Role of the C terminus Gag protein in human immunodeficiency virus type 1 virion assembly and maturation

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Lentiviral Gag polyproteins have a proline-rich protein, p6, at their C terminus. There are conflicting reports about the function of p6 in virus release. In the present work, mutants that affect p6 of human immunodeficiency virus type 1 (HIV-1) Gag polyprotein were constructed and analysed. None of the mutants prevented virus release completely; however, detachment of budding particles was less efficient as evidenced by electron microscopy. Virions of the p6 truncation mutant B2TAA had a significantly reduced number of Pol proteins (p66, p51 and p34) and an increased amount of incompletely processed Gag proteins compared with the parental virus. A mutation that altered the cleavage site between p6 and p1 did not significantly affect virus assembly, virus release or protein processing with the exception of cleavage between p6 and p1. However, virions of this mutant (B2P6C) exhibited irregular-shaped core structures that were distinct from the cone-shaped core structure seen in the parental virion. B2P6C mutant virus was non-infectious in CD4+ T cells. These results suggest that mutations in p6 affect efficient detachment of budding particles from the cell surface. Proper cleavage between p6 and p1 may be critical for the formation of the distinctive cone-shaped core structure of HIV-1 virions.

Retrovirus Gag polyproteins are essential for virus assembly. Various domains of the Gag polyprotein are associated with distinct functions of virus assembly and have been reviewed extensively (Coffin, 1990; Hunter, 1994; Wills & Craven, 1991). The Gag protein of human immunodeficiency virus type 1 (HIV-1) is first synthesized as a polypeptide of 55 kDa, then subsequently cleaved by viral protease to yield proteins MApl7, CAp24, p2, NCp7, p1 and p6, which can be detected in mature virions (Henderson et al., 1992).

The proline-rich protein (p6) is located at the C terminus of HIV-1 Gag polyprotein. This protein is separated from the NC protein by a spacer peptide, p1 (Henderson et al., 1992). Both p6 and p1 can be detected in mature virions, although their location in virions is unclear. An earlier report suggested that p6 plays an important role in virus release (Göttlinger et al., 1991). Truncation or point mutations in p6 of HIV-1 prevented virus release from transfected COS-7 cells. However, this conclusion was challenged by subsequent studies (Hoshikawa et al., 1991; Jowett et al., 1992; Paxton et al., 1993; Royer et al., 1991).

We analysed the effect of mutations at the C terminus of HIV-1 Gag polyprotein (including p6 and p1) on virus budding, release and maturation. Mutants of p6 were constructed to truncate the protein, to prevent cleavage and to analyse selected prolines that had not been previously analysed in this proline-rich molecule (Fig. 1a). B2TAA contains a premature stop codon (TAA) which replaces the codon (CAG) for the second amino acid (glutamine) of p6. B2P6C contains a mutation at the cleavage site between p6 and p1. The phenylalanine at the P1' position of the cleavage site between p6 and p1 was changed to serine and the leucine at the P1 position was changed to arginine (Fig. 1a). B2PRO contains an arginine and a glutamine substituted for the prolines at positions 5 and 7 of p6, respectively.

Virus assembly and release were initially studied in transfected COS-7 cells by monitoring virion-associated reverse transcriptase (RT) activity and by immunoblot analysis of virion proteins from cell supernatants. Equal amounts of parental (HXB2) or mutant plasmid DNA were transfected into COS-7 cells by the DEAE-dextran method. Transfection efficiency was comparable for the

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Fig. 1. Construction, virus production and infectivity of p6 mutants. (a) Mutant proviral DNA plasmids were constructed from HXB2 by site-directed mutagenesis. Top: schematic diagram of mature HIV-1 Gag products. Middle: amino acid sequence of p1 and the N-terminal region of p6 for the parental virus HXB2. The cleavage site between p1 and p6 is indicated by an arrow. (b) Virus production monitored by virion-associated RT activity. At 72 h post-transfection, virions were pelleted from the supernatants of transfected COS-7 cells. Results are representative of five independent experiments. RT activity is expressed as c.p.m./ml. (c) Virus infectivity assay. Cell-free parental and mutant viruses were prepared from the supernatants of transfected COS-7 cells. Virus infectivity was tested in Sup T1 cells as previously described (Yu et al., 1992). RT values represent samples from 1 ml culture supernatants.

Parental and mutant constructs as monitored by gp160 and gp120 expression in transfected cells (data not shown). Seventy-two hours post-transfection, culture supernatants were used for the RT assay. Comparable virion-associated RT activity was detected in parental and B2P6C-transfected cells (Fig. 1 b), suggesting that this mutant virus assembled and released as efficiently as the parental virus. B2PRO had slightly less RT activity than the parental virus (Fig. 1 b). In repeated experiments, the RT activity of B2PRO was about 50–80 % of that of the parental virus, and approximately 10-fold less than that of the parental virus for B2TAA-transfected cells (Fig. 1 b).

Infectivity of mutant viruses was evaluated. Cell-free viruses from transfected COS-7 cells were standardized by virion-associated RT activity and tested for infectivity in CD4+ SupT1 cells. B2TAA and B2P6C viruses were not infectious compared with the parental virus. No virus production was detected for the 20 day follow-up period (Fig. 1 c). B2PRO mutant virus remained infectious in SupT1 cells compared with the parental virus. Mutant virus replication was delayed by approximately 2-3 days (Fig. 1 c). B2PRO viruses produced from infected SupT1 cells were apparently not revertant as these viruses maintained the phenotype of delayed replication in SupT1 cells (data not shown).

Virus assembly and release were also studied by immunoblot as previously described (Yu et al., 1992). Virions were collected from equal amounts of transfected COS-7 cell supernatants 72 h post-transfection. Similar amounts of Gag (p24 and p17) and Pol (p66 and p51) proteins were detected in parental virus and B2P6C virus
Fig. 2. Detection of viral proteins from the supernatants of transfected COS-7 cells. (a) Ten ml of supernatant from $5 \times 10^6$ transfected COS-7 cells was used for Western blot analysis using HIV-1 positive sera. Cells were transfected with HXB2 (lanes 2 and 7), B2P6C (lanes 3 and 8), B2PRO (lanes 4 and 9) and B2TAA (lanes 5 and 10), or were mock transfected (lanes 1 and 6). Samples were transferred onto two nitrocellulose filters and blotted with either HIV-1 positive sera (lanes 1–5) or goat anti-p6 serum (lanes 6–10). (b) Transfected COS-7 cells were metabolically labelled with $[^{35}]$S-cysteine from 60–72 h post-transfection. Virus pellets were immunoprecipitated with HIV-1 positive sera (lanes 1–4) or goat anti-p7 serum (lanes 5–8). Cells were transfected with HXB2 (lanes 1 and 5), B2P6C (lanes 2 and 6), B2PRO (lanes 3 and 7) and B2TAA (lanes 4 and 8).

(Fig. 2a), which is consistent with the RT assay. Slightly fewer Gag (p24 and p17) and Pol (p66 and p51) proteins were observed in the B2PRO mutant (Fig. 2a), which is also in agreement with the RT assay. Significant amounts of p24 and p17 were detected in B2TAA mutant virus (Fig. 2a). Also, significantly more Gag precursor p55 (truncated, indicated by an arrow in Fig. 2a) and Gag intermediate p41 were detected in B2TAA mutant virus.
than in the parental virus (Fig. 2a). However, the amount of Pol proteins p66 and p51 was significantly less in B2TAA mutant virus than in the parental virus (Fig. 2a). These results suggest that B2TAA mutant viruses were assembled and released from transfected COS-7 viral proteins in released parental and mutant virions. Previous studies, we next examined the processing of parental viruses suggests that the cleavage between p7 and p24 was apparently not affected in B2P6C virus as comparable p17 and p24 were detected in parental and B2P6C mutant viruses (Fig. 2a). Detection of similar amounts of p7 in B2P6C mutant and parental viruses suggests that the cleavage between p7 and p1 was also not affected in this mutant (Fig. 2b). Two p6-related proteins were detected in B2P6C mutant viruses, whereas only one p6-related protein was detected in the parental virus (Fig. 2a). The slower migrating p6-related protein in B2P6C virions is probably the uncleaved p1 plus p6 as it is not recognized by the anti-p7 antiserum (Fig. 2b). The faster migrating p6-related protein may be the product of cleaved p6 at the mutated site or at a cryptic site (Fig. 2a). This result suggests that cleavage between p6 and p1 was affected by the mutation in B2P6C.

Gag protein processing was not significantly affected in mutant B2PRO virus compared with that in the parental virus (Fig. 2), with the exception that a Gag intermediate of p41 was detected in the mutant virus (Fig. 2b). The slightly faster migration of p6 of B2PRO compared with that of the parental virus is probably due to the amino acid substitutions in p6 (Fig. 2a). However, Gag protein processing was significantly affected in the B2TAA mutant virus. A significant amount of mutant Gag precursor p55 (truncated in size and indicated by an arrow in Fig. 2) and intermediates of Gag p41 were detected in B2TAA mutant virus as compared with the parental virus (Fig. 2). As expected, no p6 was detected in the mutant virions (Fig. 2a). A significant amount of p25 could also be detected in B2TAA mutant virus but not in the parental virus (Fig. 2a), suggestive of a defect in cleavage between p24 and p2.

Morphogenesis of mutant viruses was also studied. Sixty hours post-transfection, COS-7 cells were fixed for transmission electron microscopy. Mature virions with cone-shaped core structures were detected in parental provirus-transfected cells (Fig. 3a–c). Few budding particles could be detected in HXB2-transfected cells (data not shown). In B2TAA-transfected cells, many budding structures at various stages were detected (Fig. 3f–h). Some of the released particles were still attached to each other (Fig. 3g, arrowhead). Most of the particles had immature morphology. Detachment of the budding particles from the cell surface appeared to be affected for B2TAA.

Detachment of released particles of B2PRO mutant virus also appears to be affected (Fig. 3i–k), although compared to B2TAA the defect was not as severe. Budding particles attached to the cell surface were frequently detected and mature virions with core structures could still be detected (data not shown). Mutant virus was still infectious in certain T cells (Fig. 1c).

The defects in virus release described for B2TAA and B2PRO were not detected in B2P6C-transfected cells. Most of the virions were released from the cell surface and underwent some degree of maturation (Fig. 3d, e). However, the core structures of the mutant viruses were heterogeneous in size and shape. Most of the core structures were irregular in shape with some electron-dense material in the centre or on the side.

There have been conflicting reports regarding the role of HIV-1 p6 Gag protein in virus release (Göttlinger et al., 1991; Hoshikawa et al., 1991; Jowett et al., 1992; Paxton et al., 1993; Royer et al., 1991). Truncation or point mutations in the p6 region of an infectious molecular clone of HIV-1 prevented virus release from transfected COS-7 cells (Göttlinger et al., 1991). However, truncated Gag polyproteins that lacked p6 assembled and released efficiently when expressed in mammalian cells using the vaccinia virus vector (Hoshikawa et al., 1991) or in insect cells using baculovirus vectors (Jowett et al., 1992; Royer et al., 1991). Although the different systems used to study the effect of Gag truncations on virus release may account for the apparent discrepancies, studies using the same target cells and similar constructs also yield different results (Paxton et al., 1993). In transfected COS cells, p6 truncated Gag polyproteins still assemble into particles and release efficiently, although the incorporation of Vpr protein into released virions is blocked (Lu et al., 1993; Paxton et al., 1993).

In this study, we observed that the p6 truncation mutant of an infectious molecular HIV-1 clone, B2TAA, released less efficiently than the parental virus. Electron microscopic analysis revealed that the detachment step of virus budding may be a defect of p6 truncation (Fig. 3). However, the release of mutant virions was not completely abolished in COS cells (Figs 1 and 2), which is consistent with a previous study using a similar system (Paxton et al., 1993). The p6 truncation mutant significantly altered the ratio of mature Pol proteins to Gag proteins and the processing of mutant Gag polyprotein in the released virions (Fig. 2). Reduced processing of Gag proteins and a reduced level of Gag–Pol proteins in
Fig. 3. For legend see page 3177.
budding particles have been associated with a reduction in the release of virions from the cell surface (Kaplan et al., 1994; Park et al., 1993).

It is not clear whether the mutation in B2TAA affected the efficiency of ribosomal frame-shifting or the ability of mutant Gag protein to interact with Gag–Pol protein. Either defect would reduce the incorporation of Gag–Pol into virions and result in an accumulation of Gag precursors and intermediates. The mutations introduced into B2TAA were clearly downstream from the stem-loop structure, which is essential for frame-shifting (Jacks et al., 1988; Wilson et al., 1988). However, the
Fig. 3. Transmission electron microscopy of parental (a–c), B2P6C (d, e), B2TAA (f–h) and B2PRO (i–k)-transfected COS-7 cells. Bars represent 200 nm. Wild-type virions with typical bar-shaped cores are indicated by an arrowhead.
possibility that these mutations change the RNA structure that is required for efficient frame-shifting in vivo can not be ruled out. The mutations introduced into B2TAA may also change the structure of the mutant Gag polyprotein and therefore interfere with Gag and Gag-Pol protein-protein interactions. Evidence that truncation of p6 from HIV-1 Gag polyprotein may change the conformation of the molecule have been previously reported (Royer et al., 1991). A truncated Gag protein reacted differently to a monoclonal antibody to p24 than did the full-length Gag protein (Royer et al., 1991). Alternatively, frame-shifting of the Gag-Pol polyprotein may be normal, but truncation of p6 may alter the viral Gag polyprotein such that both Gag and Gag-Pol processing are reduced in the released virions. Further study will be required to distinguish these possibilities.

Since truncation of p6 or interference with p1–p6 processing appears to have multiple detrimental effects, the role of p6 in virus release was also studied using amino acid substitution mutants. The p6 protein of HIV-1 contains several highly conserved prolines. The prolines at positions 5 and 7 of p6 were mutated in B2PRO (Fig. 1c). These mutant virions had no significant defect on the ratio of Gag to Gag-Pol, which was seen in the B2TAA mutant (Fig. 2a). As observed by electron microscopy, the detachment step of virus budding was less efficient in B2PRO mutant-transfected COS-7 cells (Fig. 3). However, the release of mutant virions was not abolished in COS cells (Figs 1 and 2). Particles released from B2PRO-transfected COS-7 cells remain infectious in CD4+ SupT1 cells (Fig. 1c).

The organization of lentiviral Gag proteins is unique among retroviruses. Downstream from the NC protein, lentiviruses have a proline-rich protein which is not generally present in other retroviruses. The only exception is the Mason–Pfizer monkey virus, which also has a protein downstream from the NC protein (Gelderblom, 1991). Coincidentally, lentiviruses and Mason–Pfizer monkey virus have unique cone- or bar-shaped core structures (Gelderblom, 1991). The relationship between the additional C-terminal Gag proteins in lentiviruses and type D retroviruses and the formation of the cone-shaped core structure is not clear. p6 of HIV-1 is separated from the NC protein by a spacer peptide, p1. It is not clear at what stage of virus assembly or maturation that cleavage between p6 and p1 occurs. Mutation that prevent efficient cleavage between p6 and p1 (B2P6G) apparently abolish the formation of cone-shaped core structures in mutant virions (Fig. 3). Such mutations had no detectable effect on upstream cleavage of the Gag polyprotein, such as for p17 and p24, p24 and p2, or p2 and p7 (Fig. 2). This result suggests that p6, p1 or both may be important for the initiation, formation or maintenance of the cone-shaped core structure. The narrow end of the HIV-1 core structure was found to be linked to the viral membrane (Hoglund et al., 1992) to form the core envelope link (CEL). p6 was hypothesized to be involved in the formation of CEL. Such a location for p6 would be consistent with a role for p6 in detachment of budding particles from the cell surface and the formation of cone-shaped core structures. The precise function of the unique C terminus region of HIV-1 Gag protein in virus assembly, release and maturation requires further investigation.

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References


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