JOURNAL OF VIROLOGY, Mar. 1995, p. 1984–1989 Vol. 69, No. 3
0022-538X/95/$04.00+0

Virion Incorporation of Envelope Glycoproteins with Long but Not Short Cytoplasmic Tails Is Blocked by Specific, Single Amino Acid Substitutions in the Human Immunodeficiency Virus Type 1 Matrix

ERIC O. FREED* AND MALCOLM A. MARTIN

Laboratory of Molecular Microbiology, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20892-0460

Received 24 October 1994/Accepted 16 December 1994

Incorporation of envelope glycoproteins into a budding retrovirus is an essential step in the formation of an infectious virus particle. By using site-directed mutagenesis, we identified specific amino acid residues in the matrix domain of the human immunodeficiency virus type 1 (HIV-1) Gag protein that are critical to the incorporation of HIV-1 envelope glycoproteins into virus particles. Pseudotyping analyses were used to demonstrate that two heterologous envelope glycoproteins with short cytoplasmic tails (the envelope of the amphotropic murine leukemia virus and a naturally truncated HIV-2 envelope) are efficiently incorporated into HIV-1 particles bearing the matrix mutations. Furthermore, deletion of the cytoplasmic tail of HIV-1 transmembrane envelope glycoprotein gp41 from 150 to 7 or 47 residues reversed the incorporation block imposed by the matrix mutations. These results suggest the existence of a specific functional interaction between the HIV-1 matrix and the gp41 cytoplasmic tail.

The matrix (MA) domain of the human immunodeficiency virus type 1 (HIV-1) Gag protein is localized in the virion to the inner face of the viral envelope, where it associates with the lipid bilayer by means of a myristic acid moiety attached to its N terminus (17, 43). A variety of functions have been proposed for the HIV-1 MA, including roles in virus assembly (2, 14, 36, 47), virus entry (44), nuclear localization (3), and incorporation of envelope glycoproteins into virus particles (6, 45).

Retroviral envelope glycoproteins are incorporated into virions during the budding of virus particles from the plasma membrane of the infected cell. Studies performed a decade ago indicated that the MA and transmembrane envelope glycoprotein (TM) of Rous sarcoma virus could be chemically cross-linked, suggesting that an interaction between the MA and TM proteins occurred during the budding process (16). Such an interaction was supported by genetic studies of the type D Mason-Pfizer monkey virus in which mutation of the MA and TM reduced envelope incorporation into virus particles (1, 31) and by studies which concluded that deletions in the HIV-1 TM cytoplasmic tail reduced the efficiency of envelope incorporation into virus particles (7, 46). Paradoxically, however, several studies reported that deletion of the cytoplasmic tail of both the Rous sarcoma virus (29) and HIV-1 (15, 40) TMs did not impair envelope glycoprotein incorporation, suggesting that no direct interaction occurred between the cytoplasmic tail of the TM and the MA. Furthermore, the envelope glycoproteins of murine leukemia virus (MuLV) and human T-cell leukemia virus type I can be incorporated into HIV-1 particles (25, 26, 37), despite the absence of significant amino acid sequence conservation between the MAs of MuLV, human T-cell leukemia virus type I, and HIV-1. Thus, the nature of the interaction between retroviral TM and MA proteins is unclear.

* Corresponding author. Mailing address: Bldg. 4, Rm. 307, NIAID, NIH, Bethesda, MD 20892. Phone: (301) 402-3215. Fax: (301) 402-0226. Electronic mail address: Eric_Freed@nih.gov.

Two recent studies indicated that deletions and multiple amino acid changes in the HIV-1 MA blocked HIV-1 envelope incorporation into virus particles (6, 45). This effect, however, did not appear to rely on particular residues in MA, since all of the mutations introduced, with the exception of those at the extreme C terminus of MA, blocked envelope incorporation.

Lentiviruses, including HIV-1, are unique among retroviruses in having TM glycoproteins with very long cytoplasmic tails. HIV-1 and HIV-2, for example, typically express TM glycoproteins with cytoplasmic tails of approximately 150 amino acids (28). The cytoplasmic tail of the equine infectious anemia virus TM glycoprotein contains more than 200 amino acids (32). In contrast, the type C retroviruses Rous sarcoma virus and MuLV express TM proteins with cytoplasmic tails of approximately 20 and 30 amino acids, respectively (21, 30), and the type D retrovirus Mason-Pfizer monkey virus expresses a TM protein with a 38-amino-acid cytoplasmic tail (35). The unusual length of lentiviral TM cytoplasmic tails suggests that these sequences evolved to serve a specific function in the lentivirus life cycle.

To investigate the nature of a potential interaction between the HIV-1 envelope glycoproteins and the MA domain of Gag, we identified single amino acid changes in MA which blocked the incorporation of HIV-1 envelope glycoproteins into virus particles. We then analyzed the effects of these mutations on the incorporation of envelope glycoproteins which vary in the length of the TM cytoplasmic tail. The results of this study suggest that a specific functional interaction occurs between the cytoplasmic tail of HIV-1 TM envelope glycoprotein gp41 and the HIV-1 MA.

The 12LE and 30LE MA mutations block virus replication without affecting virus particle production. During previous studies, we introduced over 50 single amino acid changes into the MA of HIV-1 (10, 14). Most of these mutations did not significantly affect envelope incorporation, as determined by virus infectivity and immunoprecipitation analyses (10, 14). To assess whether single amino acid changes in the HIV-1 MA are

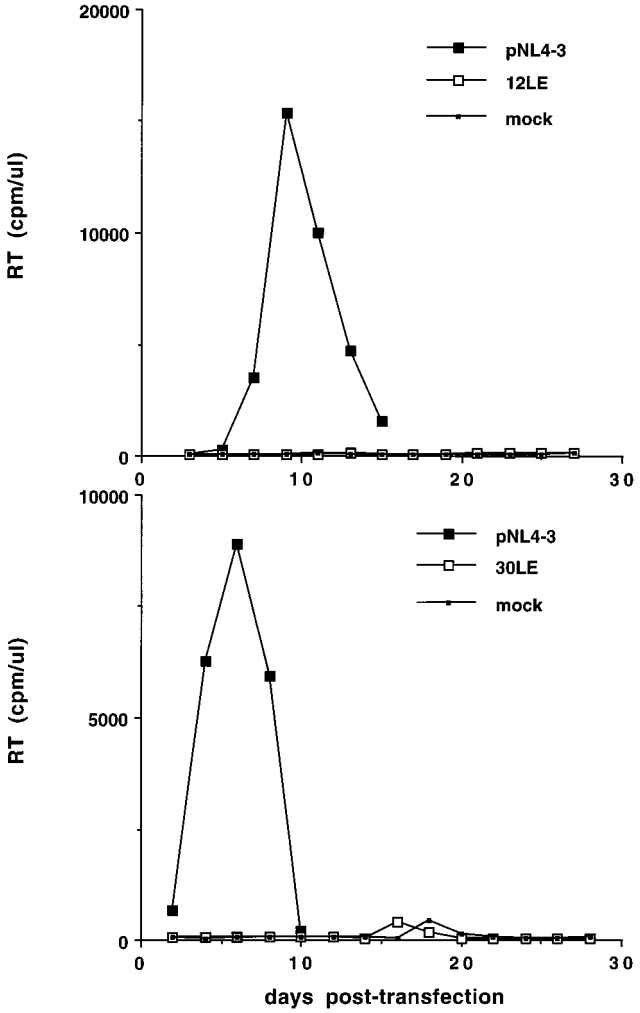

FIG. 1. Replication of wild-type and MA mutant molecular clones in the CEM(12D-7) T-cell line. The CEM(12D-7) cell line (33) was transfected with pNL4-3, pNL4-3MA/12LE, and pNL4-3MA/30LE by the DEAE-dextran procedure (14). The cells were split 1:3 every 2 days; aliquots were reserved for reverse transcriptase (RT) assays at each time point. The assays were performed as previously described (11, 42).

capable of inhibiting or blocking the incorporation of HIV-1 envelope glycoproteins into virus particles, we introduced a number of additional mutations into the HIV-1 MA as previously described (14). A mutation from Leu to Glu at MA amino acid 12 or 30 (the 12LE or 30LE mutation, respectively) blocked the establishment of a productive infection in transfected cultures of the CEM(12D-7) T-cell line (Fig. 1) without affecting virus particle production in transfected HeLa cells (data not shown). On the basis of recently published nuclear magnetic resonance data on the structure of MA (27), residues 12 and 30 would be predicted to be located close to each other in the tertiary structure of the protein.

The 12LE and 30LE MA mutations block envelope glycoprotein incorporation into virus particles. To determine whether the 12LE or 30LE mutation blocks virus replication by interfering with envelope incorporation into virus particles, we transfected HeLa cells in parallel with wild-type HIV-1 molecular clone pNL4-3 and derivatives of pNL4-3 containing the 12LE or 30LE MA mutation. Two days posttransfection, cells were plated at approximately 2×10^6/25-cm^2 tissue culture flask in 2 ml of Cys-free RPMI medium supplemented with 10% fetal bovine serum. [^{35}S]Cys (500 μCi) was added, and cells were returned to 37°C for approximately 15 h. The labeled cell supernatant was filtered and pelleted at 35,000 rpm in an ultracentrifuge (SW55 rotor) for 30 min. Cell and virus lysates were prepared and immunoprecipitated as previously described (11, 14) with human HIV immunoglobulin (catalog no. 192; NIAID AIDS Research and Reference Reagent Program). The data in Fig. 2 indicate that 12LE and 30LE MA mutant virions contain wild-type levels of p24(CA) and p17(MA) but no detectable gp120. These results suggest that mutations at MA amino acids 12 and 30 block the incorporation of HIV-1 envelope glycoproteins into virus particles.

In theory, it is possible that the 12LE and 30LE MA mutations disrupt the stable association of gp120 with gp41 on virus particles rather than incorporation of the gp120-gp41 complex into virions. This seems unlikely, however, since previously described deletion and multiple amino acid substitution mutations in HIV-1 MA blocked the appearance of both gp41 and gp120 in virus particles (6, 45).

The 12LE and 30LE MA mutations do not affect the incorporation of heterologous envelope glycoproteins with short cytoplasmic tails. As mentioned above, the cytoplasmic tails of lentiviral TM glycoproteins are very long compared with those of other retroviruses. Our finding that single-amino-acid changes in the HIV-1 MA can block incorporation of the HIV-1 envelope, together with topological considerations, led to the speculation that the residues in MA we identified as being critical for HIV-1 envelope incorporation may interact specifically with sequences within the long cytoplasmic tail of HIV-1 TM glycoprotein gp41. A prediction of this hypothesis is that the 12LE and 30LE mutations might not affect the incorporation of retroviral envelope glycoproteins with short cytoplasmic tails. To test this hypothesis, HIV-1 particles bearing the 12LE and 30LE mutations were pseudotyped with envelope glycoproteins containing short cytoplasmic tails.

Derivatives of pNL4-3, pNL4-3MA/12LE, and pNL4-3MA/30LE were constructed in which the *env* open reading frame was shifted out of frame with the previously described KFS linker insertion mutation (9) to prevent the synthesis of any HIV-1 envelope glycoprotein. These molecular clones were cotransfected into HeLa cells with vectors expressing the envelope glycoproteins of HIV-1 (pHenv; 13), HIV-2 (pCMVHIV-2env; 12), or amphotropic MuLV (pSVAMLVenv; 25). The pCMVHIV-2env vector directs the synthesis of an HIV-2 TM envelope glycoprotein lacking the C-terminal 108 amino acids (10, 12, 18). Virus pools were prepared from the cotransfected HeLa cells, and their infectivities were assayed in the HeLa-CD4-LTR-β-gal indictor cell line (23). This CD4$^+$ HeLa-derived cell line harbors the β-galactosidase gene under the transcriptional regulation of the HIV-1 long terminal repeat. Infection of these cells with HIV leads to the expression of Tat, which transactivates the integrated HIV-1 long terminal repeat and results in β-galactosidase synthesis. HIV-infected cells thus turn blue following fixation and staining. A measure of the infectious titer of each different virus stock is the number of blue cells scored following infection (Table 1). Virus particles harvested from pNL4-3KFS-transfected cells are noninfectious, since they lack envelope glycoproteins. Cotransfection of pNL4-3KFS (which expresses the wild-type MA) with vectors expressing the HIV-1 (pHenv), the truncated HIV-2 (pCMVHIV-2env), or the amphotropic MuLV (pSVAMLV env) envelope glycoprotein restored infectivity. Consistent with the results in Fig. 2, cotransfection of pNL4-3KFS/12LE or pNL4-3KFS/30LE with HIV-1 envelope expression vector pHenv resulted in production of noninfectious particles, since

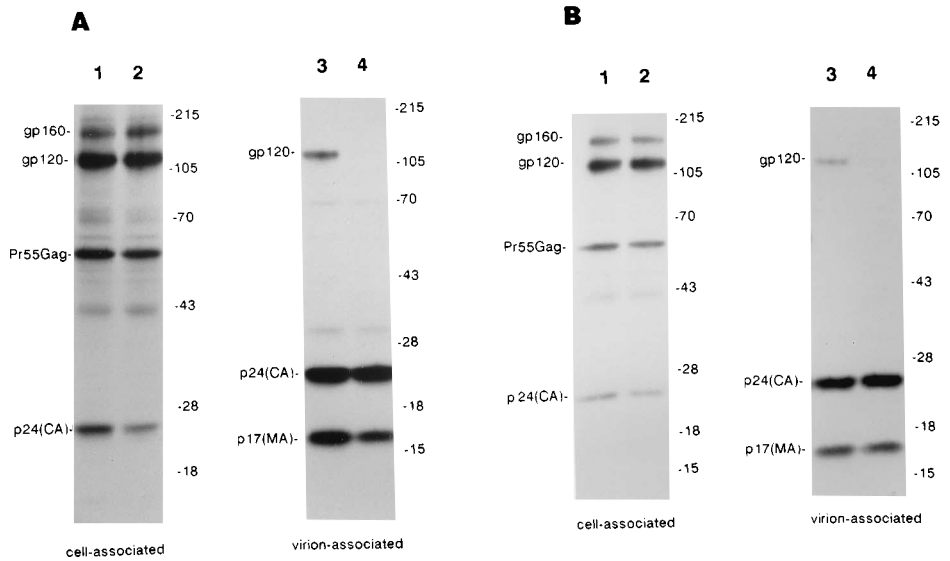

FIG. 2. Radioimmunoprecipitation analysis of HIV-1 Env incorporation into virus particles containing the 12LE and 30LE MA mutations. HeLa cells were transfected with pNL4-3, pNL4-3MA/12LE, or pNL4-3MA/30LE. Two days posttransfection, HeLa cells were metabolically labeled overnight with [^{35}S]Cys. The labeled cell supernatant was filtered and pelleted in an ultracentrifuge. Cells and virus pellets were lysed and immunoprecipitated with HIV-1-specific AIDS patient immunoglobulin as previously described (11, 41). Panels: A, pNL4-3 (lanes 1 and 3) versus pNL4-3MA/12LE (lanes 2 and 4); B, pNL4-3 (lanes 1 and 3) versus pNL4-3MA/30LE (lanes 2 and 4). The numbers to the right of the panels are molecular sizes in kilodaltons.

the 12LE and 30LE MA mutations block incorporation of the HIV-1 envelope glycoprotein. In marked contrast, cotransfection of pNL4-3KFS/12LE or pNL4-3KFS/30LE with either pCMVHIV-2env or pSVAMLVenv resulted in production of virus particles with infectivities comparable to those formed with wild-type MA. This result indicates that 12LE and 30LE virus particles with the truncated HIV-2 or MuLV envelope glycoprotein are as infectious as wild-type particles in the pseudotyping assay, suggesting that the 12LE and 30LE mutations affect only the incorporation of envelope glycoproteins, such as HIV-1 gp41, with long cytoplasmic tails.

TABLE 1. Relative infectivities of MA mutants coexpressed with the HIV-1, truncated HIV-2, or MuLV envelope glycoprotein

Transfected DNAs[a]	Pseudotyping envelope	MA	Relative no. of IU/ml[b]	
			Assay 1	Assay 2
pHenv plus:				
pNL4-3KFS	HIV-1	Wild type	100	100
pNL4-3KFS/12LE		12LE mutant	<1	<1
pNL4-3KFS/30LE		30LE mutant	<1	<1
pCMVHIV-2env plus:				
pNL4-3KFS	HIV-2	Wild type	100	100
pNL4-3KFS/12LE		12LE mutant	158	113
pNL4-3KFS/30LE		30LE mutant	90	69
pSVAMLVenv plus:				
pNL4-3KFS	MuLV	Wild type	100	100
pNL4-3KFS/12LE		12LE mutant	95	98
pNL4-3KFS/30LE		30LE mutant	160	89
pNL4-3KFS	None	Wild type	<1	<1
None (Mock infection)			<1	<1

[a] HeLa cells were cotransfected with an HIV-1 (pHenv), truncated HIV-2 (pCMVHIV-2env), or MuLV (pSVAMLVenv) envelope expression vector and *env*-negative pNL4-3 derivatives expressing wild-type MA (pNL4-3KFS) or the 12LE (pNL4-3KFS/12LE) or 30LE (pNL4-3KFS/30LE) MA mutant. Two days posttransfection, virus supernatants were harvested and used to infect the HeLa-CD4-LTR-β-gal indicator cell line. Two days postinfection, the cells were fixed and stained as previously described (23).

[b] The values shown, which are normalized for input virus-associated reverse transcriptase activity, are the relative numbers of infectious units (IU) for the virus pools. The average absolute titers were as follows: pNL4-3 plus pHenv, 4.5×10^3 IU/ml; pNL4-3KFS plus pCMVHIV-2env, 2.4×10^3 IU/ml; pNL4-3KFS plus pSVAMLVenv, 10^5 IU/ml.

To show directly that the truncated HIV-2 and MuLV envelope glycoproteins are incorporated into particles containing the 12LE and 30LE MA mutations, we radioimmunoprecipitated cell- and virion-associated proteins produced by HeLa cells cotransfected with pSVAMLVenv or pCMVHIV-2env and pNL4-3KFS, pNL4-3KFS/12LE, or pNL4-3KFS/30LE. The results were consistent with those presented in Table 1; the truncated HIV-2 and the MuLV envelope glycoproteins were readily incorporated into 12LE (Fig. 3A) and 30LE (Fig. 3B) MA mutant virus particles, as determined by the presence of the HIV-2 and MuLV surface envelope glycoproteins (gp120 and gp70, respectively) in virion-associated material.

Truncation of the cytoplasmic tail of gp41 reverses the envelope incorporation block imposed by the 12LE MA mutation. The results presented thus far suggest that incorporation of retroviral envelope glycoproteins with short cytoplasmic tails (e.g., the truncated HIV-2 or MuLV envelope) is not affected by MA mutations, while incorporation of the full-length HIV-1 envelope glycoprotein is blocked by these mutations. A prediction based on these results is that truncation of the cytoplasmic tail of HIV-1 gp41 would relieve the restriction imposed by the 12LE and 30LE MA mutations. To test this prediction, we introduced two gp41 C-terminal tail truncation mutations derived from molecular clones pNLTr712 and pNLTr752 (40; kindly provided by V. Bosch) into pNL4-3 and pNL4-3MA/12LE. The Tr752 mutation (40), which truncates the C-terminal 104 amino acids from gp41, was removed from pNLTr752 on a *Bam*HI-*Xho*I fragment and cloned into pNL4-3 and pNL4-3MA/12LE to generate pNL4-3(CTdel-104) and pNL4-3/12LE(CTdel-104), respectively. A sequence containing the Tr712 mutation (40), which truncates the C-

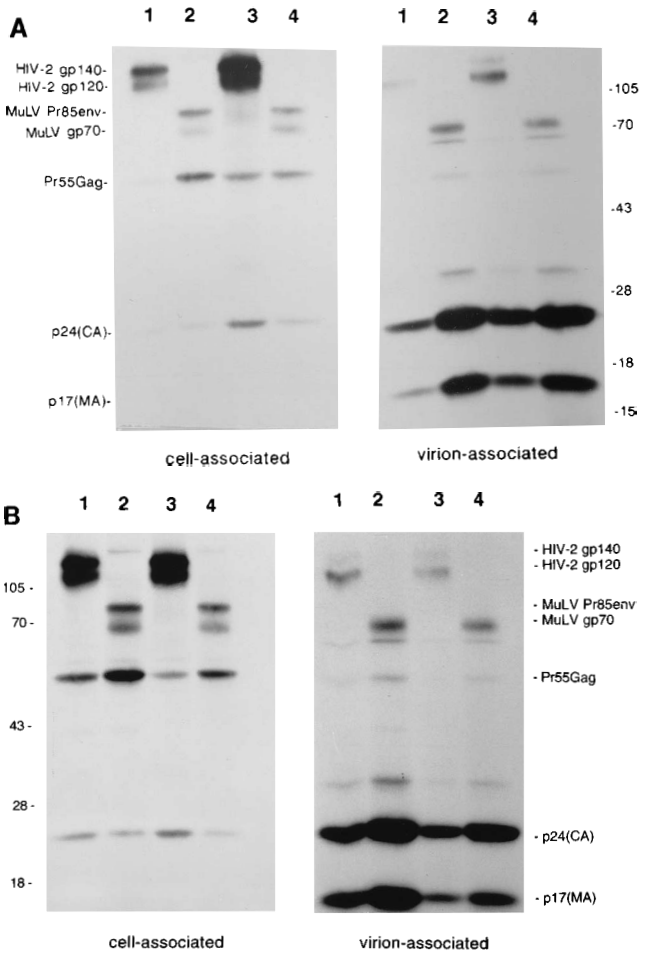

FIG. 3. Radioimmunoprecipitation analysis of truncated HIV-2 or MuLV Env incorporation into MA mutant virus particles. Panels: A, 12LE MA mutant; B, 30LE MA mutant. HeLa cells were cotransfected with pNL4-3KFS plus pCMVHIV-2env (lanes 1), pNL4-3KFS plus pSVAMLVenv (lanes 2), MA mutant pNL4-3KFS plus pCMVHIV-2env (lanes 3), or MA mutant pNL4-3KFS plus pSVAMLVenv (lanes 4). The positions of the HIV-2 Env precursor (gp140), the HIV-2 surface Env glycoprotein (gp120), the MuLV Env precursor (Pr85env), and the MuLV surface Env glycoprotein (gp70) are indicated. Transfected cells were metabolically labeled with [^{35}S]Cys, and cell- and virion-associated proteins were immunoprecipitated with a mixture of HIV-1-specific AIDS patient immunoglobulin and either HIV-2-specific AIDS patient serum (lanes 1 and 3) or anti-Raucher MuLV gp70. HIV-2-specific patient serum was obtained from the NIAID AIDS Reagent Program (catalog no. 409); anti-Raucher MuLV gp70 was from Quality Biotech Inc. (lot 81S000127). The numbers to the right of panel A and the left of panel B are molecular sizes in kilodaltons.

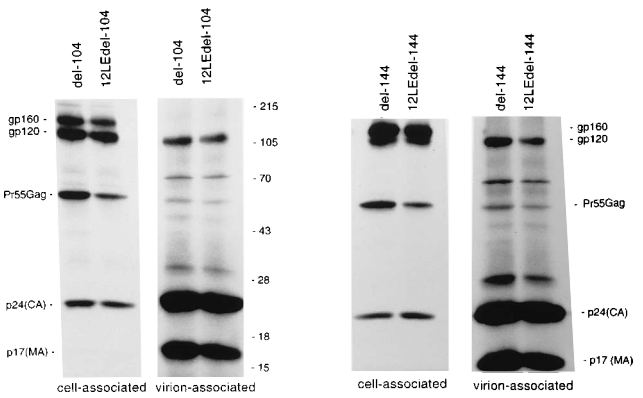

FIG. 4. Radioimmunoprecipitation analysis of truncated HIV-1 Env incorporation into MA mutant virus particles. HeLa cells were transfected with pNL4-3 derivatives expressing a truncated envelope glycoprotein (CTdel-104 or CTdel-144) and either wild-type MA or the 12LE MA mutant. Transfected cells were metabolically labeled with [^{35}S]Cys, and cell- and virion-associated proteins were immunoprecipitated with HIV-1-specific AIDS patient immunoglobulin. The numbers beside the lanes are molecular sizes in kilodaltons.

terminal 144 amino acids from gp41, was removed from pN LTr712 on a *Sal*I-*Bam*HI fragment and cloned into pNL4-3MA/12LE to generate pNL4-3/12LE(CTdel-144). For consistency, pNLTr712 was renamed pNL4-3(CTdel-144).

Virus pools were prepared from transfected HeLa cells and used to infect the HeLa-CD4-LTR-β-gal indicator cell line (23). The results (Table 2) demonstrate that the infectivities of viruses bearing gp41 C-terminal truncations are not affected by the 12LE MA mutation. The HeLa-CD4-LTR-β-gal cell titers obtained with virus bearing the gp41 C-terminal tail truncation mutations did not differ significantly from those obtained with the wild-type NL4-3 virus (data not shown).

To show directly that the truncated HIV-1 envelope glycoproteins are efficiently incorporated into virus particles, we immunoprecipitated [^{35}S]Cys-labeled virus particles from HeLa cells transfected with pNL4-3(CTdel-104), pNL4-3/12LE(CTdel-104), pNL4-3(CTdel-144), or pNL4-3/12LE(CTdel-144). As anticipated from the results in Table 2, the amounts of gp120 on NL4-3(CTdel-104) versus NL4-3/12LE (CTdel-104) and on NL4-3(CTdel-144) versus NL4-3/12LE (CTdel-144) virus particles were comparable (Fig. 4). Thus, truncation of the C terminus of gp41 restored the incorporation of HIV-1 envelope glycoproteins into virus particles containing the 12LE MA mutation.

Conclusions. In this study, we demonstrated that single-amino-acid changes at HIV-1 MA amino acids 12 and 30 block

TABLE 2. Relative infectivity of double mutants bearing the 12LE MA and gp41 C-terminal tail truncation mutations

Virus[a]	HIV-1 gp41 truncation (no. of amino acids)	MA	Relative no. of IU/ml[b]	
			Assay 1	Assay 2
NL4-3(CTdel-104)	104	Wild type	100	100
NL4-3/12LE(CTdel-104)	104	12LE mutant	125	119
NL4-3(CTdel-144)	144	Wild type	100	100
NL4-3/12LE(CTdel-144)	144	12LE mutant	105	101
NL4-3MA/12LE	None (full length)	12LE mutant	<1	<1
None (Mock infection)			<1	<1

[a] The molecular clones listed were transfected into HeLa cells. Two days posttransfection, virus supernatants were harvested and used to infect the HeLa-CD4-LTR-β-gal indicator cell line. Two days postinfection, the cells were fixed and stained as previously described (23).

[b] The values shown, which were normalized for input virus-associated reverse transcriptase activity, are the relative numbers of infectious units (IU) for the virus pools. The average absolute titers were as follows: NL4-3(CTdel-104), 4.5×10^3 IU/ml; NL4-3(CTdel-144), 4.9×10^3 IU/ml.

the incorporation of wild-type HIV-1 envelope glycoproteins into virus particles. As anticipated, this block to envelope incorporation renders virus particles containing the MA mutations noninfectious. Pseudotyping experiments performed with envelope glycoproteins with short TM cytoplasmic tails (the amphotropic MuLV and truncated HIV-2 envelope glycoproteins) indicate that the 12LE and 30LE MA mutant particles are able to incorporate heterologous envelope glycoproteins and function in biological assays. Furthermore, truncation of the C terminus of HIV-1 gp41 reverses the envelope incorporation block imposed by the 12LE mutation. Together, these results suggest the existence of a specific functional interaction(s) between the HIV-1 MA and the C terminus of gp41 that directs the incorporation of envelope glycoproteins into virions. An interaction between the HIV-1 envelope and MA is further supported by recent experiments indicating that the HIV-1 MA could direct the incorporation of HIV-1 envelope glycoproteins into chimeric HIV-1–visna virus particles (6).

It is noteworthy that the 12LE and 30LE mutations appear to block the incorporation of the full-length HIV-1 envelope glycoprotein without affecting the incorporation of envelope glycoproteins with short cytoplasmic tails. Presumably, the interaction between the long gp41 cytoplasmic tail and MA is disrupted by these MA mutations, resulting in a block to envelope incorporation. We suggest that the incorporation into HIV-1 particles of the envelope glycoproteins with short or truncated cytoplasmic tails may occur independently of MA; the 12LE and 30LE MA mutations thus have no effect on the incorporation of these envelope glycoproteins. Interestingly, not only were envelope glycoproteins with short cytoplasmic tails readily incorporated into 12LE and 30LE MA mutant particles, but these envelope glycoproteins also conferred full infectivity (relative to virus containing wild-type MA) in $CD4^+$ HeLa cells. Other investigators have previously reported that the MuLV envelope glycoprotein could be incorporated into, and render infectious, HIV-1 particles containing a 100-amino-acid deletion in MA (39).

A number of researchers have observed that passage of simian immunodeficiency virus in human cells results in the emergence of variants which lack most of the TM cytoplasmic tail (4, 20, 24). Passage of these variants in monkey cells leads to reversion to a full-length TM cytoplasmic tail (24). While the TM truncations appear to arise in part as a result of greater fusogenicity of the truncated envelope glycoproteins in human cells (38, 49), virions bearing TM glycoproteins with cytoplasmic tail truncations contain higher levels of envelope glycoprotein than do virions bearing full-length TM glycoproteins (22, 49). Variants of HIV-2 and equine infectious anemia virus (passaged in human or canine cells, respectively) which express truncated TM glycoproteins (5, 8, 19, 32, 48) have been identified, whereas premature truncation of the HIV-1 TM cytoplasmic tail is rare (34). The observation of cell type differences in the incorporation of lentiviral envelope glycoproteins into virus particles hints at involvement of cellular proteins in the envelope incorporation process.

We speculate that a specific functional interaction between MA and TM was a driving force in the evolution of the long cytoplasmic tails of lentiviral TM glycoproteins and that the interaction among MA, the TM cytoplasmic tail, and, possibly, cellular proteins, plays an important role in the virus life cycle. Since incorporation of envelope glycoproteins is essential to virus infectivity, the interaction between MA and the C terminus of gp41 may serve as a target against which effective anti-HIV therapeutics can be developed.

We thank V. Bosch for providing pNLTr712 and pNLTr752 molecular clones. The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: HIV immunoglobulin (from A. Prince), HIV-2 AIDS patient sera (from S. Osmanov), pSVAMLVenv (from N. Landau and D. Littman), and CD4-LTR-β-gal cells (from M. Emerman). We thank R. Willey, K. Strebel, L. Derr, and J. Cortner for critical review of the manuscript.

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