Generation of infectious virus particles by transient co-expression of human immunodeficiency virus type 1 gag mutants

Yu-Lin Chen, P’ei-Wen Ts’ai, Chia-Chien Yang and Chin-Tien Wang

Institute of Clinical Medicine, National Yang-Ming University, and Department of Medical Research and Education, Veterans General Hospital-Taipei, No. 201, Sec. 2, Shih-pai Road, Shih-pai, Taipei, Taiwan 11217, Republic of China

We have demonstrated that COS7 cells transiently co-expressing myristylation-defective (Myr) and protease-defective (PR) human immunodeficiency virus (HIV) mutants can release infectious virions when co-transfected with an amphotropic murine leukaemia virus envelope protein expression plasmid (SV-A-MLV-env). In contrast, no infectious virions were detected when a PR-, noninfectious HIV gag mutant was co-expressed with the Myr mutant, although the Myr mutant could still process the immature core particles in trans. This result indicates that generation of functionally normal Gag proteins is required for virus infectivity in our complementation system. A mutant with a 56-amino-acid deletion in the N-terminal region of the capsid (CA) domain could still complement the PR mutant to generate infectious virions, suggesting that the deletion mutant could provide a functional protease for processing in the PR mutant. This result is consistent with the concept that mutations within the N-terminal region of the CA domain have no major effects on Gag–Pol incorporation into particles.

The human immunodeficiency virus (HIV) gag gene encodes Gag proteins which self-assemble into virions in the late stages of the virus life cycle. The Gag protein is initially translated as a polyprotein precursor, Pr55\textsuperscript{gag}. The N terminus of Pr55\textsuperscript{gag} is co-translationally modified by myristic acid (Towler et al., 1987; Wilcox et al., 1987). Myristylation of HIV Gag proteins is required for membrane association and particle formation (Bryant & Ratner, 1990; Pal et al., 1990). During or after virus budding, Pr55\textsuperscript{gag} is proteolytically processed by a virus-encoded protease (PR) into p17 (matrix), p24 [capsid (CA)], p7 (nucleocapsid) and p6 (Leis et al., 1988; Mervis et al., 1988; Overton et al., 1989; Henderson et al., 1992). Proteolytic processing of Pr55\textsuperscript{gag} is essential for virus infectivity (Kohl et al., 1988; Gottlinger et al., 1989; Peng et al., 1989). The viral protease derives from the pol gene that is translated as a Pr160\textsuperscript{gag-pol} by −1 ribosomal frameshifting at a frequency of 5–10% (Jacks et al., 1988; Wilson et al., 1988). Dimerization of the retroviral PR during virion assembly is a prerequisite for activation of PR and subsequent proteolytic cleavage of Gag and Gag–Pol polyproteins (Le Grice et al., 1988; Burstein et al., 1991). The other cleavage products of HIV Pol are reverse transcriptase (RT), RNaseH and integrase enzymes, which are required for virus replication. Pr160\textsuperscript{gag-pol} is also myristylated at the N terminus and appears to be incorporated into the assembling virus via its N-terminal Gag determinants. Although the regions responsible for Gag–Pol assembly into virions have not been defined precisely, previous studies co-expressing Pr55\textsuperscript{gag} and Pr160\textsuperscript{gag-pol} from separate plasmids have shown that myristylation-defective (Myr\textsuperscript{−}) Pr160\textsuperscript{gag-pol} can be incorporated into immature Pr55\textsuperscript{gag} virus particles (Park & Morrow, 1992; Smith et al., 1993). Since Smith et al. (1993) have demonstrated that transient co-expression of non-myristylated Pr160\textsuperscript{gag-pol} and wild-type Pr55\textsuperscript{gag} could lead to generation of processed mature core particles, it is conceivable that co-expression of these two proteins with an additional envelope protein expression plasmid may result in production of infectious virions.

To test this possibility, we transiently co-transfected COS7 cells with a protease-defective but assembly-competent HIV mutant (PR\textsuperscript{−} or dl.NsiPst PR\textsuperscript{−}), a Myr\textsuperscript{−} HIV mutant and an amphotropic murine leukaemia virus envelope protein expression plasmid (SV-A-MLV-env); infectivity of the released virions was analysed. As described previously (Wang & Barklis, 1993) and shown in Fig. 1, the PR\textsuperscript{−} construct, which contains a linker insertion within the protease-coding region, inhibits proteolytic processing of virus particles. The Myr\textsuperscript{−} mutant lacks the myristylation signal and prevents virus assembly. The dl.NsiPst PR\textsuperscript{−} mutant was generated by introducing the protease mutation into a HIV gag mutant dl.NsiPst which contains a 56-amino-acid deletion mutation...
within the CA domain. Previous experiments have shown that the noninfectious dl.NsiPst mutant could still assemble and process particles; it also exhibited wild-type retrovirus particle density and possessed wild-type RT activity (Wang & Barklis, 1993). The backbone of our HIV-1 mutant constructs is HIVgpt (Page et al., 1990), a replication-defective, HIV proviral genome which contains the drug-resistant gene gpt (Mulligan & Berg, 1981) in the env region.

Since proteolytic cleavage of the Gag precursor is a prerequisite for virus infectivity, we first tested whether culture supernatants derived from cells co-expressing the PR− and Myr− mutants contained mature processed core particles. To do this, plasmid DNAs (15 μg) from the wild-type or PR− or Myr− mutants were transfected into COS7 cells by the calcium phosphate precipitation method. For co-transfection, 10 μg of each construct was used. At 48–72 h post-transfection, culture supernatants were collected, filtered through a 0.45-μm-pore-size filter, and centrifuged through 2 ml of 20% sucrose cushion in TSE (10 mM Tris–HCl, 100 mM NaCl, 1 mM EDTA, 0.1 mM PMSF) at 4 °C for 40 min at 274000 × g (SW41 rotor at 40000 r.p.m.). Virus pellets were suspended in IPB plus 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 0.02% sodium azide) plus 0.1 mM PMSF. Cells were rinsed with ice-cold PBS, collected in IPB plus 0.1 mM PMSF, and then subjected to microcentrifugation at 4 °C for 15 min at 13700 × g (14000 r.p.m.) to remove debris. Supernatant and cell samples were mixed with an equal volume of 2 × sample buffer (12.5 mM Tris–HCl pH 6.8, 2% SDS, 20% glycerol, 0.25% bromophenol blue) and 5% β-mercaptoethanol and boiled for 4–5 min. Samples were subjected to SDS–PAGE and then to Western immunoblotting. For immunodetection of HIV Gag proteins, a mouse anti-p24 monoclonal antibody diluted at 1:5000 was used as a primary antibody. The secondary antibody was a goat anti-mouse immunoglobulin–alkaline phosphatase conjugate. As shown in Fig. 2(a), the mature Gag protein p24 and incompletely processed products Pr55agg and p41agg were detected in the supernatant and cell samples of wild-type HIVgpt (Fig. 2a, lanes B and H). Processed Gag products of Myr− Pr55agg were observed in cell samples (Fig. 2a, lane I) but not in the supernatant (Fig. 2a, lane C). Expression of the PR− mutant released immature Pr55agg into the cell medium (Fig. 2a, lanes D and J). As expected, co-expression of PR− and Myr− mutants resulted in the appearance of mature core product p24agg in the medium supernatants (Fig. 2a, lane E), suggesting that nonmyristylated Gag–Pol proteins can be assembled into PR− virus particles and provide a functional protease. However, it is possible that the detected p24agg may derive from the intracellularly processed Gag proteins which were incorporated into assembling PR− particles as the PR− mutant budded from the cell membrane. To test this possibility, we performed another complementation experiment. COS7 cells were either transfected with wild-type, dl.NsiPst, dl.NsiPst PR− or Myr−, or co-transfected with dl.NsiPst PR− plus Myr−. Western immunoblotting analysis showed that cells transfected with dl.NsiPst alone released unprocessed and processed mutant Gag proteins, revealing a band at 49 kDa and bands at 34–36 kDa and 16–20 kDa (Fig. 2b, lanes B and M), which is consistent with a deletion of 56 amino acids. Expression of dl.NsiPst PR− in COS7 cells released unprocessed Pr49agg into the medium (Fig. 2b, lanes D and K). Cells co-transfected with both the dl.NsiPst PR− and Myr− mutants released both unprocessed Pr49agg and the processed Gag products p16 to p20 (Fig. 2b, lanes C and L). However, p24agg was only detected inside cells transfected either with Myr− alone (Fig. 2b, lanes E and J) or with Myr− plus dl.NsiPst PR− (Fig. 2b, lane L) and not seen in the medium supernatants of co-transfectants (Fig. 2b, lane C). In contrast, Gag proteins p16 to p20 derived from proteolytic processing of Pr49agg were observed in the co-transfectant supernatant sample (Fig. 2b, lane C) but not seen in the cell sample (Fig. 2b, lane L)

These results support our hypothesis that the p24 proteins observed in the co-transfectant culture media are not derived from intracellularly processed Gag proteins but most likely result from proteolytic processing of Pr55agg by the incorporated nonmyristylated Gag–Pol which provides a functional protease in trans during particle assembly and budding.

![Fig. 1. HIV gag mutant constructs. As described previously (Wang & Barklis, 1993), the Myr− mutant, generated from a mutant provided by L. Ratner (Bryant & Ratner, 1990), contains a second codon glycine → alanine mutation which blocks particle assembly and release; PR− is a prorotease mutant with a linker insertion at Bcl-2-429; dl.NsiPst is a capsid mutation with a deletion from Nsi-1251 to Pst-1418. The dl.NsiPst PR− is a double mutant construct generated from recombination of dl.NsiPst and PR−. All HIV mutants were expressed in the HIVgpt backbone which was provided by D. Littman (Page et al., 1990).](image_url)
Infectivity of co-expressed HIV gag mutants

Fig. 2. Expression and release of wild-type and mutant HIV Gag proteins. COS7 cells were either transfected or co-transfected with the designated constructs. At 72 h post-transfection, supernatants and cells were collected and prepared for protein analysis as described in the text. Supernatant samples corresponding to 50% of the total samples and cell samples corresponding to 5% of the total cell were fractionated by SDS-PAGE and electroblotted onto a nitrocellulose filter. HIV Gag proteins were detected with a mouse anti-p24 monoclonal antibody, followed by a secondary alkaline phosphatase-conjugated goat anti-mouse antibody and alkaline phosphatase activity was determined. (a) Molecular size markers are indicated on the right. Lanes A and G, mock; lanes B and H, wild-type; lanes C and I, Myr\(^-\); lanes D and J, PR\(^-\); lanes E and K, PR\(^-\) + Myr\(^-\). (b) Molecular size markers (lanes G and H) are indicated in the middle and HIV Gag proteins Pr\(55^\alpha\), p\(41^\alpha\) and p\(24^\alpha\) are shown on the right. Lanes A and N, mock; lanes B and M, dl.NsiPst; lanes C and L, dl.NsiPst PR\(^-\) + Myr\(^-\); lanes D and K, dl.NsiPst; lanes E and J, Myr\(^-\); lanes F and I, wild-type.

Previous studies have reported that 90% of virus-associated Gag proteins can be recovered when centrifuged through a 2 ml 20% sucrose cushion at 4 °C for 40 min at 274 000 × g (Wang & Barklis, 1993). We believed that our Gag proteins recovered from supernatants should be virus-associated. We performed a sucrose density-gradient fractionation experiment to analyse whether the p\(24^\alpha\) released from cells co-expressing PR\(^-\) and Myr\(^-\) was associated with retrovirus particles. Supernatants from COS7 cells co-transfected with PR\(^-\) and Myr\(^-\) were centrifuged through linear sucrose gradients (20–60%). Fractions were collected, measured for density and analysed for Gag proteins by Western immunoblotting. Peak p\(24^\alpha\) fractions banded at densities of 1±14–1±18 g/ml, which is consistent with wild-type retrovirus particle density (data not shown).

Since co-expression of PR\(^-\) and Myr\(^-\) in COS7 cells could produce virions with wild-type retrovirus particle density, we then tested the infectivity of virus particles released from the co-transfectants. To do so, mutant HIV\(gpt\) constructs were co-transfected into COS7 cells with SV-A-MLV-env (Page et al., 1990). At 48–72 h post-transfection, virus-containing supernatants were collected, filtered and used to infect HeLa cells. Adsorption of virions was allowed to proceed at 37 °C in the presence of 4 µg/ml polybrene. Selection of mycophenolic-acid-resistant colonies followed the protocol as described previously (Wang & Barklis, 1993). Colonies were fixed and stained with 50% methanol plus 0±5% methylene blue. Numbers of drug-resistant colonies were converted into titres (c.f.u./ml). Infectivity was determined by the ratio of the mutant titre to the titre of wild-type HIV\(gpt\) in parallel
reported that the HIV PR RNA packaging in our system; however, a previous study infective (Table 1) despite assembly of the Myr−Pr160 have no major effects on the incorporation of the mutant consistent with a previous study suggesting that deletion 1993). Although the amount of incorporated Myr−proteins are functionally involved in the post-assembly and studies of HIV−produce infectious virions. In support of this concept, previous is necessary in our complementation experiments in order to generation of functionally equivalent normal Gag proteins and provide a functional protease in trans.‡ This result supports the notion that the myristylation signal is not absolutely required for HIV Gag−Pol incorporation into virus particles. The fact that the dl.NsiPst can complement PR−particles with a functional protease in trans. This result supports the notion that the myristylation signal is not absolutely required for Gag−Pol incorporation into PR−particles. One recent study has shown that several domains within HIV pol are involved in particle processing and maturation (Quillent et al., 1996). Our system of complementation experiments to generate infectious virions may provide a tool to investigate the potential effects of mutant Gag−Pol on the process of virus maturation and infectivity.

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## References


### Table 1. Infectivity of HIV gag mutants

<table>
<thead>
<tr>
<th>Construct*</th>
<th>Titre (c.f.u./ml)</th>
<th>HIVgpt titre (c.f.u./ml)†</th>
<th>Infectivity (%)‡</th>
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* Each construct was co-transfected with SV−A−MLV−env into COS7 cells. At 48−72 h after transfection, supernatants were used to infect HeLa cells. Infection and selection of drug-resistant colonies were performed as described in the text.
† HIVgpt titres were determined in parallel experiments. Duplicate experiments were performed with different DNAs and/or for different times.
‡ Infectivities for each mutant were determined by the ratio of its titre versus the wild-type HIVgpt titre in a parallel experiment.
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