Ultrastructural characterization of human immunodeficiency virus type 1 Gag-containing particles assembled in a recombinant adenovirus vector system

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The human immunodeficiency virus type 1 (HIV-1) Gag protein was expressed in A549 cells infected with recombinant adenovirus types 4 and 7, each carrying the HIV-1 gag and pro genes. The Gag protein was assembled into enveloped virus-like particles that budded from plasma and vacuolar membranes. The particles, isolated by precipitation and isopycnic density centrifugation, contained both processed and unprocessed Gag-associated proteins.

Introduction

The major internal proteins of human immunodeficiency virus type 1 (HIV-1), like those of other retroviruses, are generated through proteolytic cleavage of a core precursor. Prior to viral morphogenesis, the precursor, Pr55\textsuperscript{gag}, undergoes myristoylation at its N-terminal glycine (Veronese et al., 1988; Göttinger et al., 1989), a step that appears to be necessary for its association with cellular membranes, further processing and assembly into mature virions (Jacobs et al., 1989; Bryant & Ratner, 1990). Processing, for which the viral protease is necessary and sufficient (Kramer et al., 1986; Debouck et al., 1987; Kohl et al., 1988), probably begins concomitantly with the budding of virus particles from host cell membranes (Munn et al., 1985; Palmer et al., 1985; Goto et al., 1990), but maturation may not be completed until the virus has been released (Katsumoto et al., 1987; Gelderblom et al., 1989). Two transitional, alternative cleavage products (Pr41; Mervis et al., 1988; Veronese et al., 1988) are processed further to yield four mature proteins: p17, the N-terminal product of the precursor, is an envelope-associated matrix protein (Ratner et al., 1985; Gelderblom et al., 1987a); p24 is arranged to form an electron-dense, cone-shaped core shell (Gelderblom et al., 1987a); p6 and p9 are nucleic acid-binding proteins that are derived from the C-terminal end of the precursor (Mervis et al., 1988) and are associated with the viral genome within the core.

The precursor and the protease are encoded by two overlapping genomic open reading frames (ORFs) that can be translated through ribosomal frameshifting (Ratner et al., 1985; Wain-Hobson et al., 1985; Kramer et al., 1986; Jacks et al., 1988). Pr55\textsuperscript{gag} is the primary translational product of the gag ORF, the protease, encoded by the 5' end of the pol ORF (pro), is an autocatalytic cleavage product of a 160K gag-pol fusion protein synthesized by the frameshifting mechanism (Ratner et al., 1985; Kramer et al., 1986; Debouck et al., 1987; Farmerie et al., 1987; Ashorn et al., 1990).

Pr55\textsuperscript{gag} expressed in a number of recombinant systems is assembled into particles whose morphogenesis mimics that of HIV (Gheysen et al., 1989; Göttinger et al., 1989; Karacostas et al., 1989; Peng et al., 1989; Smith et al., 1990). The particles, like the virus, form by budding, acquire envelopes and are released into culture fluids. Although expression of the precursor alone was sufficient for particle production in recombinant vaccinia virus systems (Hu et al., 1990; Shioda & Shibuta, 1990), the coding sequence for the viral protease was present in other reported systems.

The formation of Gag-containing, non-infectious virus-like particles provides an opportunity to employ such particles as antigen-bearing vehicles in vaccines, particularly in those targeted against AIDS, the causative agent of which is HIV. The particles can incorporate other co-expressed viral proteins (Haffar et al., 1990; Smith et al., 1990) and might be expected to ameliorate epitope presentation to the immune system, in comparison to a subunit vaccine.

We report here the synthesis of Gag-containing particles in a recombinant adenovirus vector system, using serotypes that have been incorporated in live virus
vaccines targeted against adenovirus-related respiratory disease (Edmondson et al., 1966; Gutekunst et al., 1967; Top et al., 1971).

Methods

Construction of recombinant adenoviruses. The construction of recombinant adenoviruses containing the gene for the HIV-1 envelope protein has been described (Chanda et al., 1990); similar procedures were used to incorporate gag and pro. Briefly, a DNA fragment containing the entire gag and pro coding regions (bp 335 to 2165) of HIV-1 strain LAV (Wain-Hobson et al., 1985) was constructed with a unique SalI site in front of the AUG codon of the gag gene and an XbaI site at bp 2165, for the insertion of the viral rev-responsive element (cre; bp 7178 to 7698). A 2.37 kb SalI fragment containing the three HIV-1 sequences was inserted at a SalI site in an expression cassette containing the adenovirus type 7 (Ad7) major late promoter (MLP), the tripartite leader (TPL) with an intervening sequence between the first and second leaders, and the hexon polyadenylation site (poly A), as described previously (Chanda et al., 1990). The cassette was inserted 159 bp from the right end of an Ad7 genome (Susenbach, 1984) containing the HIV-1 rev gene (Feinberg et al., 1986; Sodroski et al., 1986) in a deleted [79.5 to 88.4 map units (m.u.)] E3 region (Chanda et al., 1990).

A similar expression cassette containing analogous Ad4 sequences and the three HIV-1 coding regions was inserted at a site 139 bp from the right end of an Ad4 genome which contained HIV-1 rev in an E3 deletion between 76 and 86 m.u.

Infection of cells. A549 cells were grown in Dulbecco's MEM supplemented with 10% foetal bovine serum and antibiotics. Monolayer cultures were washed and infected with wild-type or recombinant viruses at an m.o.i. of 2.5 to 10 p.f.u./cell. Infected cells were maintained in the same medium with or without 2% gamma globulin-free foetal bovine serum.

Electron microscopy. For immunoelectron microscopy, infected cells were harvested by scraping or trypsinization, collected and washed by centrifugation, and fixed with 0.5% glutaraldehyde in phosphate-buffered saline (PBS) for 30 min. Fixed cells were washed, dehydrated in graded ethanol solutions, embedded in Lowicryl K4M and sectioned. Gag antigen was detected by floating sections sequentially on ‘blocking’ solution (1% bacitracin in PBS), anti-Gag monoclonal antibody (MAb) (Epitope Inc.), PBS and goat anti-mouse IgG coupled to 10 nm or 20 nm colloidal gold (E-Y Labs). Following a final wash in PBS and air-drying, the sections were stained with uranyl acetate and Reynolds' lead citrate. For transmission electron microscopy, infected cells were harvested as above, fixed sequentially with 2.5% glutaraldehyde and 1% OsO4, in 0.1 m-sodium cacodylate buffer pH 7.2, then treated for 1 h with 1% tannic acid and washed with 1% sodium sulphate (Simionescu & Simionescu, 1976). The cells were dehydrated in graded ethanol solutions and propylene oxide, embedded in Poly/Bed 812 (Poly-science), sectioned and doubly stained with uranyl acetate and Reynolds' lead citrate.

Samples were negatively stained by applying them drop-wise to carbon-coated copper specimen grids, rinsing the grids with water or 0.1% ammonium acetate, and staining with 1% sodium phosphotungstate or 1% ammonium molybdate. Some adsorbed samples were fixed in situ with 2% glutaraldehyde for 5 min before being stained.

For critical-point drying, samples were fixed for 10 min with 2.5% glutaraldehyde in cacodylate buffer and applied to specimen grids. The grids were washed, floated on OsO4 fixative, washed again and dehydrated in graded ethanol solutions, including half-saturated uranyl acetate in 50% (v/v) ethanol. They were then critical-point dried from liquid CO2.

Specimens were examined in a JEOL 100CX electron microscope at magnifications of 8500 x to 100000 x, using an accelerating voltage of 60 kV.

Isolation of Gag-containing particles. The fluids were harvested from infected cell cultures at 72 h post-infection and clarified by low speed centrifugation. Gag protein was precipitated by dissolving 0.3 g (NH4)2SO4/ml in clarified culture fluid and incubating the solution overnight at 4 °C. The precipitate was collected by centrifugation at 6000 g for 90 min and redissolved in sufficient PBS to produce a 50-fold to 75-fold concentration of the original fluid volume. The concentrate was overlaid onto a cushion of 45% (w/v) Nycodenz (Nycomed AS) in PBS and centrifuged at 150000 g for 90 min in a Beckman SW50.1 or SW40 swinging-bucket rotor. The sedimented material was overlaid with step gradients consisting of 35% (w/v), 27% 18% and 9% Nycodenz, and PBS alone. The flotation gradients were centrifuged at 115000 g for 18 h and fractions were collected from the top. Densities were calculated from refractive indices.

Dissolution of soluble and particle-associated Gag antigen. To determine the relative proportions of particle-associated and soluble Gag, 5 ml aliquots of samples were overlaid onto two-step gradients consisting of 4-75 ml 20% (w/w) sucrose in PBS and 2-75 ml 60% sucrose, and then centrifuged for 3 h at 80000 g in a Beckman SW41 rotor. The antigen remaining in the supernatant fluid (soluble) and that harvested from the interface of the sucrose layers (particulate) were measured by ELISA.

Assays. To detect Gag (p24 equivalent), ELISA was performed with a DuPont HIV-1 p24 Core Profile ELISA (E.I. DuPont de Nemours) using an anti-p24 capture MAb and human HIV-seropositive serum as the secondary antibody. Protein concentrations were determined with BCA Protein Assay Reagent (Pierce Chemical), according to the manufacturer's instructions. Samples were subjected to PAGE in 10%, 11% or 12% reducing gels (Laemmli, 1970), which were stained with Coomassie blue or Western-blotted (Towbin et al., 1979). Bands on electroblotted membranes were detected with human HIV-1-1seropositive serum. Densitometric scans of stained gels were done using an Electrophoresis Data Center (Helena Laboratories).

Results

Generation of recombinant adenoviruses

Ad4 and Ad7 recombinant viruses expressing the gag/pro genes (Ad4-Gag, Ad7-Gag) were generated by homologous recombination (Chanda et al., 1990) and the structure of each viral genome was confirmed by restriction endonuclease analysis of DNA extracted from infected cells by the method of Hirt (1967). The genomic structures of the recombinant viruses are shown in Fig. 1.

Electron microscopy of infected cells

Cultures of A549 cells infected with Ad4-Gag or Ad7-Gag contained virus-like particles ranging in diameter from 110 nm to 150 nm, with an average diameter of
Assembly of HIV-1 Gag-containing particles

A procedure was devised to isolate the particles and to preserve their structural integrity. Preliminary investigations showed that 30% to 50% of Gag in clarified fluids from Ad4-Gag-infected cultures could be recovered between the two sucrose layers after sedimentation in sucrose step gradients. Incubation of the sedimented Gag with 0.5% Triton X-100 converted it to a lighter species that remained in the supernatant fluids of the gradients; therefore, the antigen was judged to be in particulate form.

Various concentrations of ammonium sulphate were employed in an attempt to concentrate the antigen from culture fluids; Gag was found to be precipitated quantitatively by the addition of 0.3 g/ml ammonium sulphate, with less than 2% of the original antigen remaining in the supernatant fluid. Sedimentable antigen was further concentrated by centrifugation of redissolved precipitates onto cushions of 45% Nycodenz.

Following the isopycnic flotation of the concentrated particles into Nycodenz gradients, Gag was recovered in a peak within a density range of 1.09 g/ml to 1.16 g/ml as determined by ELISA (Fig. 4). In most experiments, the antigen recovered at a buoyant density of 1.10 g/ml to 1.12 g/ml represented at least 30% of the total Gag detected in the original clarified culture fluids. These major peaks normally yielded 1 to 2 μg Gag/10⁶ cells. Occasionally, small peaks were recovered at higher densities, but these never contained more than 5% to 10% of the total Gag-related protein detected in the gradients.

Electron microscopy demonstrated that the major antigen peak contained a population of smooth, round particles that ranged in diameter from 100 nm to 150 nm (Fig. 5a). These particles were judged to be equivalent to the Gag-containing particles observed in thin sections of infected cells; they were not observed in parallel experiments in which cultures were infected with wild-type Ad4. When the particles were penetrated by negative stain, striated ring-like structures sometimes were observed within the envelopes (Fig. 5b and c).

PAGE followed by staining or Western blotting indicated that fractions in the major Gag peak from Nycodenz gradients contained approximately equal amounts of Pr55ng and Pr41; p24 and p17 were

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**Assembly of recombinant adenoviruses carrying the HIV-1 gag/pro coding sequences.**

Ad4-Gag

<table>
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<tr>
<th>E3</th>
<th>rec</th>
<th>MLP/TPL</th>
<th>gag/pro</th>
<th>poly A</th>
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<td>199 bp</td>
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Ad7-Gag

<table>
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<th>rec</th>
<th>MLP/TPL</th>
<th>gag/pro</th>
<th>poly A</th>
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</thead>
<tbody>
<tr>
<td>79.5 m.u.</td>
<td>58.4 m.u.</td>
<td>159 bp</td>
<td>100 m.u.</td>
<td></td>
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</tbody>
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**Immunoelectron microscopy**

To confirm that the particles contained Gag, thin sections of infected cells were reacted with anti-HIV MAbs. As shown in Fig. 3, the particles could be labelled with MAb directed against Gag; they were not labelled when incubated with MAb directed against the HIV envelope protein or with normal mouse IgG. No particles were detected in cultures infected with wild-type viruses or with a recombinant Ad7 expressing the HIV-1 envelope antigen (Chanda et al., 1990).
detectable also (Fig. 7). When highly concentrated samples were analysed, p24 appeared to migrate as a doublet. Although some Gag-associated protein also was detectable at higher densities, the relative proportion of processed products decreased in denser fractions. Scanning densitometry of stained gels indicated that Gag-associated proteins always made up at least 50% of the total protein present in the major gradient peak.

**Discussion**

These studies demonstrate that Gag-containing virus-like particles are assembled in cultured cells following the expression of HIV gag and pro gene products by recombinant adenovirus vectors. The particles were assembled as they budded from both periplasmic and vacuolar membranes, and the particle envelopes were
Assembly of HIV-1 Gag-containing particles

Fig. 3. Detection of Gag in virus-like particles by immunoelectron microscopy. (a) Extracellular particles labelled with anti-Gag MAb and colloidal gold (20 nm) following infection with Ad4-Gag. (b and c) Rare particles (arrows) with central electron-dense material condensed into core-like structures. These sections were stained with uranyl acetate but were not treated with antibody. (d) Particles labelled with anti-Gag MAb and 10 nm colloidal gold, after infection with Ad7-Gag. (e) Same as (d), showing labelled intravacuolar particles. Particles were not labelled by anti-Env MAb. Bar marker represents 100 nm.

derived from these membranes. In these respects, the particles described here are morphogenetically similar to those produced in mammalian cells using other reported recombinant systems (Karacostas et al., 1989; Peng et al., 1989; Smith et al., 1990).

Fig. 4. Isopycnic centrifugation of Gag-containing particles in a Nycodenz gradient. The position of the Gag antigen peak corresponded to the position of a hazy, light band in the gradient. (○), Gag concentration; (●), buoyant density.

Fig. 5. Virus-like particles in a Gag peak recovered at a density of 1.10 g/ml after isopycnic centrifugation in Nycodenz. (a) Although the particles were fixed before being negatively stained with ammonium molybdate, they appear to have partially collapsed after drying. (b and c) Particles into which stain has penetrated contain ring-like structures that appear to be striated (arrows). Bar markers represent 100 nm.

The regularly spaced projections on the surfaces of some particles in Ad4-Gag-infected cultures superficially resemble the projections often observed on both HIV virions and 'immature' virus-like particles (Palmer et al., 1985; Gelderblom et al., 1987a; Hockley et al., 1988;
Fig. 6. Virus-like particles prepared by critical-point drying. (a) The inner components have collapsed and separated from the envelope. (b) The arrow indicates an internal structure that appears to be equivalent to the radially striated layer observed in sectioned particles. (c and d) Two particles containing structures that resemble immature viral cores. Bar marker represents 100 nm.

Fig. 7. Electrophoretic analysis, in a 12% acrylamide gel, of a typical preparation of particles banded in a Nycodenz gradient. Lane 1 was stained with Coomassie blue, whereas lane 2 is a Western blot. The identities of the minor bands have not been established. The figures on the left indicate the positions of Mr markers.

Özel et al., 1988; Smith et al., 1990). Generally, the projections are considered to be products of the viral env gene; however the appearance of projections on particles that were assembled in the absence of the env gene suggests that micrographs showing such projections must be interpreted with caution. At least some projections may represent non-viral cellular proteins, such as the histocompatibility antigens previously shown to be associated with virions (Gelderblom et al., 1987b; Henderson et al., 1987).

Striations in the electron-translucent layer of the particles were discernible only in the thinnest sections cut. The striated ring-like structures observed within negative stain-penetrated particles and rarely in critical point-dried particles probably represent the same structural entity. In this regard, the images of Gag-containing particles were similar to those of immature particles observed by Hockley et al. (1988) in purified preparations of various HIV isolates. Although those investigators suggested that the striations might have a helical arrangement, we believe it is more likely that the striations represent a radial array of Gag protein, the periodicity and clarity of which usually were obscured in two-dimensional images. The occasional separation of the striated rings from particle envelopes suggests that the rings have a structural integrity that is separate from, but protected by, the envelope.

The proposition that Pr55\textsuperscript{agg} is distributed on particle radii is an attractive one. If the N terminus of the protein were oriented externally in such an array, processing of the precursor would leave the mature p17 in a position contiguous to the envelope, where it is located in the HIV virion (Gelderblom et al., 1987a). All of the segments of the precursor would be arranged in the same relative positions that they are believed to occupy after maturation: p24, the core shell protein, would be external to p6 and p9, nucleic acid-binding proteins associated with viral RNA (Ratner et al., 1985; Gelderblom et al., 1989). Thus, the processed products could assume their final intraviral positions efficiently.

The paucity of visible core structures in extracellular particles implied that Pr55\textsuperscript{agg} was not being efficiently processed to mature internal proteins. This conclusion was confirmed by PAGE, which demonstrated that most of the particle-associated Gag protein consisted of Pr55\textsuperscript{agg} and Pr41. Any diminution or disruption of precursor processing would be expected to result in quantitatively or qualitatively abnormal core morphogenesis. Among the possible causes of Gag processing deficiencies are conformational changes leading to insufficient protease activity (Bryant & Ratner, 1990; Stewart et al., 1990) or to Gag protein instability (Rhee & Hunter, 1990), inhibition of Gag processing by myristoylation in the absence of other HIV proteins (Flexner et
although non-infectious Gag-al., occasionally in HIV-1-infected H-9 lymphocyte cells the protease expressed in the adenovirus system was of infected cells (Stewart et al., 1989). Avian leukosis virus have been observed within vacuoles of infected cells (Palmer et al., 1985; Fekovic et al., 1986). Similarly, wild-type virions, as well as protease-defective virions, of avian leukosis virus have been observed within vacuoles of infected cells (Palmer et al., 1990); however, no intravacuolar budding particles were seen. Furthermore, the protease expressed in the adenovirus system was sufficient to process at least some of the expressed Pr55~. Therefore, the cause of vacuolar budding may not involve viral protease activity.

Particles were isolated by a procedure designed to preserve their structural integrity, and the advantages of using Nycodenz as a density gradient medium have been discussed (Ford & Rickwood, 1982; Rickwood et al., 1982). The particles were floated into the gradients to preclude the potential disruption of the envelopes by shearing forces encountered during sedimentation (Lawrence, 1976; Polson & Stannard, 1970). Electron microscopy, sedimentation through sucrose step gradients and recovery of high yields of Gag antigen in Nycodenz gradient peaks indicated that the sequential sedimentation and flotation steps did not result in detectable particle disruption.

Similar particles produced in other recombinant systems have been recovered after centrifugation in sucrose (Karacostas et al., 1989; Haffar et al., 1990; Smith et al., 1990) or in potassium tartrate (Shioda & Shibuta, 1990) gradients. However, electron micrographs of isolated particles produced by recombinant vaccinia viruses (Karacostas et al., 1989; Shioda & Shibuta, 1990) did not suggest that homogeneous populations of well-preserved particles had been obtained.

Recovery of particle-associated purified Gag protein from Ad4-Gag-infected cells was comparable to the yields reported for other recombinant systems. Concentrations as high as 0.67 μg p24/10^6 cells were released from BSC-40 cells infected with one of the recombinant vaccinia viruses constructed by Hu et al. (1990). As much as 10 μg p24/10^6 cells was released from CMT3 COS cells infected with a Gag-expressing simian virus 40 late replacement vector (Smith et al., 1990). Whether all p24 released in those systems resided in Gag-containing particles is not known.

Although the Gag antigen recovered in Nycodenz gradient peaks appears to be entirely particle-associated, determination of the purity of the antigen was problematic. PAGE of isolated particles followed by either Western blotting or staining resulted in the appearance of minor bands that could not be correlated with known Gag-related proteins. It is possible that the human serum used for blotting recognized residual adenoviral proteins that might have contaminated the Gag antigen peaks. Migration of p24 as a doublet has been observed by other investigators; the phenomenon has been attributed to the existence of alternative cleavage sites or to phosphorylated forms of p24 (Mervis et al., 1988; Veronese et al., 1988; Gowda et al., 1989; Overton et al., 1990). Nevertheless, at least some of the minor bands observed in stained gels may represent unidentified particle-related proteins; the particle envelopes may include surface proteins or other proteins of cellular origin. Thus, the minor bands could not be identified either as contaminants or as particle constituents.

Non-infectious antigen-bearing particles such as those described here may provide an alternative to killed virus or subunit vaccines targeted against AIDS. In combination with incorporated, co-expressed viral antigens, their use might obviate any need to potentiate immunogenicity by construction of antigen-bearing liposomes (Allison & Gregoriadis, 1974) or immunostimulatory complexes (Morein et al., 1984). Furthermore, the particles’ lack of an infectious HIV genome and their potential for continual synthesis through a live virus vector make them attractive vaccine candidates.

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References


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