of viral Env proteins into mature virions. Since mutations in p17 did not affect the synthesis, processing, and transport of viral Env proteins, our data suggest that MA protein interaction with viral Env proteins during assembly is more likely to be responsible for the incorporation of viral Env proteins. It is not clear which region of p17 directly interacts with the viral Env protein. However, a tertiary structure is likely to be involved since five deletion mutants in two separate regions of p17 all impaired the incorporation of viral Env protein.

Retrovirus Gag assembly could occur in the absence of viral Env proteins (5, 9, 12, 21, 25a, 29, 30). However, recent studies suggest that assembly and release of virus particles under normal physiological conditions require the presence of viral Env and Gag proteins. When HIV-1 Gag proteins alone were expressed in polarized epithelium cells, virus particles were released from both the apical and basolateral sites (16). In contrast, when Gag proteins were coexpressed with viral Env proteins in the same cells, virus particles were released only from the basolateral site (16). This suggests that the viral Env proteins determine the site of virus release. The p17 mutants D1, D2, D3, D7, and D8 apparently lost their ability to interact with viral Env proteins. At the same time, they appeared to assemble and release virus particles more efficiently than the WT virus (Fig. 1 and 2c). One possible explanation for this phenomenon is that in the absence of viral Env and Gag protein interaction, the assembly of viral capsid proteins is deregulated. It is also noticeable that the Gag polyprotein p55 of these mutants is also processed more quickly than the WT p55 (Fig. 2d), suggesting that an interaction between Gag and Env proteins may stabilize Gag polyprotein in the cells. It is not known where the Gag polyproteins and Env proteins start to interact in the infected cells. The Gag polyproteins of MuLV have been found to be associated with intracellular membrane compartments (10). In the presence of monensin, which blocks vesicular transport, the release of virus particles and Gag proteins was inhibited, suggesting that the transport of Gag polyprotein is through the vesicular system (10). Therefore, the interaction between viral Env and Gag proteins may occur in the intracellular membrane compartments, where the proteins are then cotransported to the site of virus assembly. Alternatively, the viral Env and Gag proteins may travel via separate routes from their synthesis sites to the site of virus assembly.

The assembly of retrovirus particles requires interaction among the Gag polyproteins. The key region responsible for this interaction is located in the CA protein. Deletions or linker insertions in the CA region of MuLV did not block the targeting of the Gag polyprotein to the plasma membrane but inhibited virus particle formation (10, 28). Mutations in MA proteins, with the exception of the myristoylation mutant, usually do not affect their ability to interact with other Gag polyproteins, as they can form virions with WT Gag polyproteins (36). Our cotransfection study indicated that the p17 deletion mutants, like p24 mutants (32), can be used as negative dominant mutants to interfere with the replication of WT virus. One possible explanation for the observed interference may be the result of interaction among p17 mutant Gag polyproteins and WT Gag protein. Virions formed by the mutant and WT Gag polyproteins would be impaired in their ability to incorporate viral Env proteins and would have impaired virus infectivity.

Since incorporation of viral Env proteins is a critical step toward the generation of infectious virions, and this step is highly unique to virus assembly, strategies designed to interfere with the incorporation of viral Env proteins into mature virions, such as blocking the interaction between the MA protein and viral Env protein, may be useful for blocking HIV replication.

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