Comparative morphology of Gag protein structures produced by mutants of the gag gene of human immunodeficiency virus type 1

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Six mutants that differ in the extent of their carboxy-terminal sequences and two deletion mutants of the gag gene of HIV-1 have been characterized morphologically following their expression in Spodoptera frugiperda cells using recombinant baculoviruses. Electron microscopy has revealed distinct morphological forms of the Gag protein that can be classified as either (i) particulate, three-dimensional, spherical or tubular shells or (ii) non-particulate, two-dimensional, flat, curved or convoluted sheets. Progressive truncation of the carboxy terminus of Gag was accompanied by changes in the morphology and formation of spherical particles from predominantly C-type assembly and budding at the plasma membrane, through B-type intracytoplasmic assembly, to A-type assembly with budding mainly into cytoplasmic vacuoles. Deletions within the Pr24 CA domain of Gag abolished particle formation but retained association of the protein with the plasma membrane. All of the observed morphologies of the mutant Gag proteins could be accommodated within an icosahedral model for the organization of spherical particles and a basic hexagonal arrangement of assembled Gag protein monomers.

Introduction

The gag gene of human immunodeficiency virus (HIV) encodes the major structural proteins of the virus particle; it is translated as an M r 55K precursor protein (Gag) which is cleaved by the viral protease into the 17K matrix (MA), 24K capsid (CA) and 15K nucleocapsid (NC) domains. The NC domain is further cleaved to produce p7 and p6 (Wills & Craven, 1991). When the gag gene is expressed in eukaryotic expression systems in the absence of the viral protease, virus-like 'Gag particles' are produced which are analogous to the early, uncleaved, budding stages of HIV (Gheysen et al., 1989; Karacostas et al., 1989; Overton et al., 1989). Expression of truncation mutants of the HIV gag gene has delineated a number of 'signals' within Gag that are involved in the assembly and budding processes. At the amino (N) terminus of Gag, myristylation of the N-terminal glycine in association with a distinct MA-encoded sequence is required for transport of the protein to the host cell plasma membrane (Gheysen et al., 1989; Göttlinger et al., 1989; Overton et al., 1989; Royer et al., 1991; Yuan et al., 1993). Deletion of the entire Pr15 NC domain from the carboxy (C) terminus of the Gag precursor molecule has no effect on membrane targeting but abolishes particle formation (Gheysen et al., 1989; Hoshikawa et al., 1991) whereas shorter deletions within the NC domain have been reported variously to affect particle assembly and release. Göttlinger et al. (1991) have reported that deletion of Pr6 prevented the release of particles from the host cell membrane. In the majority of similar studies, however, deletion of the Pr6 domain has not prevented particle release (Hoshikawa et al., 1991; Royer et al., 1991; Jowett et al., 1992) which indicates a possible co-factor-dependent role for the Pr6 domain in the final stages of budding. The Gag Pr7 domain is required for the capture and incorporation of RNA into the budding particle (Gorelick et al., 1990; Clavel & Orenstein, 1990) but deletion of much of Pr7 does not prevent particle formation (Royer et al., 1991; Jowett et al., 1992). At least one key boundary in the sequences required for particle assembly has been mapped to the junction of the Pr7 and Pr24 domains (Royer et al., 1991; Jowett et al., 1992). Less is known about signals within the Pr24 CA and Pr17 MA domains that are required for Gag/Gag interaction and particle assembly. The majority of scanning deletions within MA have shown little effect on particle assembly (Yu et al., 1992a, b; Luban et al., 1993; Wang & Barklis, 1993; Fäcke et al., 1993) whereas in the CA domain, point
mutations and deletions that affect particle formation have been described (Trono et al., 1989; Luban et al., 1993; Wang & Barklis, 1993; von Poblotzki et al., 1993; Zhao et al., 1994). All mutants that prevent particle formation and release represent stages of the assembly and budding process beyond which the altered Gag molecules cannot progress, thus analysis of Gag mutants by electron microscopy (EM) can provide morphological information on the nature of the Gag interactions that lead to assembly. We show in this study that all the observed forms of Gag protein can be accommodated within a recently proposed model for the structure of the Gag particle (Nermut et al., 1994) and support the hypothesis that HIV is an icosahedral particle with hexagonally arranged Gag monomers.

Methods

Cells and viruses. The baculovirus expression vector system and Spodoptera frugiperda (SF9) cells were used for the production of recombinant Gag proteins. Cells and viruses were propagated as previously described (Jowett et al., 1992; Overton et al., 1989; Summers & Smith, 1987). Several of the gag gene truncations were identical to those described by Jowett et al. (1992) and thus the same methods of DNA manipulation and the same designations for the mutants and their products were used, namely Pr55, Pr46, Pr45, Pr44, Pr42, Pr41.5 and Pr41. Additional gag gene deletions named D1 and D2 were as described by Zhao et al. (1993). The terminal amino acid number of each mutant and its position in relation to the carboxy terminus of the gag open reading frame is shown in Fig. 1. SF9 cells infected with the recombinant baculoviruses together with normal uninfected SF9 cells and cells infected with wild-type baculovirus (Autographa californica nuclear polyhedrosis virus) and non-Gag (CD4 and herpes virus) recombinants were prepared for electron microscopy at 2 days post-infection, when protein expression was high and cell structure was still intact.

Electron microscopy. Cells for scanning electron microscopy were attached to poly-L-lysine treated coverslips and fixed in 2.5% glutaraldehyde in cacodylate buffer as described by Hockley et al. (1988). The specimens were processed in a continuous-flow apparatus using a sequence of 1% osmium tetroxide, 0.5% tannic acid and 1% osmium tetroxide, with intermediate washes, followed by rapid dehydration in ethanol (Hockley et al., 1988). The specimens were dried by the critical point method using carbon dioxide and then sputter coated with a 5 nm layer of gold/palladium. Specimens were examined with a Philips scanning electron microscope 501B operating at 30 kV.

Cells for conventional Araldite embedding and sectioning were fixed, prepared and examined as described by Hockley et al. (1988) and Jowett et al. (1992). Measurements were made from micrographs (negatives) of sectioned specimens using a video camera, computer and Global Lab Image software (Data Translation, Wokingham). Only particles with a distinct membrane were measured. In order to make valid size comparisons the micrographs used for measurements were all made within a few days and at the same magnification with the specimens accurately placed at the eucentric height. The magnification of the microscope was calibrated with a diffraction grating replica.

Cells for immunolabelling studies were fixed in 3% formaldehyde in PBS which had been freshly prepared from paraformaldehyde. Fixation continued for 1 h at room temperature followed by 18 h at 4 °C in the above fixative with the addition of 0.5 M sucrose. The cell pellet was spread thinly onto 5 mm square pieces of Whatman No. 1 filter paper, placed in a Reichert MM80E slammer (Leica) and impact frozen on a polished copper block cooled with liquid nitrogen (Grief et al., 1994). Frozen specimens were substituted with methanol containing 0.5% uranyl acetate. Freeze substitution was continued for 36 h at −90 °C and the specimens were finally embedded in Lowicryl HM20 resin at −45 °C (Grief et al., 1994). Sections on gold grids were incubated in anti-Pr55/Pr46Gag (HIV-1) monoclonal antibody (MRC AIDS Directed Programme Reagent No. 313) and goat anti-mouse antibodies conjugated to 5 nm colloidal gold (Biocell Research Laboratories) with appropriate blocking and washing (Nermut & Nicol, 1989).

Results

Sections of normal uninfected SF9 cells and cells infected with wild-type baculovirus or with non-Gag recombinants displayed a typical morphology as described by Van der Wilk et al. (1987), Matsuura et al. (1987) and Carstens et al. (1992). No structures resembling budding or immature HIV were seen.

Pr55Gag and Pr46Gag

Scanning electron microscopy (SEM) of cells infected with recombinant baculovirus containing the complete gag gene (Pr55) typically showed the surface of the cells to be covered with spherical Gag particles (Fig. 2a, b and c) and confirmed other reported observations of sectioned Gag particles. Sections of cells infected with recombinant virus containing the complete gag gene or the Pr46gag truncation mutant both showed identical budding Gag particles and images of all the well characterized stages of the budding process were observed (e.g. Gheysen et al., 1989). Typical free particles were circular in section, approximately 120 nm in diameter and contained a peripheral electron-dense layer of Gag protein 12 to 15 nm in thickness (Fig. 2d). The
innermost part of the protein layer was often the most electron-dense region (Fig. 2d) but no consistent substructure was recognized in the protein layer. The centre of the particles consisted of material similar in appearance to the cytoplasm of the cells (Fig. 2d) and ribosomes were occasionally seen inside Gag particles.

**Pr45Gag and Pr44Gag**

SEM of cells infected with these truncation mutants revealed that the majority of cells displayed both spherical particles and filamentous or tubular projections on their surface (Fig. 3a, b and c). The filaments were up to 3 μm in length and uniform in diameter (Fig. 3b). The spherical particles were similar to the particles on Pr55gag- and Pr46gag-infected cells and larger in diameter than the filaments. Dimensions from sections are given in Table 1. The filaments exhibited two distinct forms of morphology in that they either stood up from the cell surface or lay flat on the cell (Fig. 3a, b and c); this was clearly evident in side views (Fig. 3a) and stereomicrographs (not shown). Most of the filaments appeared to be tubular in form but some had the appearance of ridges or folds on the surface of the cells (Fig. 3b). Flattened filaments were most often straight but with occasional sharp angular changes of direction (Fig. 3c).

The nature and size (Table 1) of the Pr45Gag and Pr44Gag particles and filaments were investigated further in sectioned cells. The tubular filaments were approximately 68 nm in diameter and the spherical particles present on the same cells were approximately 105 nm in diameter (Fig. 3d). The dense protein layer in both spherical and tubular Pr45Gag and Pr44Gag particles...
Fig. 3. Cells infected with Pr45gag and Pr44gag recombinant baculoviruses. (a to c) Scanning electron micrographs showing Pr44Gag cells covered with filamentous Gag particles. (a and b) The filaments are mostly flattened on the cell surface but some stand erect. (b) Spherical particles are present amongst the filaments and some filaments have the appearance of ridges or folds (arrows) on the cell surface. (c) Flattened filaments are typically straight but show some sharp, angular (arrows) changes in direction. (d to f) Sectioned Pr45Gag cells. (d) Filamentous particles have an outer tubular layer of Gag protein and contain cytoplasmic material. Spherical particles (arrow) have a greater diameter than the tubular filaments. (e) Flattened filaments have an incomplete tubular layer of Gag protein and are attached to the cell where the protein is absent. (f) Some filaments are attached by a long narrow fold of the host cell plasma membrane. Bar markers represent 1 μm (a to c) and 100 nm (d to f).
Table 1. Comparative sizes of Gag particles (nm)

<table>
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<tr>
<th>Gag particles</th>
<th>Average diameter</th>
<th>Standard deviation</th>
<th>Maximum diameter</th>
<th>Minimum diameter</th>
<th>Number of particles</th>
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<td>Pr55 spherical particles</td>
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<td>160.0</td>
<td>105.0</td>
<td>62</td>
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<td>Pr45 &amp; Pr44 tubular filaments</td>
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<tr>
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<td>6.6</td>
<td>128.0</td>
<td>91.0</td>
<td>100</td>
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<tr>
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<td>17.0</td>
<td>190.0</td>
<td>103.0</td>
<td>75</td>
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Fig. 4. Sectioned Pr45gag- and Pr44gag-infected cells. (a) Pre-formed Gag shells ‘budding’ into cytoplasmic vacuole in Pr45Gag-infected cell, filamentous particles are present on cell surface. (b) Spiral Gag shells associated with the plasma membrane of Pr45Gag cell. (c) Assembly of Gag shells in cytoplasm, compare with Fig. 8 (c to e). (d) Section of Pr44Gag cell immunolabelled with anti-Pr55/24 monoclonal antibody, spherical and filamentous particles are labelled; unlabelled parts of filament are totally within the section and thus not exposed to antibodies. Bar markers represent 100 nm.

was approximately 15 nm in thickness, the same dimension as in Pr55 particles: both types of particles contained cytoplasmic material (Fig. 3d). Sections also showed what appeared to be budding and free particles which were only 68 nm in diameter, the same size as the filaments (Fig. 3e). The budding ‘mini-particles’ were attached to the cell by a stalk which could be up to 250 nm in length (Fig. 3f). From the evidence of SEM, which showed only spherical particles that were larger than the filaments, the mini-buds and 68 nm diameter particles were interpreted as transverse sections of tubular filaments. Since many of these transverse sections were attached to the cell by a thin stalk it would appear that the flattened filamentous structures seen by SEM
were attached to the host cell along much of their length; such structures could also appear as ridges or folds by SEM.

Ring-like profiles, identical in morphology to the Gag protein in budding spherical particles, were seen in the cytoplasm of cells which exhibited abundant filamentous budding (Fig. 4a). These structures were presumably spherical or at least nearly so since, in many sections, the protein did not form a complete ring but instead formed a circular profile part of which was formed from two overlapped layers (Fig. 4b and c). These pre-formed 'Gag shells' were only occasionally observed in association with the plasma membrane (Fig. 4b) but were seen more often in association with cytoplasmic vacuoles (Fig. 4a) and some free particles were formed by this route.

Immunolabelling studies on sections of cells infected with the Pr44gag truncation mutant showed labelling with anti-Pr55/24 monoclonal antibody of all spherical and filamentous particles (Fig. 4d) and of intracellular Gag protein shells.

**Pr42Gag**

The majority of cells infected with the Pr42gag mutant displayed a form and arrangement of the Gag protein which was not seen in sections of the other specimens. A small number of spherical Gag particles were associated with the cells but there was no indication of typical budding and the particles were free and either detached on the surface of the cell or enclosed in large intracytoplasmic vacuoles (Fig. 5a). The large vacuoles were often closely applied to the plasma membrane and caused a protrusion at the cell surface (Fig. 5a). Some vacuoles were open to the exterior of the cell (Fig. 5b) so that Gag particles were released and were free on the surface of the cell. The cell and vacuole membranes were in many instances separated only by an electron-dense protein layer 15 to 20 nm in thickness (Fig. 5c). The Pr42Gag particles present in the vacuoles were approximately 130 nm in diameter (Table 1) with a protein layer about 15 nm in thickness; thus the dimensions were not significantly different to those of Pr55Gag particles. The Pr4 particles differed, however, in that typically they were empty (Fig. 5b) and did not contain the cytoplasmic material which was seen in Pr55 particles. The Pr42Gag particles have been shown not to bind RNA (Jowett et al., 1992) and it is interesting to speculate that cytoplasmic material is incorporated into Gag particles via the non-specific capture of RNA. Pre-formed Gag shells were numerous in the cytoplasm of some cells and entry of Gag shells into small vacuoles deep in the cytoplasm was also observed. There was morphological evidence (not shown) that the large surface vacuoles full of Gag particles arose by migration and fusion of small vacuoles with each other and then with the plasma membrane.

Some of the Gag particles either free on the cell surface or within surface vacuoles were unusual in that they had both an inner and an outer membrane with, again, an empty central area (Fig. 5d). These particles were often slightly larger than the 120 nm diameter Pr55Gag particles and the protein layer trapped between the two membranes was 15 to 20 nm in thickness. The formation of these double-membraned particles was clearly related to the process whereby a layer of protein was trapped between the large, particle-containing vacuoles and the plasma membrane. A similar layer of protein trapped by a small vacuole would result in a double-membraned particle if the small vacuole protruded and became cut off from the cell (e.g. as in Fig. 5e).

Approximately one in ten cell profiles from the sectioned pellet of Pr42 cells showed normal surface budding of 120 nm diameter particles which was consistent with the earlier report of Pr42 being the most severe deletion that retained particle competence (Jowett et al., 1992). Pre-formed cytoplasmic Gag shells were seen very rarely in cells which exhibited normal surface budding. No filamentous particles were observed on any of the Pr42Gag cells. Immunolabelling studies of the Pr42 cells with anti-Pr55/24 monoclonal antibody showed specific labelling of all forms of Gag particles as well as trapped layers of protein associated with surface vacuoles (Fig. 5f).

**Pr41.5Gag and Pr41Gag**

No spherical or tubular Gag particles or filaments were produced by these mutants but two morphological forms of the protein were detected in both specimens. The most usual arrangement of the protein was as a layer 15 to 20 nm in thickness trapped between the plasma membrane and the cytoplasmic surface of sub-membrane vacuoles; a similar arrangement to the vacuoles and protein layers found with Pr42Gag but without any particles in the vacuoles. The vacuoles showed considerable variation in size and shape, and large areas of the cell surface often had many small adjacent vacuoles (Fig. 6a). Several vacuoles could be adjacent to the same portion of plasma membrane so that several adjacent layers of trapped protein were formed between vacuolar and plasma membranes and between adjacent vacuolar membranes (Fig. 6b). A similar protein layer was also seen between adjacent vacuoles deep in the cytoplasm (Fig. 6c).

Pr41.5Gag and Pr41Gag proteins also occurred in a second morphological form which was simply a uniform layer of protein approximately 10 nm in thickness.
Fig. 5. Sectioned Pr42gag-infected cells. (a) Large vacuoles protruding at the surface of a cell, spherical Gag particles are present within the vacuoles. (b) Surface vacuole open to the exterior allowing release of Gag particles, the particles are typically empty and do not contain cytoplasmic material. (c) An electron-dense layer of protein (arrow) is found between the plasma and vacuole membranes where the vacuole is closely applied to the cell surface. (d) Gag particle with inner and outer membranes (arrows). (e) Small vacuole at surface of cell which, if released, might give rise to particle with inner and outer membrane. (f) Section of Pr42Gag cell immunolabelled with anti-Pr55/24 monoclonal antibody, spherical particles and the dense layer of protein between plasma and vacuolar membranes are labelled. Bar markers represent 1 μm (a) and 100 nm (b to f).
beneath the plasma membrane of the host cell and not associated with any vacuoles (Fig. 6d). This layer was most often found where the cell surface was formed into projections and it was also incorporated into some budding baculoviruses. Occasionally a similar 10 nm layer of protein was seen on the cytoplasmic face of vacuolar membranes, without any association with a second membrane (Fig. 6d).

The 'trapped' protein layers of Pr41.5Gag and Pr41Gag, and indeed Pr42Gag, have a greater thickness (15 to 20 nm) than the protein layers of Pr41.5Gag and Pr41Gag associated with a single membrane (10 nm). These dimensions indicate that a double layer of protein was trapped between two membranes (Fig. 6d) and that the truncated Gag protein molecules may have an affinity for each other in addition to their known affinity with membranes.

Immunolabelling studies of the Pr41 cells with anti-Pr55/24 monoclonal antibody showed labelling of the dense protein layers between vacuolar and plasma membranes are labelled. Bar markers represent 1 μm (a) and 100 nm (b to e).
Morphology of HIV Gag protein structures

Fig. 7. Sectioned D2gag- and D1gag-infected cells. (a) D2 cell outer surface is convoluted and has a continuous layer of protein beneath the plasma membrane. (b) Some portions of the D2 protein layer show a central region of reduced electron density. (c) D1 cell has discrete crescent-like patches of protein beneath the plasma membrane. (d) Spike-like projections are present in the plasma membrane specifically over some D1 protein crescents. Bar markers represent 100 nm.

often there was some labelling of plasma and vacuolar membranes even where adjacent vacuoles and a dense protein layer were absent.

D2Gag

Cells infected with the D2gag deletion mutant typically had a highly irregular and convoluted surface (Fig. 7a), which was not seen in cells infected with the other recombinant baculoviruses. Beneath the plasma membrane of the most convoluted cells there was an almost continuous, uniform layer of protein 15 to 20 nm in thickness (Fig. 7a). Some sections showed a region of reduced electron density in the centre of the protein layer (Fig. 7b) which might indicate a double layer of protein. No vacuoles were associated with the outer protein layer but occasionally a protein layer was seen associated with vacuoles deeper in the cytoplasm. In some cells the protein layer was present in small discrete patches beneath the plasma membrane but there was no indication of crescent-like protrusions or any further progress towards particle formation.

D1Gag

The D1Gag protein was present in cells as a 15 to 20 nm thick layer beneath the plasma membrane; the protein was typically found in discrete patches which formed crescent-like protrusions (120 to 130 nm in diameter) from the cell surface (Fig. 7c) in contrast to the
continuous convoluted layer of protein in D2Gag cells. When large amounts of protein were present several crescents were often grouped together on a cell protrusion. All protrusions contained cytoplasmic components and some were connected to the cell by only a narrow stalk. Free Gag particles were seen infrequently; they were usually large and irregular in shape and were probably cell projections which appeared to be cut off because of the plane of the section.

One feature seen with the D1Gag protein crescents and not identified so clearly on any of the other Gag proteins was the presence of surface spikes on the cell membrane overlying the protein crescent. Spikes were seen on only a small proportion of Gag protein crescents but when present they were numerous and regularly arranged specifically over the Gag protein (Fig. 7d). Similar spikes were seen more frequently on budding baculovirus particles in the same specimens and they probably represent the major surface glycoprotein (gp67) of the baculovirus.

**Discussion**

Eight different mutations of the gag gene of HIV have been shown in this study to produce six characteristic forms of Gag protein, usually in association with the host cell plasma membrane. The Gag protein molecular assemblies exhibit two basic morphological forms: (i) particulate, three-dimensional, spherical or tubular shells or (ii) non-particulate, two-dimensional, flat, curved or convoluted sheets. The change in structure from particulate to non-particulate has been related directly to alterations in the primary amino acid sequence by Jowett et al. (1992) but EM was used in these studies mainly to score mutations as positive or negative for particle formation. More detailed analyses of the phenotypes have now been provided and comparison of the abnormal morphology with normal budding can reveal further details of the processes of transport, assembly and targeting of Gag molecules. Ideally, the results should be interpreted not only in terms of sequences in the gag gene but should also take into account the known molecular organization of the Gag protein shell of virus-like particles and HIV (Nermut et al., 1993, 1994).

Three basic mechanisms of assembly and release of retroviruses were recognized by Bernhard (1958) and led to the classification of A-, B- and C-type particles. Spherical A-type particles are assembled in the cytoplasm; they may then bud into cisternae of the endoplasmic reticulum but the particles are not released to the exterior of the cell. Similarly, B-type assembly of the Gag protein also involves intracytoplasmic formation of a spherical shell which later becomes enveloped and released at the plasma membrane. In contrast, C-type assembly is characterized by a stepwise accumulation of Gag protein at the plasma membrane concomitant with budding and release. The Gag proteins of all known retroviruses are assembled and released from the host cell by either the B- or C-type methods; lentiviruses (HIV) have C-type assembly. A-type assembly probably results in abnormal or defective particles.

The Gag truncation mutants described here illustrate a change from typical C-type assembly in Pr55Gag cells to a mixture of A-, B- and C-type assembly in Pr45 and Pr44Gag cells. The intracytoplasmic Gag shells in Pr45 and Pr44Gag cells generally acquired an envelope at intracellular vacuoles which resembles A-type assembly but occasionally budding was also observed at the plasma membrane (B-type assembly). Many of the Pr42Gag cells showed only A-type assembly with accumulation of Gag shells in vacuoles; release of these particles to the exterior of the cell occurred by opening of the vacuole at the cell surface. Thus progressive truncation of the Pr7Gag domain produced a change in assembly of particles from C- to B- and A-type i.e. redirection of the Gag molecules to a new intracytoplasmic site of assembly and re-targeting of the particles from the plasma membrane to vacuolar membranes. Clearly more than one domain in the primary structure of the protein is responsible for the same morphological effect since a similar change in the pathway of morphogenesis from C-type to mainly A-type was produced by large deletions in the MApl7 coding region of the HIV gag gene (Fücke et al., 1993; Spearman et al., 1994). The interdependence of domains in the primary sequence of the Gag molecule and their non-exclusive effect on morphology is also shown by further truncation of the molecule to Pr41Gag in which particle formation was abolished but plasma membrane targeting of the Gag protein was retained in addition to vacuolar targeting. Many studies have shown individual parts of the Gag molecule that are essential for components of the transport, assembly and targeting processes (see Introduction) but it is now becoming clear that mutations at different sites can produce similar morphological changes and that a single change, such as non-N-myristylation, can affect more than one part of the assembly process (Chazal et al., 1994). Full understanding of particle assembly depends not just on knowledge of the primary amino acid sequence but also on an understanding of secondary and higher orders of structure and on the conformational changes brought about by mutations.

The morphologies of Gag protein assemblies shown here by SEM and in sections are compatible with the network of hexagonal rings formed from radially arranged rod-like Gag molecules which have been found in spherical Gag particles by negative staining (Nermut et al., 1994). Pentagonal or five co-ordinated rings were
Fig. 8. Diagrams illustrating the formation of convoluted sheets and icospiral shells from hexagonal units. (a) Addition of a heptagon (x) causes curvature in a flat sheet of hexagons. (b) Addition of a heptagon (x) and pentagons (y) cause complex curvature or convolutions in a sheet of hexagons (redrawn from Iijima et al., 1992). (c to e) Icospiral shells formed by asymmetrical addition of pentagons to a sheet of hexagonal units (redrawn from Kroto, 1988).

also seen occasionally (Nermut et al., 1994) which is consistent with expectations as no system of hexagons alone can enclose space (Thompson, 1961; Caspar & Klug, 1962). A net consisting of hexagons in combination with twelve pentagonal units can form an icosahedron and, although the icosahedral shape has not been seen directly in immature HIV or in Gag particles, there is evidence that mature HIV has an icosahedral organization (Marx et al., 1988; Ozel et al., 1988; Nermut et al., 1993). Indeed, the icosahedron is a fundamental shape for many viruses and the core shell (Gag protein) of mature murine leukaemia (C-type) retrovirus has been shown to be icosahedral (Nermut et al., 1972). If the core shell or membrane-associated protein of mature HIV has an icosahedral structure then the same icosahedral arrangement is almost certainly present in immature HIV and, by analogy, in Gag particles produced by expression systems since all stages contain the same Gag protein either in the cleaved or precursor form. Different sizes of approximately spherical particles can be formed as icosahedra that contain different numbers of hexagonal units or as other approximately spherical shapes containing irregular arrangements of hexagons and pentagons.

The morphologies of abnormal phenotypes can also be accommodated in the hexagonal network model. Hexagonal units alone can form tubular filamentous structures but they remain as curved sheets or as open cylinders unless closed at the end by half of an icosahedron (i.e. six pentagonal units). Such a tube would be a longitudinally rigid structure which is consistent with the tubular filaments associated with mutants Pr45Gag and Pr44Gag. The tubular filaments would probably have a helical arrangement of hexagons since helical addition of hexagonal units would account for longitudinal growth (Iijima, 1991). Introduction of pentagonal or heptagonal units into the tubular structures would produce precise and consistent angular changes in direction of the tubes (Iijima et al., 1992) such as have been reported here in the filamentous particles observed by SEM. The reverse curvature of Gag protein layers which may occur on the outside of cytoplasmic vacuoles can be also accounted for by the addition of heptagons (Fig. 8a). Pentagons and heptagons introduce opposite curvatures and thus, when both are present in close association, they nullify each others overall effect but leave a structure with a complex subcurvature (Fig. 8b) (Iijima et al., 1992) such as is found in the non-particulate, convoluted protein layers of D2Gag. Intracytoplasmic Gag shells with partly overlapping layers of Gag protein (Fig. 4b and c) can be similarly explained by an icosahedral arrangement of hexagons. Such a structure would begin to form as an icosahedron but asymmetrical addition of hexagons or pentagons would prevent closure and result in an icospiral shell (Fig. 8c, d and e) which is a combination of a helix and an icosahedron (Kroto, 1988).

The hexagonal organization of Gag protein molecules can explain the observed morphologies of mutant proteins but the molecular changes that occur within the Gag monomers and result in different shapes of particles and differing curvature remain to be determined. Evidence deduced from mutant proteins may help to explain the formation and location of pentagonal units in normal icosahedral particles. The assembly of curved and closed shells of Gag protein in the cytoplasm of host cells indicates that association with a membrane is not essential for the curvature of Gag. The formation of Gag particles and other assemblies of Gag protein presumably occurs wherever Gag molecules accumulate at a concentration sufficient for self-assembly, as has been suggested for other retroviruses (Rhee & Hunter, 1990). Curvature may be an intrinsic property of the protein itself and may possibly be explained by the shape of the Gag molecule and/or the arrangement of bonds that link
Gag molecules together. Conical molecules closely bound together along their tapering sides would form curved structures (Hewitt, 1977). A fuller understanding of the molecular morphology of Gag protein structures should aid our understanding of the assembly of HIV and promote the design of agents that block assembly or induce dis-assembly.

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