Distinct signals in human immunodeficiency virus type 1 Pr55 necessary for RNA binding and particle formation

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The human immunodeficiency virus type 1 (HIV-1) gag gene product Pr55 self-assembles to form virus-like particles when expressed in Spodoptera frugiperda cells using recombinant baculoviruses. The particles resemble immature HIV and are released from the infected cell into the culture medium. Using this system we have progressively truncated the gag open reading frame from the C terminus and examined each deleted gag protein for its particle-producing capability. We show that deletion of Pr6 and deletions that progressively remove the distal region of the Pr7 domain, including one Cys–His box thought to function as an RNA capture signal, do not affect particle formation. However deletion of two Cys–His boxes causes production of slightly larger particles with altered sedimentation properties. Sequence-specific North-Western assays using an RNA probe representative of the HIV-1 packaging signal revealed specific RNA binding by all mutants that maintained both Cys–His boxes. However, deletion of one Cys–His box reduced RNA binding substantially and loss of two Cys–His boxes abolished binding entirely. We conclude that HIV-1 gag particle formation per se does not require viral RNA encapsidation, but that it may act as a cofactor in the condensation of the immature core. Further deletion of gag sequences upstream of the Cys–His boxes led to the abolition of particle-forming ability, and we show that one boundary of the gag sequence necessary for particle formation lies within eight amino acids spanning one of the known protease cleavage sites at the C terminus of Pr24.

Introduction

The human immunodeficiency virus (HIV) group-specific antigen (gag) gene product represents the major component of the virus particle. It is first synthesized as a precursor protein of 55K (Pr55) and forms the structural basis of the retrovirus core. During or following virus release, Pr55 is cleaved by the viral protease to yield three gag-related components, Pr17 (also known as MA protein), Pr24 (also known as CA) and Pr15 (also known as NC protein) (for reviews see Kieny, 1990; Wills & Craven, 1991). The Pr15 molecule undergoes further cleavage to Pr7 (sometimes known as Pr9) and Pr6 (Veronese et al., 1987, 1988), and low frequency internal initiation within the Pr55 open reading frame can result in the production of a Pr41gag product of unknown significance (Mervis et al., 1988). At a rate of about 5%, ribosomes translating the gag mRNA undergo a frameshift event near the Pr7/Pr6 junction, resulting in the deletion of the Pr6 domain and the production of a much larger (Pr160) gag–pol fusion protein which is also packaged into forming virions (Jacks et al., 1988). In this way, the enzymatic functions necessary for virus infectivity (the protease, reverse transcriptase, RNase H and integrase) are incorporated into the budding particle. When expressed alone in a number of eukaryotic expression systems, Pr55 produces virus-like particles very similar to the early budding immature particles seen in HIV-infected cells (Gheysen et al., 1989; Karacostas et al., 1989; Overton et al., 1989; Hu et al., 1990; Luo et al., 1990; Shioda & Shibuta, 1990; Smith et al., 1990; Haffar et al., 1991; Hoshikawa et al., 1991; Royer et al., 1991; Mergener et al., 1992). The ability of similar core antigens from other retroviruses to self-assemble into particle structures is now well documented (Delchambre et al., 1989; Rasmussen et al., 1990; Morikawa et al., 1991).

Pr55 is normally a myristylated protein and when myristylation occurs, particles bud from the cell surface. Lack of myristylation (by mutagenesis of the N terminus of Pr55) does not affect particle formation, but does prevent particle budding and release (Gheysen et al., 1989; Overton et al., 1989).
In contrast to Pr55, the expression of permanently frameshifted gag-pol fusion protein does not allow particle formation (Shioda & Shibuta, 1990; Mergener et al., 1992), a result which is in keeping with earlier work using other retroviruses (Felsenstein & Goff, 1988). This might suggest a role for Pr6 in particle formation or release because gag-pol fusions lack the gag Pr6 domain. Indeed, truncations of the Pr6 domain within HIV proviral clones have been reported to allow particle formation but to prevent virion release from infected cells (Gottlinger et al., 1991). However, equivalent deletions in a vaccinia virus expression system producing solely gag antigen allow particle formation and release (Hoshikawa et al., 1991). However, in this case, there is the possibility that vaccinia virus itself contributes a function that can substitute for the missing Pr6 domain.

Pr55 is a multifunctional protein and, in addition to driving particle formation and the co-incorporation of gag-pol fusion proteins, it is also responsible for the capture and incorporation into the core of the viral genomic RNA. This function resides in the gag Pr7 domain and is associated with two Cys-His motifs thought to function as the RNA capture signal. Point mutations within these motifs reduce RNA incorporation and virus infectivity (Clavel & Orenstein, 1990; Gorelick et al., 1990). Expression of gag proteins lacking the Pr7 domain prevents particle formation (Gheysen et al., 1989; Hoshikawa et al., 1991) suggesting that, in addition to RNA binding, the essential signals for gag-gag interaction also lie within, or very close to, Pr7. This result has been taken to suggest a link between RNA binding and particle formation (Gelderblom, 1991). Intriguingly, mutations in HIV genomic RNA that lead to poor genome incorporation also result in particles with altered morphology (Clavel & Orenstein, 1990) and, in other systems, a direct role for Cys-His boxes in subunit interaction as well as nucleic acid-binding has been demonstrated (Loeber et al., 1991). Moreover, from a virus point of view, a link between successful RNA capture and particle formation could be beneficial, helping to minimize the formation of empty virus particles.

To localize the sequences necessary for particle formation in finer detail, and to investigate the association with RNA binding, we have expressed a detailed series of gag truncation mutants and assessed their ability to form and release particles, and to bind HIV RNA.

Methods

DNA manipulation. Routine manipulations of DNA, plasmid preparation, restriction digests and subcloning were all as described (Sambrook et al., 1989). Gag gene truncations Pr45, Pr44 and Pr42 were all prepared by site-directed mutagenesis as described by Kunkel (1985) using the following oligonucleotides: for Pr45, 5'- GCCTGCTC-CTAAGTACATCT 3'; for Pr44, 5'- CACAACCCCATTTTCCA- GGG 3'; for Pr42, 5'- TGAAACACAAACATCTTT 3'. Genes encoding truncations Pr41, 5 and Pr41 were made by the polymerase chain reaction (PCR) using the forward primer 5'- CCGGGAGCTCA-GAGATGTTGCGAGACTGC' and reverse primers as follows: for Pr41.5, 5'- CCGTCTAGACTATGTATTTGTTTACTGCT- CAT3' and for Pr41, 5'- CCGTCTAGACTACAAATCTGGC- TTATGCC 3'. Amplified products were eluted from a gel, cleaved with SacI and XbaI, and cloned into the baculovirus transfer vector pAcCL29.1 (Livingstone & Jones, 1989). All mutants and PCR products were sequenced prior to their use for expression. Selection of recombinant baculoviruses was done using linearized baculovirus DNA as described (Kitts et al., 1991).

Cells and viruses. Spodoptera frugiperda (Sf9) cells were propagated at 28°C in TC-100 medium (Overton et al., 1989) containing 10% foetal calf serum. AcNPV-Bgal, AcPAK6 (for transfections) and recombinant viruses were grown and assayed in confluent monolayers of Sf9 cells as described (Summers & Smith, 1987).

RNA binding. RNA binding by each gag mutant was assayed by North-Western blotting under the conditions described by Luban & Goff (1991). The probe was prepared by in vitro transcription of an HIV fragment spanning nucleotides 673 (a SacI site) to 840 (an XmnI site) of HIV-1LAI. Non-specific probe was transcribed from the negative strand.

Electron microscopy. For negative staining, Sf9 cells infected with one of the truncated gag mutants were harvested 2 days post-infection, washed once in PBS and fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2 for at least 24 h. Fixed cells were embedded in 1% low melting point agarose, and the agarose blocks were briefly fixed in glutaraldehyde and washed in cacodylate buffer before being treated with 1% osmium tetroxide (in cacodylate buffer) for 2 h and 0.5% uranyl acetate for a further 3 h. After dehydration in ethanol, cells were embedded via propylene oxide in Araldite. Sections were cut with a diamond knife on a Reichert-Jung Ultratrac E ultramicrotome, post-stained with uranyl acetate and lead citrate, and examined with a Philips CM12 electron microscope operating at 80 kV.

Results

Expression of truncated gag antigens

The initial series of gag deletion mutants used in this study is shown in Fig. 1. For the first construct (Pr46) the SacI-BglII fragment spanning most of the gag ORF of HIV-1LAI was cloned into the baculovirus expression vector pAcCL29.1 (Livingstone & Jones, 1989). This fragment encodes the gag precursor protein up to and including the frameshift signal, but the protein is deleted downstream of amino acid 437 (the Pr6 domain). Three further mutations truncating the upstream Pr7 domain were prepared in the same expression vector by site-directed mutagenesis, introducing a stop codon (TAG) at the position shown. These three mutations resulted in the deletion of the gag ORF downstream of (i) amino acid 427 (just downstream of the Cys-His boxes), (ii) amino
acid 410 (between the two Cys–His boxes) and (iii) amino acid 390 (just before the Cys–His boxes). Based on the sequence changes made, these mutants were predicted to encode proteins of Mr 45K, 44K and 42K and were named accordingly (Fig. 1). All mutations were confirmed by sequence analysis before their co-transfection into cells with viral DNA to yield recombinant baculoviruses (Kitts et al., 1991).

Recombinant viruses producing gag protein were screened for and identified by Western blotting with a mix of monoclonal antibodies (MAbs) reactive with HIV gag Pr17 and Pr24 antigens. Plaque-purified mutant viruses from each transfection were then used to infect a culture of SF9 cells at a multiplicity of 10, and the cultures were harvested 2 days post-infection and examined by SDS–PAGE and Western blotting (Fig. 2). Cell lysates from each truncation mutant produced stainable quantities of a new protein (a) at a mobility corresponding to the predicted Mr of the gag protein. Each gag protein was also stained specifically with anti-gag MAbs (b). Wild-type virus-infected (lane 6) or mock-infected cells (lane 7) showed no evidence of any cross-reactive material. Wild-type Pr55 protein (lane 1) exhibited some breakdown, as described previously (Gheysen et al., 1989), but the truncation mutants showed much less breakdown suggesting that the majority of cellular proteases cleave from the C terminus of gag.

Particle formation and release
To assess the ability of each of our truncated gag mutants to produce HIV-like particles, SF9 cells were infected with virus at high multiplicity and the supernatants were harvested 2 days post-infection before appreciable cell lysis had occurred. After clarification, supernatants were applied to a sucrose density gradient as described (Gheysen et al., 1989) and the gradient was fractionated and analysed by Western blotting using anti-gag MAbs (Fig. 3). We found that truncation mutants Pr46 (a), Pr45

![Fig. 1. Construction of gag truncation mutants. The position of each mutant made is shown in relation to the 3'-terminal end of the gag ORF. The SacI-BglII fragment of HIV-1 was cloned directly into a baculovirus transfer vector to produce Pr46 which encodes a protein deleted downstream of amino acid 437. Site-directed mutation introduced a stop codon at codons 427, 410 and 390, producing constructs Pr45, Pr44 and Pr41, respectively. The position of the Cys–His boxes in relation to the mutations is shown.](image)

![Fig. 2. Expression of gag truncations and reactivity with anti-gag MAbs. Recombinant viruses were grown to high titre and used to infect SF9 cells at a multiplicity of 10. Two days post-infection cells were washed, lysed and fractionated on 10% SDS–polyacrylamide gels. Gels were either stained directly with Coomassie blue (a) or transferred to nitrocellulose and incubated with gag-specific MAbs (b). Lane 1, Pr55; 2, Pr46; 3, Pr45; 4, Pr44; 5, Pr42; 6, wild-type baculovirus-infected; 7, mock-infected. M, s were taken from pre-stained standards run at the same time.](image)

![Fig. 3. Sucrose gradient analysis of gag particles. Supernatants from cultures infected at high multiplicity were harvested 2 days post-infection and layered onto preformed gradients of 20% to 60% sucrose in PBS. The gradients were centrifuged at 36000 r.p.m. for 100 min and fractionated from the top. Aliquots of each fraction were electrophoresed on 10% SDS–polyacrylamide gels and Western blotted with anti-Pr24 MAb. p46, p45, p44 and p42. (a) to (d) respectively.](image)
Fig. 4. Electron micrographs of truncated gag mutant-infected Sf9 cells. Cells were processed as described 2 days after infection with Pr46 (a), Pr45 (b), Pr44 (c) or Pr42 (d). Surface vacuole formation was observed only with Pr42 and is marked V. Free Pr42 particles are shown (FP). The bar marker represents 100 nm.

(b) and Pr44 (c) all produced gag-containing particles that migrated to a position similar to Pr55 (45% sucrose). Truncation mutant Pr42 (d) also produced core-like particles, but they sedimented in 25 to 30% sucrose as compared to 45% sucrose for wild-type.

Electron microscopy

Deletion of gag C-terminal sequences was clearly not incompatible with expression and secretion of gag protein that could be banded by velocity gradient centrifugation (Fig. 3). However, the exact macromolecular state of this protein could not be deduced by gradient analysis alone. Accordingly, we examined the culture supernatant by negative staining and also prepared thin sections through infected Sf9 cells for direct visualization of the particle budding process. Typical virus-like particles were found in all samples (Fig. 4). Particle morphology and budding for Pr55 was similar to that described earlier and that Pr46 (a), Pr45 (b) and Pr44 (c) was similar.

Generally particle formation occurred at the plasma membrane, producing roughly spherical particles about 120 nm in diameter. There was some evidence of intracytoplasmic gag 'ring' structures typical of the structures formed in the absence of gag myristylation (Gheysen et
al., 1989; Overton et al., 1989; Gelderblom, 1991), but the number of these structures was small. However, Pr42 in addition to normal budding at the cell surface, showed some budding into intracellular and cell surface vacuoles (marked V in Fig. 4d) as well as from the plasma membrane. Particles formed within vacuoles had essentially normal morphology but showed a greater range of size than the surface particles (average approx. 130 nm). The increase in particle diameter associated with Pr42 may partly explain the altered banding properties of this mutant in velocity gradients (Fig. 3d).

We conclude from the data presented in Fig. 3 and 4 that, within the baculovirus expression system, deletion of the gag Pr6 domain and a large proportion of the Pr7 domain including the two Cys–His boxes does not prevent gag particle assembly or release.

A link with RNA binding?

Although particle assembly was clearly independent of the presence of the Cys–His domain of gag, a link with RNA binding could not be ruled out. For example, in some retroviruses specific RNA binding has been shown to be associated with the matrix domain of the gag protein (Katoh & Yoshinaka, 1990), which is present in all our deletion mutants. Therefore we examined the ability of each of our truncated gag proteins to capture RNA representative of the HIV genome. The exact extent of the cis-acting sequences (the psi site) necessary for the efficient incorporation of genomic RNA into HIV particles is not yet clear. The region of the molecule from the 3' end of the 5' long terminal repeat to the beginning of the gag ORF is clearly necessary, but has not yet been shown to be sufficient for efficient packaging (Lever et al., 1989; Clavel & Orenstein, 1990).

To assess the truncated gag proteins for RNA binding we used North-Western blotting with a probe encompassing the supposed psi site and some of the 5' end of gag. This system has been shown recently to give specific RNA binding to Pr55 (Luban & Goff, 1991). The protein profile observed on the blot was similar to that shown in Fig. 2(a).

We found efficient RNA binding associated with Pr55 and with each of the Pr55 breakdown products (Fig. 5, lane 1) suggested earlier to lack C-terminal sequences (Fig. 2 and discussion thereof). Consistent with this we also observed RNA binding with Pr46 and Pr45 (Fig. 5, lanes 2 and 3). However, we observed substantially reduced RNA binding associated with Pr44 (one Cys–His box; lane 4) and none with Pr42 (no Cys–His boxes; lane 5). Background binding to a set of cellular proteins can be discerned in wild-type baculovirus- and mock-infected cells (lanes 6 and 7), but was poor compared to gag-related activity and did not obscure any of the truncated gag bands. One breakdown product of Pr55 with an $M_\text{r}$ of approximately 20K also bound probe but was absent from the gag truncation mutants, suggesting it may represent a Pr15-containing fragment. North-Western blotting with a non-specific RNA probe gave only background binding (data not shown). This result confirms that Cys–His boxes are essential for RNA capture, although it does not rule out the involvement of other gag sequences. It is also clear that particle formation, demonstrated for each truncation mutant, is not linked to the ability to bind RNA, as represented by the probe used for our blotting experiments. Although we cannot wholly rule out co- incorporation of non-specific RNA fragments into the developing particle, the fact...
Fig. 7. Electron micrographs of Si9 cells infected with Pr41.5 (a) and Pr41 (b). The micrographs shown are typical of many sections examined. Gross cell surface distortion is apparent in both infections, but no particles are apparent in either. The bar marker represents 200 nm.

that non-specific probes do not bind to gag protein suggests that this is unlikely.

**Endpoint mapping of sequences involved in particle formation**

Previous work using baculovirus (Gheysen et al., 1989) and vaccinia virus expression systems (Hoshikawa et al., 1991) has suggested that truncation of the HIV gag-coding sequence at the distal end of the Pr24 domain abolishes particle formation. However, one recent report has claimed that deletions in this area still allow particles to be formed, although the evidence presented in favour of this was scant (Royer et al., 1991). The HIV protease can cleave at two distinct sites at the end of the Pr24 domain, VLAE (amino acids 362 to 365) and IMMQ (amino acids 376 to 379) (see Fig. 6). This can lead to some confusion over the exact C terminus of Pr24. We found that one of the two reports detailing particle abolition had truncated the gag coding sequence from the upstream site whereas the report detailing continued particle formation had deleted sequences C-terminal to the alternative downstream site. Thus, the 3' limit of gag sequences necessary for particle formation might lie between these two sites, both of which are upstream of the gag construct, Pr42 (Fig. 1), with the largest deletion which still forms particles. To confirm the boundary for particle formation, we constructed two more gag truncation mutants (Pr41 and Pr41.5; Fig. 6). The first is deleted from the upper of the two Pr24 C-terminal protease cleavage sites (amino acid 363) and the second from amino acid 372, located between the two possible cleavage sites. The level of expression of each mutant was similar to that of the constructs already described, but no antigen was found in the tissue culture superna-

tant (data not shown). When analysed by electron microscopy, neither mutant showed evidence of particle formation (Fig. 7). Electron-dense material typical of gag protein was found just beneath the plasma membrane of infected cells. The cells had abundant large surface vacuoles, with the dense protein layer found between the vacuole and the cell surface membranes, and these electron-dense layers labelled specifically with an anti-gag MAb and gold conjugate (not shown). Similar structures have been observed in earlier studies (Gheysen et al., 1989). Based on these results and those of Royer et al. (1991), we deduce that the C-terminal boundary for the sequences involved in HIV gag particle formation lies between amino acids 372 and 379, and spans the known downstream protease cleavage site at the end of the Pr24 domain.

**Discussion**

The ability of gag gene products to self-assemble following expression has been established for a number of retroviruses, bovine (Rasmussen et al., 1990), feline (Morikawa et al., 1991), human (Gheysen et al., 1989; Karacostas et al., 1989; Overton et al., 1989; Hu et al., 1990; Luo et al., 1990; Shioda & Shibuta, 1990; Smith et al., 1990; Haffar et al., 1991; Hoshikawa et al., 1991; Royer et al., 1991; Mergener et al., 1992) and simian immunodeficiency viruses (Delchambre et al., 1989). However, the boundaries of the gag sequences necessary for particle formation have not been systematically determined. Gag-pol fusion proteins expressed in similar systems have failed to yield particles and, as the frameshift event producing the gag-pol fusion protein also removes the Pr6 domain of gag, a role for the Pr6
domain in particle formation was feasible. However, we have shown that deletion of Pr6 leads to the production of gag particles that are indistinguishable from those produced by Pr55. Therefore, the failure of gag-pol fusion proteins to assemble is most likely due to steric effects imposed by the presence of the large pol domain. These results suggest that incorporation of the HIV gag-pol precursor into forming virions is gag-driven as shown for other retrovirus systems (Felsenstein & Goff, 1988).

Gottlinger et al. (1991) have shown that deletion of the Pr6 domain within HIV proviral clones results in HIV particle formation but suppression of particle release from transfected COS-7 cells. They suggested that lack of Pr6 might prevent the final stages of particle assembly which, in turn, leads to prevention of particle release. From our own findings and those recently published (Hoshikawa et al., 1991; Royer et al., 1991) we suggest that particle assembly and release are separable events. Lack of Pr6 evidently does not affect particle assembly per se, but does appear to prevent particle release when all other HIV-encoded proteins are present. During the expression of gag products only, and irrespective of the expression system used (recombinant baculoviruses, this work and Royer et al., 1991; recombinant vaccinia viruses, Hoshikawa et al., 1991), gag proteins lacking the Pr6 domain are efficiently released from infected cells. This finding argues that as yet undefined HIV-encoded products may be actively involved in HIV budding from permissive cells once formation of the membrane-associated protein shell is complete, as suggested for other retroviruses (Luftig et al., 1990). Alternatively, vaccinia virus or baculoviruses could provide the functions necessary for gag particle release in the absence of the Pr6 domain.

We also investigated the role of the Pr7 domain in gag particle assembly. Pr7 is undoubtedly the nucleocapsid protein of HIV as mutations in the Cys-His arrays within this domain severely depress the incorporation of genomic RNA into forming virions (Gorelick et al., 1990). Gheysen et al. (1989) originally showed that complete deletion of Pr7 leads to loss of particle assembly, a result recently confirmed by Hoshikawa et al. (1991). Thus, in addition to the RNA-binding activity, the C-terminal boundary of the region enabling gag particle formation is also within the Pr7 domain. To determine the endpoint of such sequences and to investigate a possible link with RNA binding, we produced a series of truncations through Pr7 up to and including the endpoint originally described by Gheysen et al. (1989). Deletion of both Cys-His motifs in these constructs led to a complete loss of specific RNA binding, with loss of the downstream motif (construct Pr44) causing a 70% reduction in binding. These results are consistent with mutation studies that disrupt one or other of the Cys-His motifs (Gorelick et al., 1990; Luban & Goff, 1991). Particle formation was apparent despite removal of one (Pr44) or two (Pr42) Cys-His arrays, indicating that the signals necessary for particle formation do not include either motif. However, it is worth noting that Pr42 demonstrated some cell surface vacuolation in addition to typical particle formation, suggesting that a proportion of the expressed gag protein in this construct fails to assemble successfully into particles. Pr42 particles are marginally larger (about 10%) on average than the particles produced by constructs that retain RNA-binding capability. Therefore, it is possible that RNA incorporation acts as a condensing agent helping the gag core to adopt a tighter conformation. Absence of RNA might also contribute to the altered sedimentation of Pr42 particles in sucrose gradients.

As Pr42 retains particle formation, the downstream sequence essential for particle assembly lies N-terminal to amino acid 390. Royer et al. (1991) have recently proposed that deletion of gag sequences downstream of amino acid 379 (their mutant AcNPVgag14myr) does not affect particle morphogenesis. However, the electron micrograph of cells infected with this truncation mutant shows no free particles when compared to those of the other gag truncations described, but does show evidence of plasma membrane vacuolation, similar to our Pr42 mutant. These data suggest that both mutants encroach upon the signals necessary for particle formation, although they do not delete them entirely.

Two further truncations (Pr41 and Pr41.5) N-terminal to Pr42 and AcNPVgag14myr fail to show any evidence of particle assembly, producing only cell surface vacuoles with associated layers of protein. This result confirms and extends the observations of Gheysen et al. (1989) to locate the downstream boundary for particle formation to between amino acids 372 (our mutant Pr41.5) and 379 (AcNPVgag14myr; Royer et al., 1991). The identification of this region as overlapping the downstream cleavage site for the HIV protease at the Pr24/Pr7 junction is fitting as activation of the protease at this site during HIV budding but prior to release would result in the abolition of particle formation. Further deletion analysis of Pr55 should allow the definition of the minimum sequences required for particle assembly, as has been reported for other retroviruses (reviewed in Wills & Craven, 1991).

Gag-only particles of the type described have a number of uses as 'particle carriers', for example of the HIV env protein, to produce non-replicating vaccine candidates (Haffar et al., 1991). We suggest that if this technology comes into widespread use for the production of recombinant vaccines, the use of constructs similar to Pr42 be encouraged as they would prevent the possible transduction of RNA to recipient cells.
I M J thanks Y M for many things. This work was supported by the UK Medical Research Council's AIDS Directed Programme.

References


(Received 24 June 1992; Accepted 4 August 1992)