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Truncation of the Human Immunodeficiency Virus Type 1 Envelope Glycoprotein Allows Efficient Pseudotyping of Moloney Murine Leukemia Virus Particles and Gene Transfer into CD4$^+$ Cells

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Human immunodeficiency virus type 1 (HIV-1) can readily accept envelope (Env) glycoproteins from distantly related retroviruses. However, we previously showed that the HIV-1 Env glycoprotein complex is excluded even from particles formed by the Gag proteins of another lentivirus, visna virus, unless the matrix domain of the visna virus Gag polyprotein is replaced by that of HIV-1. We also showed that the integrity of the HIV-1 matrix domain is critical for the incorporation of wild-type HIV-1 Env protein but not for the incorporation of a truncated form which lacks the 144 C-terminal amino acids of the cytoplasmic domain of the transmembrane glycoprotein. We report here that the C-terminal truncation of the transmembrane glycoprotein also allows the efficient incorporation of HIV-1 Env proteins into viral particles formed by the Gag proteins of the widely divergent Moloney murine leukemia virus (Mo-MLV). Additionally, pseudotyping of a Mo-MLV-based vector with the truncated rather than the full-length HIV-1 Env allowed efficient transduction of human CD4$^+$ cells. These results establish that Mo-MLV-based vectors can be used to target cells susceptible to infection by HIV-1.

Retroviral envelope (Env) glycoproteins are synthesized in the form of a precursor which is cleaved by a cellular protease to yield the surface glycoprotein and the transmembrane glycoprotein components of the mature Env protein complex (12). Although dispensable for viral particle formation, the Env proteins are efficiently recruited into budding virions by a mechanism which is only poorly understood. Env protein incorporation is selective, since most cellular proteins are excluded from retroviral particles (12). However, retroviral particles can readily accommodate heterologous viral Env proteins. For instance, Moloney murine leukemia virus (Mo-MLV) particles can functionally incorporate the surface glycoproteins of human T-cell leukemia virus type I, gibbon ape leukemia virus, and vesicular stomatitis virus (6, 33, 36). Conversely, the Env glycoproteins of murine retroviruses are readily accepted by other retroviruses such as human immunodeficiency virus type 1 (HIV-1) (16, 17, 29).

In view of the efficient formation of pseudotypes even between widely divergent retroviruses, we were surprised to find that the HIV-1 Env protein complex is excluded from particles formed by the Gag proteins of visna virus (4), one of the closest known relatives of HIV-1 among nonprimate lentiviruses (30). Similarly, attempts to pseudotype Mo-MLV particles with HIV-1 Env have not yielded any evidence for infectious virion formation (36), although Mo-MLV can form infectious hybrid virions with the Env proteins of several other nonmurine retroviruses (15, 33, 36). Interestingly, visna virus particles acquired the ability to efficiently incorporate HIV-1 Env protein upon replacement of the N-terminal matrix (MA) domain of the visna virus Gag polyprotein by the HIV-1 MA domain (4). The key role of the MA domain in Env incorporation is also apparent from the finding that alterations in HIV-1 MA which did not affect particle formation prevented the incorporation of autologous Env protein (4, 38) but not that of amphotropic murine leukemia virus (A-MLV), a widely divergent type C retrovirus (18, 34).

Recent reports indicate that these differences in the requirements for the incorporation of HIV-1 versus A-MLV Env are attributable to differences in the length of the cytoplasmic domain of the transmembrane glycoprotein (8, 18). The cytoplasmic tail of the HIV-1 transmembrane glycoprotein gp41 comprises about 150 amino acids, while the transmembrane glycoproteins of oncoretroviruses such as A-MLV have much shorter cytoplasmic tails of less than 50 amino acids. Remarkably, C-terminal truncation of the HIV-1 Env cytoplasmic tail restored not only the incorporation of the autologous Env glycoprotein complex into HIV-1 MA mutant particles but also virus replication (8, 18). These observations suggested that the Env glycoprotein complex of HIV-1 is excluded from heterologous retroviral particles because its long cytoplasmic tail cannot be accommodated. To test this hypothesis, we examined whether the efficiency of incorporation of HIV-1 Env protein into Mo-MLV particles can be increased through truncation of the cytoplasmic tail of gp41.

To obtain an expression construct for the Mo-MLV structural proteins, we generated NCA$_{gag}$, an *env*-deficient version of the previously described chimeric proviral construct HXBH10-NCA$_{gag}$ (11), which carries the Mo-MLV *gag* and protease coding regions in an HIV-1 background. NCA$_{gag}$ is identical to HXBH10/NCA$_{gag}$, except that the HIV-1 *env* initiation codon was replaced by ACG and a frameshift mutation was introduced at the *Kpn*I site in *env*. To provide wild-type HIV-1 Env in *trans*, we used the mutant HIV-1 proviral clone HXBH10-*gag*$^-$ (4), which is unable to express internal struc-

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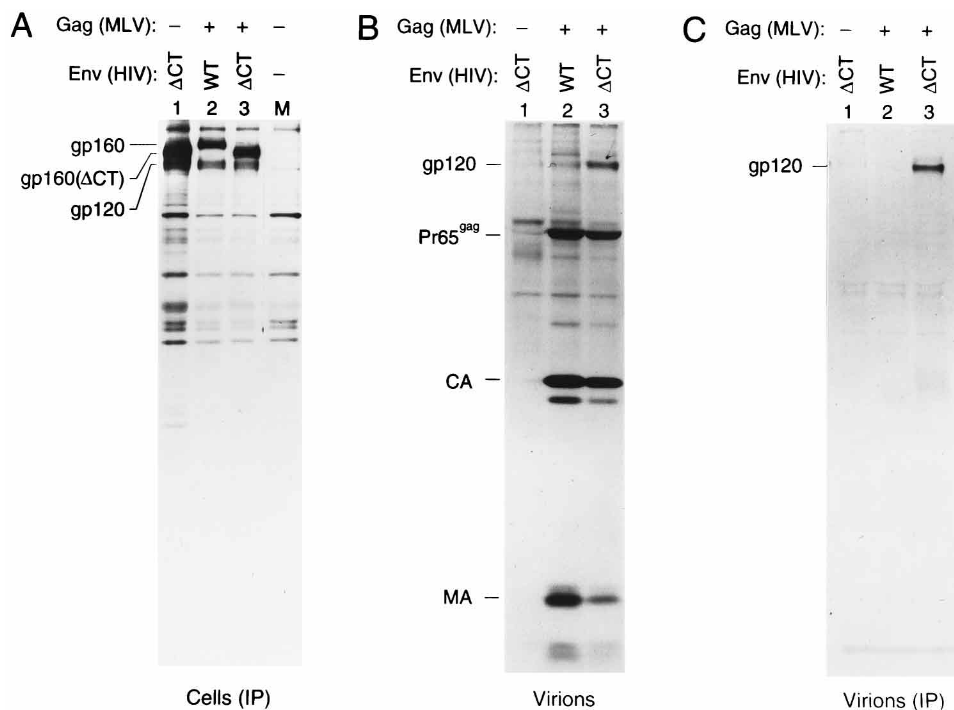

FIG. 1. Cytoplasmic tail truncation allows HIV-1 Env protein incorporation into Mo-MLV particles. HeLa cells were transfected with the mutant HIV-1 Env expression construct HXBH10-*gag*$^-$/ΔCT (lanes 1) or cotransfected with the Mo-MLV Gag expression construct NCA$_{gag}$ along with the wild-type (WT) HIV-1 Env expression construct HXBH10-*gag*$^-$ (lanes 2) or the mutant HIV-1 Env expression construct HXBH10-*gag*$^-$/ΔCT (lanes 3). The transfected cells were metabolically labeled with [^{35}S]methionine plus [^{35}S]cysteine and lysed, and HIV-1 Env proteins were immunoprecipitated from the cell lysates with patient serum (A). Viral particles released into the supernatant were pelleted through 20% sucrose cushions and disrupted in RIPA buffer. Aliquots were either analyzed directly by SDS-PAGE (B) or immunoprecipitated with serum from a patient infected with HIV-1 prior to SDS-PAGE (C). Lane M, mock transfection. IP, immunoprecipitation.

tural HIV-1 proteins. A modified version of HXBH10-*gag*$^-$ with a premature termination codon (TAA) in place of codon 713 of the *env* gene (HXBH10-*gag*$^-$/ΔCT) yielded an expression construct for C-terminally truncated HIV-1 Env. The HXBH10-*gag*$^-$/ΔCT construct was expected to produce an HIV-1 Env protein which lacks the 144 C-terminal amino acids of gp41 and retains a cytoplasmic tail of only 7 amino acids.

The Mo-MLV Gag expressor construct NCA$_{gag}$ was transfected into HeLa cells together with the wild-type HIV-1 Env expressor HXBH10-*gag*$^-$ or with the truncated HIV-1 Env expressor HXBH10-*gag*$^-$/ΔCT. To compare the levels of Env protein synthesis, the cultures were metabolically labeled with [^{35}S]methionine plus [^{35}S]cysteine from 48 to 60 h posttransfection. The cells were then lysed in RIPA buffer (140 mM NaCl, 8 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.05% sodium dodecyl sulfate [SDS]), and HIV-1-encoded proteins were immunoprecipitated from the cell lysates with serum from a patient infected with HIV-1. Although the intracellular steady-state levels of HIV-1 Env appeared somewhat reduced upon cotransfection of the NCA$_{gag}$ construct, perhaps due to promoter competition (Fig. 1A, lanes 1 and 3), the levels of the wild-type and truncated forms of the HIV-1 Env precursor were comparable (Fig. 1A, lanes 2 and 3). Also, the truncation of the cytoplasmic tail of gp41 did not affect cleavage of the Env precursor to yield the surface glycoprotein gp120 (Fig. 1A, lanes 2 and 3).

To examine whether HIV-1 Env proteins expressed in *trans* were incorporated into Mo-MLV particles, viral particles released during the labeling period were pelleted through 20% sucrose cushions (in phosphate-buffered saline) for 90 min at 4°C and 27,000 rpm in a Beckman SW41 rotor. Pelleted virions were lysed in RIPA buffer, and aliquots were analyzed directly by SDS-polyacrylamide gel electrophoresis (PAGE) and in parallel by immunoprecipitation with serum from an individual infected with HIV-1. As expected, the Mo-MLV Gag polyprotein Pr65^{gag} and mature Mo-MLV Gag proteins were detectable in the pelletable fraction upon transfection of NCA$_{gag}$ (Fig. 1B, lanes 2 and 3). It is noteworthy, however, that the levels of Mo-MLV particle production reproducibly were about three- to fivefold lower when the truncated rather than the full-length version of HIV-1 Env was coexpressed. Despite the lower particle yield, a protein with the expected mobility of gp120 was clearly more prominent in the particulate fraction when the truncated form of HIV-1 Env was coexpressed with Mo-MLV Gag (Fig. 1B, lane 3).

To verify the association of C-terminally truncated HIV-1 Env with Mo-MLV particles, HIV-1-encoded proteins were immunoprecipitated from the particulate fractions with serum from an HIV-1-infected patient. Neither the HIV-1 surface glycoprotein gp120 nor the unprocessed Env precursor was detected in the particulate fraction when truncated HIV-1 Env was expressed alone or when wild-type HIV-1 Env was coexpressed with Mo-MLV Gag (Fig. 1C, lanes 1 and 2). These results indicate that proteins with similar electrophoretic mobilities which were visible only after direct analysis of the pelleted material by SDS-PAGE (Fig. 1B, lanes 1 and 2) are unrelated to gp120 or to the Env precursor. In contrast, significant amounts of gp120 were immunoprecipitated from the particulate fraction when C-terminally truncated HIV-1 Env was coexpressed with Mo-MLV Gag (Fig. 1C, lane 3). In addition to gp120, a small amount of the truncated Env precursor was immunoprecipitated (Fig. 1C, lane 3). Thus, while wild-

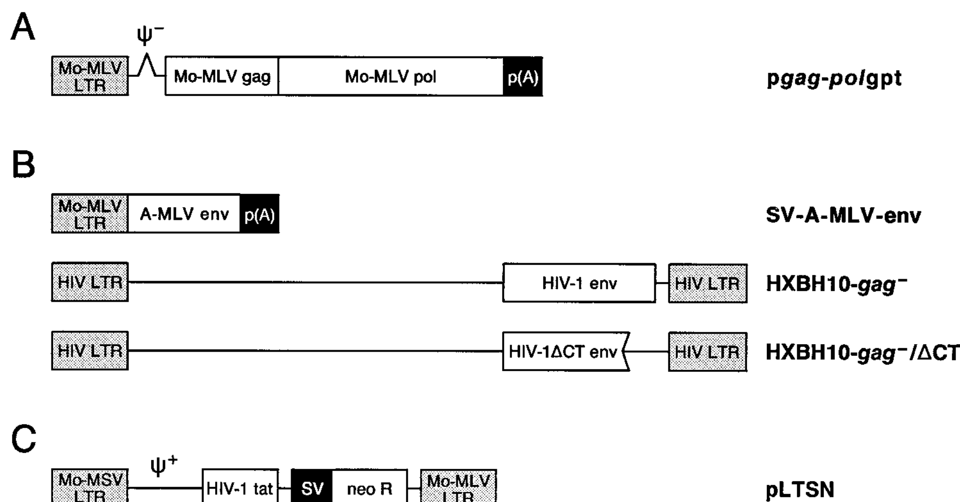

FIG. 2. Schematic representation of plasmids used to produce pseudotyped Mo-MLV-based vectors by transient transfection. (A) Mo-MLV Gag-Pol expression construct. (B) A-MLV and HIV-1 Env expression constructs. (C) Tat transfer vector. The Mo-MLV packaging signal (Ψ) is deleted in p*gag-pol*gpt but is present in pLTSN. In the HIV-1 proviral constructs HXBH10-*gag*$^-$ and HXBH10-*gag*$^-$/ΔCT, the solid line between the 5′ LTR and the *env* gene illustrates the absence of functional *gag*/*pol* reading frames. Shaded boxes indicate retroviral LTR sequences; open boxes indicate protein coding sequences; solid boxes indicate simian virus 40 sequences. Mo-MLV, Moloney murine sarcoma virus; p(A), simian virus 40 polyadenylation signal; SV, simian virus 40 promoter; neo R, neomycin phosphotransferase gene.

type HIV-1 Env protein was not incorporated at detectable levels, the removal of the cytoplasmic domain of gp41 allowed the efficient incorporation of HIV-1 Env protein into Mo-MLV particles.

To determine whether the truncated HIV-1 Env protein was incorporated into Mo-MLV particles in a functional manner, the infectivity of pseudotyped Mo-MLV particles was tested. Virus particles were produced by transient transfection of 293T human kidney cells with p*gag-pol*gpt (19), which encodes the Mo-MLV internal structural proteins and enzymes but lacks most of the *env* gene as well as the Ψ packaging signal (Fig. 2A). Env proteins were provided in *trans* by cotransfection of SV-A-MLV-env (23), HXBH10-*gag*$^-$, or HXBH10-*gag*$^-$/ΔCT, which encode, respectively, wild-type A-MLV Env, wild-type HIV-1 Env, or C-terminally truncated HIV-1 Env (Fig. 2B). In addition, a packageable transcript was provided by cotransfection of pLTSN (Fig. 2C), a derivative of the murine leukemia virus-based retroviral vector pLXSN (20) which harbors the HIV-1 *tat* gene under the transcriptional control of the Moloney murine sarcoma virus long terminal repeat (LTR) (26a). As target cells, we used a CD4$^+$ HeLa cell line which harbors a β-galactosidase gene under the transcriptional control of HIV-1 LTR sequences, including the Tat-responsive region. In this setting, infectious pseudotyped viral particles were expected to transduce the *tat* gene and thus induce the synthesis of β-galactosidase in the target cells (14).

Pseudotyped virions were generated by transfection of 293T cells with 10 μg of the Mo-MLV Gag-Pol expression construct p*gag-pol*gpt, along with 10 μg of the transducing vector pLTSN and 0.1 to 10 μg of the different Env expression constructs. Fresh medium was added to the cultures 12 h before the virus was collected. Culture supernatants were harvested 60 h post-transfection, and cellular debris was removed by low-speed centrifugation and passage through 0.45-μm-pore-size filters (Millipore). Reverse transcriptase activity in the filtered supernatants was determined as described previously (10) to control for virus particle yield. To assess the ability of the pseudotyped virions to transduce CD4$^+$ cells, serial dilutions of the filtered supernatants were applied to HeLa-CD4-LTR-β-Gal cells (14), which had been seeded into 24-well culture plates the day before infection at 3×10^4 cells per well. Polybrene (8 μg/ml) was added to the wells, and the cells were kept in a total volume of 0.5 ml. After 12 h at 37°C, 1.5 ml of medium was added to dilute the Polybrene; 36 h later, the cells were washed, fixed, and stained as previously described (14), and blue cells expressing Tat were counted with a light microscope.

Both the use of A-MLV Env and that of the C-terminally truncated HIV-1 Env resulted in the appearance of foci of stained target cells after incubation with the β-galactosidase substrate X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), indicating successful vector transduction. The A-MLV Env, which served as a positive control, yielded titers in the order of 10^5 transducing units/ml (Table 1). Titers were not markedly affected when the amount of A-MLV Env expression plasmid was reduced from 10 to 0.5 μg (Table 1), consistent with a previous report that homologous Env protein is efficiently incorporated into Mo-MLV particles even when expressed at relatively low levels (31).

Initial experiments revealed that the HIV-1 Env expressor constructs, in contrast to the A-MLV expression construct, had

TABLE 1. Infectivity of Mo-MLV particles pseudotyped with A-MLV or HIV-1 Env proteins

Expt no.	Transducing units/ml[a]			
	A-MLV Env	HIV-1 WT Env	HIV-1 ΔCT Env	HIV-1 Env$^-$
1	3.7×10^5 (10)	4 (1)	4×10^3 (0.5)	0 (1)
2	6×10^5 (10)	0 (0.1)	4.4×10^4 (0.1)	0 (0.1)
3	1.7×10^5 (10)	ND[b]	3.3×10^4 (0.1)	0 (0.1)
4	2×10^5 (5)	0 (0.1)	4.6×10^3 (0.1)	0 (0.1)
5	1×10^5 (0.5)	ND	ND	ND

[a] On HeLa-CD4-LTR-β-Gal indicator cells. Pseudotyped virions were produced in 293T cells by cotransfection of p*gag-pol*gpt (10 μg), pLTSN (10 μg), and the amounts (in micrograms) of SV-A-MLV-env (A-MLV Env), HXBH10-*gag*$^-$ (HIV-1 WT Env), HXBH10-*gag*$^-$/ΔCT (HIV-1 ΔCT Env), or HXBH10-*gag*$^-$/*env*$^-$ (HIV-1 Env$^-$) indicated in parentheses. WT, wild type.

[b] ND, not determined.

a negative effect on viral particle production when transfected along with the Mo-MLV Gag-Pol expressor p*gag-pol*gpt (data not shown). However, this effect, which was particularly pronounced with HXBH10-gag^-/ΔCT, could be avoided by reducing the amount of HIV Env expressor plasmid in cotransfection experiments to 0.5 μg or less. Titers obtained with 0.5 or 0.1 μg of the truncated HIV-1 Env expression construct HXBH10-gag^-/ΔCT ranged from 4.0×10^3 to 4.4×10^4 transducing units/ml (Table 1). In marked contrast, none or only very few stained target cells were observed when comparable amounts of the wild-type HIV-1 Env expression construct HXBH10-gag^- were used (Table 1). No stained cells were detected when HXBH10-gag^-/env^- (Table 1), an *env*-deficient version of HXBH10-gag^- which has the *env* initiation codon replaced by ACG and in addition harbors a frameshift mutation at the *Kpn*I site in *env*, was used.

It is well documented that cell culture-adapted isolates of HIV-1 can be efficiently neutralized with soluble CD4 (sCD4) (3, 7, 13, 28, 32). Recombinant sCD4 competitively inhibits the interaction between viral gp120 and cellular CD4 and at higher concentrations also induces dissociation of the gp41-gp120 complex on the virion surface (21, 22). To verify that the entry of Mo-MLV particles pseudotyped by truncated HIV-1 Env was dependent on the interaction between gp120 and CD4, pseudotypes were incubated with sCD4 prior to the infection of target cells. Pretreatment of Mo-MLV particles pseudotyped by truncated HIV-1 Env resulted in a complete loss of infectivity, despite a relatively high initial infectious titer of 4.4×10^4 transducing units/ml. Under identical conditions, sCD4 had no significant effect on the infectivity of particles pseudotyped by A-MLV Env, demonstrating the specificity of the inhibitory effect of sCD4 for pseudotypes bearing HIV-1 Env (data not shown). Polybrene was crucial for efficient vector transduction when A-MLV Env was used for pseudotyping, while this reagent had no apparent effect when the truncated HIV-1 Env protein was used (data not shown).

Our results provide evidence that the long cytoplasmic tail of the HIV-1 transmembrane glycoprotein is responsible for the poor incorporation of the HIV-1 Env protein complex into heterologous viral particles. While we did not detect incorporation of wild-type HIV-1 Env into Mo-MLV particles, a truncated form which lacks 144 C-terminal residues and retains a cytoplasmic tail of only 7 amino acids was readily incorporated. The efficient recruitment of the truncated but not of the full-length HIV-1 Env glycoprotein into Mo-MLV particles confirms that the cytoplasmic tail does not contain determinants which are crucial for incorporation. Rather, it supports the view that a bulky cytoplasmic tail causes the exclusion of HIV-1 Env due to steric hindrance unless an MA domain which can accommodate the tail is present.

It has been suggested that the HIV-1 Env cytoplasmic tail, while dispensable for Env incorporation into virions, is important at an early step in the virus life cycle between CD4 binding and formation of the DNA provirus (9). Our results argue against a central role of the cytoplasmic tail in virus entry, because a truncated HIV-1 Env which lacks 144 C-terminal amino acids could efficiently complement Mo-MLV particles in *trans* and support a single round of virus transmission. Interestingly, HIV-1 mutants which essentially lack the Env cytoplasmic tail retain the ability to replicate in MT4 cells but do not spread in other human $CD4^+$ cell lines (5, 35). The basis for this restricted host range in the absence of the Env cytoplasmic tail remains unclear. Since the removal of the 144 C-terminal amino acids of HIV-1 Env increases the membrane fusion capacity of the mutant glycoprotein (35), it seems possible that increased cytopathogenicity is at least in part responsible for the inability of the mutant to replicate in most cell types.

Our study demonstrates that complementation of a Mo-MLV-based vector by a C-terminally truncated HIV-1 Env allows gene transfer into $CD4^+$ cells. HIV-1-based vectors which specifically target cells susceptible to HIV-1 and which could in principle serve as gene transfer vehicles for intracellular immunization against HIV-1 have been described elsewhere (1, 2, 23–27, 37). However, for eventual use in humans vector systems which allow targeted transduction of $CD4^+$ cells but which are, at least in part, derived from nonhuman retroviruses would appear preferable. Additionally, the combination of structural components from evolutionarily divergent retroviruses, as well as the use of a mutant Env which does not support virus spread in most cell types, should reduce the likelihood that replication-competent virus can arise. Further experiments will be required to clarify the basis for the negative effect of the C-terminally truncated HIV-1 Env on Mo-MLV particle yield. Significant improvements in titer may be possible if this limiting factor can be overcome.

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