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Human Immunodeficiency Virus Type 1 Capsid Formation in Reticulocyte Lysates

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The Gag polyprotein of human immunodeficiency virus (HIV) ($Pr55^{Gag}$) contains sufficient information to direct particle assembly events when expressed within tissue culture cells. HIV Gag proteins normally form particles at a plasma membrane assembly site, in a manner analogous to that of the type C avian and mammalian leukemia/sarcoma viruses. It has not previously been demonstrated that immature HIV capsids can form without budding through an intact cellular membrane. In this study, a rabbit reticulocyte lysate translation reaction was used to recreate HIV capsid formation in vitro. Production of HIV-1 $Pr55^{Gag}$ and of a matrix-deleted Gag construct resulted in the formation of a subset of Gag protein structures with an equilibrium density of 1.15 g/ml. Gel filtration chromatography revealed these Gag protein structures to be larger than 2×10^6 Da, consistent with the formation of large multimers or capsids. These Gag protein structures were protease sensitive in the absence of detergent, indicating that they did not contain a complete lipid envelope. Spherical structures were detected by electron microscopy within the reticulocyte lysate reaction mixtures and appeared essentially identical to immature HIV capsids or retrovirus-like particles. These results demonstrate that the HIV Gag protein is capable of producing immature capsids in a cell-free reaction and that such capsids lack a complete lipid envelope.

During retrovirus assembly, multiple coordinated events occur and result in the production of extracellular, enveloped virions. Previous studies have shown that the *gag* gene products of a number of retroviruses, when produced in the absence of all other viral gene products, are sufficient to catalyze the formation and release of enveloped virions from cells (5, 12, 16, 27). Retroviral Gag polyproteins, therefore, contain sufficient information to direct Gag-Gag protein interaction, intracellular transport, plasma membrane interaction, and budding when produced within appropriate tissue culture cells. The sequence and intracellular location of these assembly events, however, are not uniform between retroviral species.

Human immunodeficiency virus type 1 (HIV-1) Gag proteins interact at the plasma membrane of infected cells, where particles are first detected by electron microscopy as a dense plaque underlying the plasma membrane (10). Gag protein plaques progress to form protruding buds which separate from the plasma membrane to form the nascent particle. A plasma membrane site of assembly is also a feature of the life cycle of the type C oncoviruses and the human T-cell leukemia viruses. In contrast, the type B mouse mammary tumor virus and type D Mason-Pfizer monkey virus (M-PMV) first assemble capsids at an intracellular site (4, 11). Mouse mammary tumor virus or M-PMV intracellular capsids then travel to the plasma membrane of the cell, where envelopment and budding takes place. Although the assembly pathway of the type B and D viruses appears to be radically different from that of HIV and the type C oncoviruses, evidence that the pathways have common characteristics exists. Specific mutations within Gag proteins which follow one assembly pathway may redirect assembly to more closely resemble the alternate pathway. For example, a single amino acid change in the matrix protein region of the M-PMV Gag polyprotein was shown to change its assembly from the type B/D pathway to assembly on the plasma membrane (22). Similarly, some mutations within the HIV matrix protein region of the HIV Gag polyprotein have resulted in the formation of immature intracellular particles (3, 8) or intracellular capsid-like structures (25). It is possible that HIV and other type C pathway retroviruses require interactions with cellular membranes in order to initiate capsid assembly. Alternatively, the plasma membrane assembly site may be a consequence of other events, such as the efficient transport of polyproteins to this site, where a critical concentration of Gag proteins is then achieved. An in vitro system which is capable of recreating capsid formation would facilitate the understanding of these crucial steps in the virus life cycle.

Cell-free assembly of viral capsids has been reported for papovaviruses (13, 23), picornaviruses (17), herpesviruses (20), and several species of bacteriophage (1, 15). Recently, retroviral capsid-like structures were produced in vitro by using a bacterial production system expressing the Gag polyprotein of M-PMV (14). We have studied cell-free formation of capsids by HIV Gag proteins utilizing a reticulocyte lysate-based coupled transcription/translation reaction. Analysis was performed with complete $Pr55^{Gag}$ as well as with $Pr55^{Gag}$ containing a large deletion in the matrix protein region. Both the intact precursor polyprotein and the matrix-deleted protein formed immature capsids in this cell-free system. The appearance of the in vitro-synthesized capsids was remarkably similar to the immature structures formed by these proteins when expressed in mammalian cells (25). This represents the first evidence that retroviruses which follow the type C assembly pathway are capable of capsid formation in a cell-free reaction mixture. Although no exogenous membranes were added to the reticulocyte lysate reaction, membranes present within the reticulocyte lysate itself may have contributed to successful capsid production.

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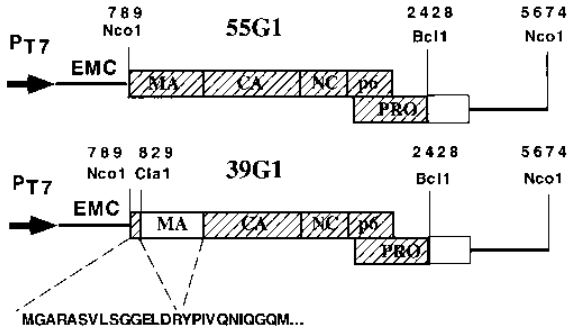

FIG. 1. Schematic diagram of HIV Gag expression constructs. Relevant restriction sites used in construction are indicated. Expressed regions are indicated by shading. The nucleotide numbering above the restriction sites is from the GenBank database under accession no. K03455. Plasmid p39G1 contains a deletion of most of the matrix protein region; the amino acid sequence of the junction is shown below the genetic map. P_{T7}, T7 promoter, EMC, encephalomyocarditis virus leader sequence of expression plasmid pTM3.

Gag protein expression using a reticulocyte-lysate coupled transcription/translation reaction. The expression plasmid pTM3 (19), which contains the long untranslated region from encephalomyocarditis virus and the T7 RNA polymerase promoter, was used to facilitate high-level expression of HIV *gag* gene constructs. The HIV *gag* expression plasmids p55G1 and p39G1 are shown schematically in Fig. 1. The construction of these plasmids has been detailed previously (25). Briefly, site-directed mutagenesis was used to introduce an *Nco*I site at the *gag* initiation codon of the proviral clone pHXB2gpt2 (21), and then an *Nco*I-to-*Nco*I fragment was ligated in the *Nco*I site within the polylinker of pTM3 to create plasmid pGPG1. p55G1 was derived from pGPG1 by creation of a frameshift mutation downstream of the *gag* stop codon (using the *Bcl*I site, codon 71 of protease-coding region). p39G1 was derived by a PCR method from pGPG1 to express the initial 15 amino acids of MA fused in-frame to the final codon of MA (the tyrosine 132 codon of the *gag* open reading frame), thus deleting all of MA downstream from the initial 15 amino acids while expressing the rest of $Pr55^{Gag}$. These two expression constructs were extensively characterized previously by restriction digestion, sequencing, and expression utilizing the vaccinia virus/T7 RNA polymerase hybrid protein expression system. p55G1 expression in mammalian cells results in a myristylated, 55-kDa Gag polyprotein which produces efficient budding of enveloped particles from the plasma membrane; p39G1 expression produces a myristylated, 39-kDa Gag protein with a large deletion within the matrix protein region and forms intracellular, capsid-like structures in mammalian cells (25). Neither construct expresses an intact protease protein.

The Gag protein constructs described above were expressed using a coupled transcription/translation reaction based on a rabbit reticulocyte lysate (TNT Lysate Translation System; Promega, Madison, Wis.). Reactions were carried out according to the manufacturer's instructions with 1 μg of uncut plasmid DNA, 25 μl of reticulocyte lysate, 2 μl of reaction buffer, 1 μl of T7 RNA polymerase, 1 μl of a 1 mM amino acid mixture without methionine, 4 μl of [^{35}S]methionine (1,200 Ci/mmol, 10 mCi/ml), 1 μl of RNasin RNase inhibitor, and 15 μl of deionized water. The reaction mix was incubated at 30°C for 1 h and then immediately analyzed for capsid formation.

A subset of Gag protein translation products demonstrates an equilibrium density of 1.15 g/ml. Gag protein translation products were initially analyzed for capsid formation by equilibrium centrifugation on sucrose gradients. The entire translation product mixture was loaded on the top of a 20 to 60% (wt/vol) linear sucrose gradient in NTE buffer (100 mM NaCl, 10 mM Tris-HCl [pH 8.0], 1 mM EDTA) and subjected to centrifugation in an SW 28.1 rotor (Beckman) at 20,000 rpm for 16 h. Equal fractions were collected from the bottom of the tube. Analysis was performed by immunoprecipitation of each fraction with pooled HIV patients' sera, followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. The refractive index was also measured for each gradient fraction with a refractometer and used to calculate the corresponding sucrose density. Both the $Pr55^{Gag}$ molecule and the p39G1 product demonstrated a peak of specific signal at 1.15 g/ml and a broad shoulder in lighter fractions of the gradient (data shown for 39G1 in Fig. 2A). The faster-migrating bands seen on the gel in Fig. 2A probably represent the products of premature termination of translation; these products were found only in the upper fractions of the gradient. In order to confirm that the fraction with a density of 1.15 g/ml (the 1.15-g/ml fraction) contained Gag protein structures which had reached equilibrium density, half of the 1.15-g/ml fraction from an initial 16-h centrifugation procedure was diluted in 10% sucrose and loaded at the top of a second 20 to 60% linear sucrose gradient. Centrifugation of the 1.15-g/ml peak on a second sucrose gradient was carried out under identical conditions for 32 h and then analyzed by immunoprecipitation and autoradiography as described previously. The appearance of the peak of Gag protein in the 1.15-g/ml fraction following a 32-h centrifugation step (Fig. 2B, fraction 5) established that this was a true equilibrium density measurement for these Gag protein structures.

The p39G1 product has been shown to form intracellular retrovirus capsid-like structures when expressed in a mammalian cell line. Intracellular particles produced by this construct resemble immature HIV cores when examined by electron microscopy (25). Equilibrium centrifugation experiments were performed with these intracellular structures for comparison with the centrifugation profile of the putative in vitro capsid structures. BSC-40 cells (African green monkey kidney cell line) were first infected with 10 PFU of recombinant vaccinia virus VTF 7-3 (9) per cell to express T7 RNA polymerase and then transfected with 10 μg of p39G1 DNA via cationic lipid-mediated transfection (Lipofectin; Gibco BRL, Gaithersburg, Md.). After 4 h of incubation in complete Dulbecco's modified Eagle medium (D-MEM) with 10% fetal calf serum, cells were starved for 15 min in D-MEM lacking methionine. Labelling was then performed for 2 h with [^{35}S]methionine (50 μCi/ml). The cells were then washed twice in NTE buffer and lysed in hypotonic media (10 mM Tris [pH 8.0], 1 mM EDTA with protease inhibitors) by Dounce homogenization. The lysate was normalized to 100 mM NaCl–1 mM $MgCl_2$, and then nuclei and unbroken cells were removed by centrifugation at 1,000 × *g* for 10 min. The resulting postnuclear supernatant was loaded on the top of a 20 to 60% linear sucrose gradient and subjected to centrifugation and analysis identical to that of the reticulocyte lysate products. Examination of these [^{35}S]methionine-labelled 39G1 intracellular structures revealed a pattern similar to that of the reticulocyte lysate product (compare Fig. 2C and B). The peak amount of Gag protein as determined by densitometric analysis occurred at a sucrose density of 1.16 g/ml.

In order to demonstrate that the equilibrium density pattern of Gag protein from reticulocyte lysate reactions was not an artifact of the in vitro reaction which might be seen with unrelated proteins, a control plasmid (pTM1/LacZ, kindly provided by P. Olivo, Washington University, St. Louis, Mo.) was used to express the complete *Escherichia coli* β-galactosidase

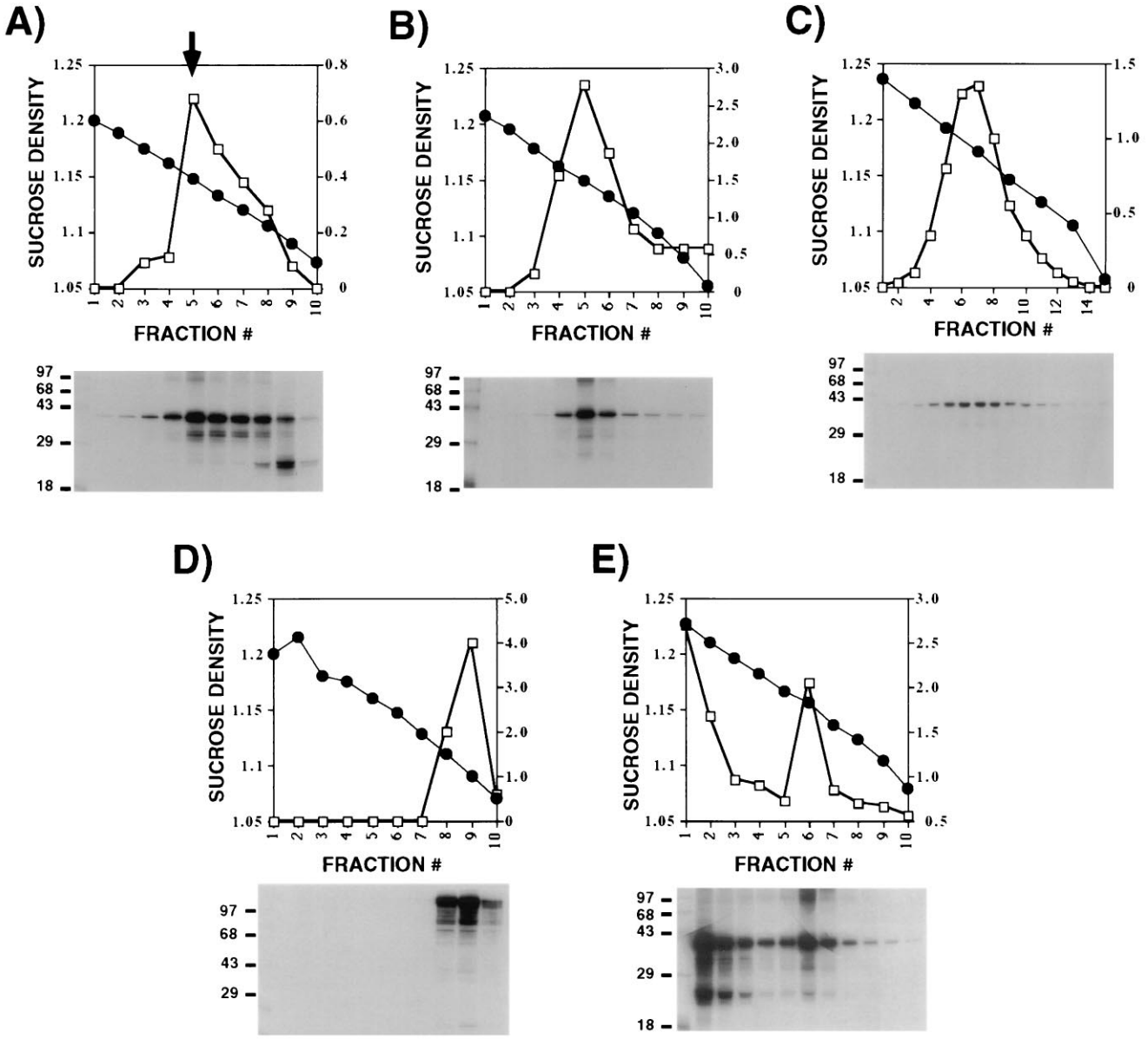

FIG. 2. Sucrose gradient analysis of p39G1 product. (A) The matrix-deletion construct p39G1 was used to produce ^{35}S-labelled Gag protein in a coupled transcription/translation reaction. The reaction mix was layered on the top of a 20 to 60% (wt/vol) sucrose gradient and centrifuged at 20,000 rpm for 16 h in an SW 28.1 rotor. Ten equal fractions were collected and analyzed by immunoprecipitation with HIV patients' sera followed by SDS-PAGE and autoradiography. The sucrose density of each fraction is shown (closed circles; density [left ordinate] expressed in grams per milliliter) plotted against laser densitometry results (open boxes; absorbance [right ordinate]). The corresponding autoradiograph is shown below the plot. The arrow indicates the 1.15-g/ml fraction of the gradient. (B) Half of the 1.15-g/ml fraction from an initial 16-h gradient centrifugation was diluted to 10% sucrose and layered over a second 20 to 60% gradient. Centrifugation was carried out under conditions identical to those described for panel A but extended to 32 h. Analysis was done as described for panel A. (C) Intracellular Gag protein structures produced by p39G1 transfection of BSC-40 cells were isolated as described in the text and analyzed by methods identical to that for panel A. (D) β-Galactosidase was produced in a coupled reticulocyte lysate transcription/translation reaction and analyzed as described previously. Immunoprecipitation was performed with a monoclonal antibody to β-galactosidase (Amersham). (E) Equilibrium flotation gradient centrifugation was performed by normalizing the 39G1 in vitro translation product to 60% sucrose (wt/vol) and loading the entire sample on the bottom of a 20 to 60% gradient. Centrifugation, collection, and analysis of collected fractions were carried out in the manner described for panel A. The positions of molecular mass markers (in kilodaltons) are indicated at the left of each autoradiogram.

protein for comparison with Gag protein expression and centrifugation profiles. In vitro transcription and translation were carried out as described above and produced a product with a size of 116 kDa. Centrifugation was performed for 16 h in an identical manner to that already described for translated Gag proteins, and the resulting gradient fractions were immunoprecipitated with a monoclonal antibody to β-galactosidase (Amersham, Arlington Heights, Ill.) and examined by SDS-PAGE and autoradiography. As expected for soluble proteins, which do not reach equilibrium under these centrifugation conditions because of their low sedimentation coefficients, the β-galactosidase protein remained in the lightest fractions of the gradient (Fig. 2D, lanes 8 to 10).

As additional evidence that a subset of translated Gag protein formed structures with an equilibrium density of 1.15 g/ml, equilibrium flotation centrifugation analysis was performed.

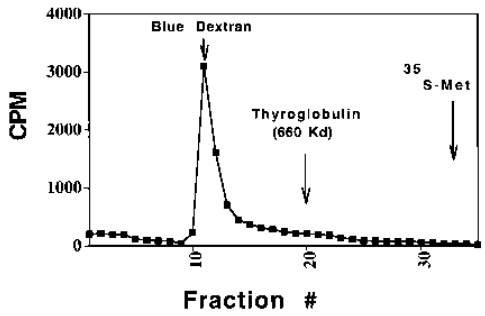

FIG. 3. Size-exclusion chromatography of Gag protein structures. The 1.15-g/ml fraction from equilibrium density centrifugation of $Pr55^{Gag}$ (p55G1 product) was analyzed by size-exclusion chromatography on a CL-6B column. Shown is a plot of [^{35}S]methionine-labelled protein eluted from the column as determined by liquid scintillation counting (*y* axis, counts per minute [CPM]) versus fraction number (*x* axis). The elution peaks of Blue Dextran (to delineate the excluded volume), thyroglobulin (a marker protein; Sigma, St. Louis, Mo.), and free [^{35}S]methionine (^{35}S-Met) (to delineate the total elution volume) are indicated by arrows. Kd, kilodaltons.

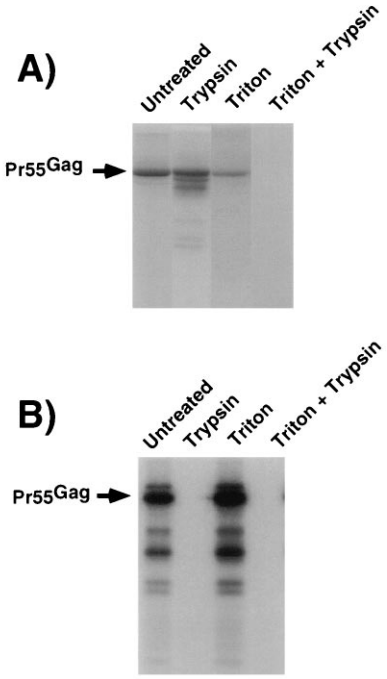

FIG. 4. Comparison of susceptibilities of lipid-enveloped Gag protein particles and in vitro-translated Gag proteins to trypsin digestion in the presence or absence of detergent. (A) Retrovirus-like particles produced with the vaccinia virus/T7 RNA polymerase expression system were collected from BSC-40 cellular supernatants and purified by sucrose gradient centrifugation as described in the text. The particle-containing fraction was divided into four equal parts, which were subjected to incubation at room temperature for 30 min with no additives prior to immunoprecipitation (untreated lane), treatment with 200 μg of trypsin per ml (trypsin lane), treatment with 1% Triton X-100 (Triton lane), or treatment with 200 μg of trypsin per ml and 1% Triton X-100 (Triton + Trypsin lane). (B) [^{35}S]methionine-labelled $Pr55^{Gag}$ from a reticulocyte lysate translation reaction was divided into four equal fractions and treated as described for panel A. The position of $Pr55^{Gag}$ is indicated by the arrow; lower bands represent products of premature termination of translation.

The Gag protein was translated as described before, and then the translation mix was adjusted to 60% sucrose (wt/vol) and loaded at the bottom of a 20 to 60% linear sucrose gradient. Centrifugation was performed for 16 h in an SW 28.1 rotor at 20,000 rpm, and fractions were collected and analyzed as described above. A peak of the Gag protein was also demonstrated by this method at a sucrose density of 1.15 g/ml, demonstrating again that this was a true equilibrium density for this subset of the in vitro-translated Gag protein.

The 1.15-g/ml structures formed by Gag proteins were not disrupted by treatment with pancreatic RNase (100 μg/ml) for 30 min at 30°C or RQ1 DNase (100 U/ml) for 30 min at 30°C, or by treatment with 10 mM EDTA (data not shown). Similarly, treatment with 0.05% Triton X-100 failed to disrupt the 1.15-g/ml fraction of the Gag protein (data not shown).

Size exclusion chromatography of the 1.15-g/ml Gag protein fraction. Size exclusion chromatography was next utilized to estimate the size of the Gag protein structures from fractions taken from sucrose gradients. The 1.15-g/ml sucrose fraction from gradients described above was diluted in buffer containing 0.2 M NaCl, 10 mM Tris (pH 8.0), 1 mM EDTA and run by gravity flow over a Sepharose CL-6B column. Blue dextran was used to demonstrate the void volume, thyroglobulin was used as a 660-kDa size marker, and free [^{35}S]methionine was used to delineate the total elution volume. Fractions collected from the column were analyzed by counting in a liquid scintillation counter. Analysis of the 1.15-g/ml fraction from sucrose density centrifugation of the p55G1 translation product on a CL-6B size exclusion chromatography column revealed that the Gag protein structures eluted in the void volume of this column together with blue dextran, indicating a size of greater than 2×10^6 Da for globular proteins (Fig. 3). The peak of ^{35}S-labelled protein (shown as ^{35}S counts in Fig. 3) in the void volume contained the p55G1 product as determined by immunoprecipitation and autoradiography (data not shown). These data indicate that the peak-specific Gag protein signal seen in the 1.15-g/ml fraction represents the formation of large Gag protein structures and is consistent with Gag protein multimerization or capsid formation.

Gag protein structures from reticulocyte lysates are sensitive to proteolytic digestion in the absence of detergent. Complete retroviral particles are resistant to digestion with proteases such as trypsin unless detergents are utilized to solubilize the lipid envelope (26). Because the equilibrium density of the in vitro-translated Gag protein structures was similar to that of a complete retroviral particle (1.16 to 1.18 g/ml [6]), we performed experiments to test whether these structures were protected by a lipid envelope. Retrovirus-like particles produced with the vaccinia virus/T7 RNA polymerase expression system and expression plasmid p55G1 were used as a lipid-enveloped control. $Pr55^{Gag}$ was produced in BSC-40 cells with this expression system as previously described (25) and labelled for 4 h with 200 μCi of [^{35}S]cysteine/methionine per ml, and the cellular supernatants were pelleted through a 20% sucrose cushion in an SW 28.1 rotor at 28,000 rpm for 1 h. The resulting pellet was resuspended in phosphate-buffered saline (PBS) and loaded on the top of a linear 20 to 60% sucrose gradient for centrifugation at 20,000 rpm for 16 h. The 1.16-g/ml fraction (containing purified retrovirus-like particles) from this gradient was then collected, diluted in PBS, and divided into four equal portions. The particle preparations were incubated for 30 min at room temperature with 200 μg of trypsin per ml, 1% Triton X-100, 200 μg of trypsin per ml–1% Triton X-100, or no additives. Each fraction was then analyzed by immunoprecipitation with pooled HIV patients' sera followed by SDS-PAGE and autoradiography. The intact Gag protein was demonstrated when the particle-containing fraction was treated with trypsin alone, with Triton X-100 alone, or with no added protease or detergent (Fig. 4A, first three lanes). In the presence of 1% Triton X-100, trypsin was able to

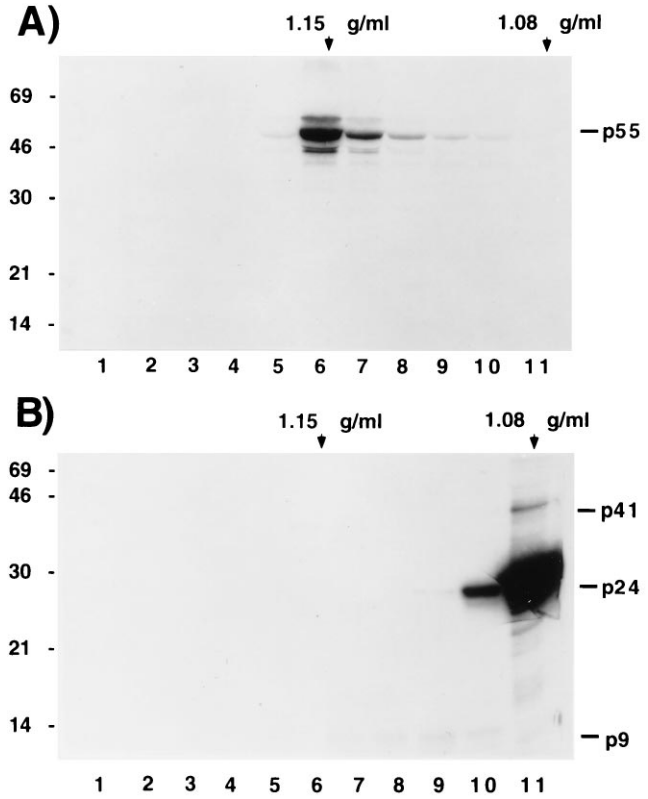

FIG. 5. Digestion of Gag protein structures with HIV protease. (A) The [^{35}S]methionine-labelled p55G1 product from a reticulocyte lysate transcription/translation reaction was examined by equilibrium centrifugation on a linear sucrose gradient as described in the text. The 1.15-g/ml fraction was divided in half and analyzed following a second centrifugation step. (A) Autoradiograph of sample with no protease treatment. Fractions were immunoprecipitated with HIV patients' sera and examined by autoradiography. (B) The remaining half of the 1.15-g/ml fraction was digested with HIV protease and subjected to a centrifugation step and analysis identical to those for panel A. Size markers (in kilodaltons) are shown on the left.

digest the Gag protein (Fig. 4A, last lane). The in vitro-translated Gag protein programmed with the p55G1 expression plasmid and labelled with [^{35}S]methionine was then subjected to an identical series of treatments. The reticulocyte lysate-translated Gag protein was completely digested by trypsin in the absence of detergent (Fig. 4B), indicating the lack of a protective lipid coat.

The Gag protein structures (putative capsids) with a density of 1.15 g/ml were also susceptible to specific cleavage by HIV protease in the absence of detergent, as shown in Fig. 5. A coupled transcription/translation reaction was first performed with plasmid p55G1 to express ^{35}S-labelled Pr55^{Gag}. The reaction products were subjected to equilibrium centrifugation analysis on a 20 to 60% sucrose gradient for 16 h as already described. The 1.15-g/ml fraction was then collected from the gradient and divided into two equal portions. One portion was diluted to 10% sucrose with NTE buffer and subjected to a second identical round of equilibrium sedimentation centrifugation, followed by immunoprecipitation with pooled HIV patients' sera and analysis by SDS-PAGE and autoradiography. Pr55^{Gag} appeared in the 1.15-g/ml fraction, confirming previous results (Fig. 5A, fraction 6). The second half of the 1.15-g/ml fraction from the initial centrifugation step was diluted to 10% sucrose and digested with recombinant HIV protease (kindly provided by M. Bryant, Searle Laboratories, Chesterfield, Mo.) at a concentration of 5.5 μg/ml for 30 min at 30°C. This digested product was then layered on top of a second 20 to 60% sucrose gradient for ultracentrifugation and analysis exactly as previously described. The digestion products visualized include p24 (CA), p9 (NC), and a small amount of p41 (Fig. 5B, lanes 10 and 11). The matrix protein (MA) was not visualized in this experiment because of the label ([^{35}S]methionine) employed. The cleavage products appeared at the top of the gradient in a position characteristic of free proteins, indicating that the multimerized Gag proteins (capsids) were completely disrupted by specific protease digestion in the absence of detergent.

Analysis of immature capsid formation by electron microscopy. Transmission electron microscopy was utilized to examine the morphology of the HIV Gag protein structures produced in vitro. For these experiments, the components of the transcription/translation reaction were doubled, yielding a final reaction volume of 100 μl. The resulting translation products were prepared for electron microscopic analysis by dilution in 200 μl of NTE (100 mM NaCl, 10 mM Tris-Cl [pH 8.0], 1 mM EDTA) and centrifugation in a 1.5-ml microcentrifuge tube at 100,000 × *g* for 1 hour in a Sorvall RCM120EX microultracentrifuge. The resulting pellet was fixed with 2% gluteraldehyde in phosphate buffer, postfixed with 1% osmium tetroxide, stained with 1% uranyl acetate, dehydrated in ethanol, and embedded with propylene oxide and Spurr resin. Thin sections were cut with a diamond knife, stained with uranyl acetate and lead citrate, and analyzed on a Philips model 3000 electron microscope. Reactions performed with the addition of p55G1 and p39G1 plasmid DNA were analyzed; control reactions were performed with the expression vectors pTM3, pTM1, or with no added DNA. Examination of control lysate reactions revealed mostly a homogeneous pattern of ribosomes (data not shown). However, membrane sheets were apparent in some control sections (Fig. 6A), as well as a few irregular, thin-walled structures with an ovoid shape. In sections pelleted from lysates producing matrix-deleted Gag protein (Fig. 6B and C) or intact Pr55^{Gag} (Fig. 7A), structures resembling immature retroviral capsids were identified. These structures were spherical and stained more densely than the background material. Clusters of multiple spherical structures were found, often near the edge of the pelleted lysate (Fig. 6B). The size and appearance of these structures were then compared directly with those of the corresponding immature capsids produced by transfection of an African green monkey kidney cell line (BSC-40 cells). The diameter of the reticulocyte lysate-produced capsid structures was typically 90 to 100 nm but with significant variation in size. The mean diameter as determined from measurement of a random sampling of 50 observed capsid-like structures was 99 nm for p55G1-programmed lysates (with a standard deviation of 17 nm) and 93 nm for p39G1-programmed lysates (with a standard deviation of 18 nm). Close examination revealed an apparent outer lipid bilayer with underlying dense material (best seen in Fig. 6C). Comparison with sections from cells expressing the same Gag protein constructs revealed the particles to be very similar in morphology and in size, although the particles produced in cells were approximately 5 to 10 nm larger (compare Fig. 6B and C and Fig. 7A and B). Occasional incomplete forms were noted for the p39G1 reticulocyte lysate product (Fig. 6C, marked by asterisk), a finding identical to that described when this protein was produced in mammalian cells (25). This evidence, together with the sucrose gradient data already presented, supports the conclusion that HIV Gag proteins are capable of forming immature capsids in a cell-free system.

The formation of capsids in a cell-free system has recently

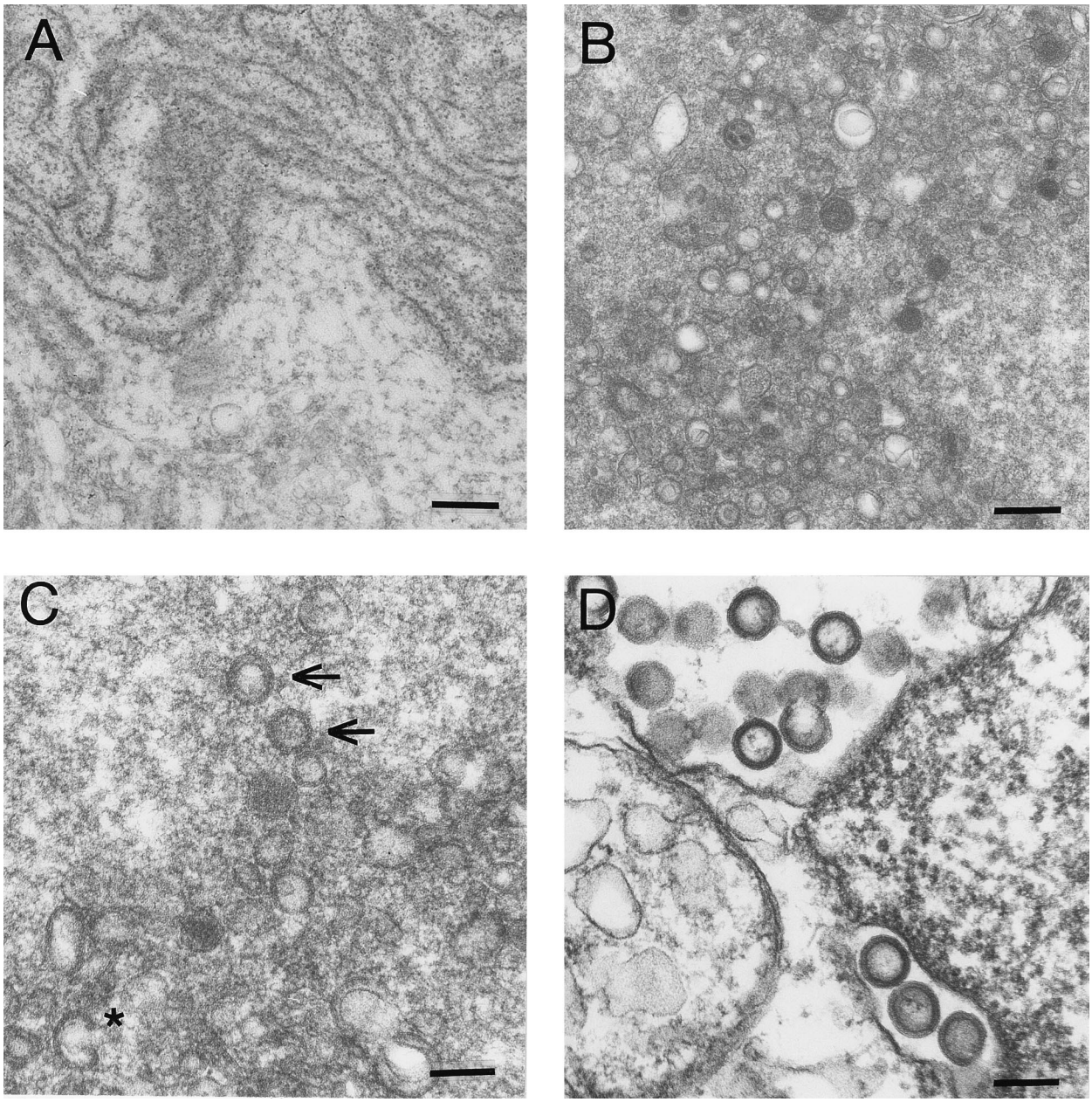

FIG. 6. Transmission electron microscopy of cell-free capsid formation. Reticulocyte lysates were diluted in NTE buffer and pelleted in a microultracentrifuge prior to processing for electron microscopy. (A) Negative control reticulocyte lysate (expression plasmid pTM3). Magnification, ×31,375; bar, 320 nm. (B) Cluster of 90- to 100-nm spherical structures in lysate expressing 39G1 product. Magnification, ×31,375; bar, 320 nm. (C) Immature capsids produced by expression of p39G1 in reticulocyte lysates (arrows). The asterisk indicates the position of incomplete forms. Magnification, ×62,750; bar, 159 nm. (D) Retrovirus-like particles in an intracellular compartment produced by expression of p39G1 in BSC-40 cells with the vaccinia virus/T7 RNA polymerase transient protein expression system. Magnification, ×62,750; bar, 159 nm.

been demonstrated for the Gag protein from the type D virus, M-PMV (14). Because this virus normally assembles intact capsids within cells, self-assembly of Gag proteins without a requirement for added cellular membranes is perhaps not unexpected. It is known that HIV CA protein in purified form will self-assemble into rod-shaped structures (7) but not into the form of an immature spherical capsid. It has also been recently reported that HIV CA-NC-p6 protein purified from bacteria can at high concentrations form sheets, cylinders, and occasional spherical structures and that efficient cylinder formation requires the addition of RNA (2). We now demonstrate for the first time that Gag proteins from HIV, a retrovirus which follows the type C assembly pathway, will self-assemble into immature capsid-like structures which closely resemble retrovirus-like particles produced in cells. The bouyant density of the capsids in this study (1.15 g/ml) is very similar to that of an intact, enveloped retrovirus particle (1.16 to 1.18 g/ml [6]). A mature retroviral core would be expected to have a density of 1.24 to 1.26 g/ml (6), while immature M-PMV cores produced in vitro demonstrate an equilibrium density of 1.19 g/ml (14). The lighter density of the capsids demonstrated in this study suggests that they are complexed with lipid. However, suscep-

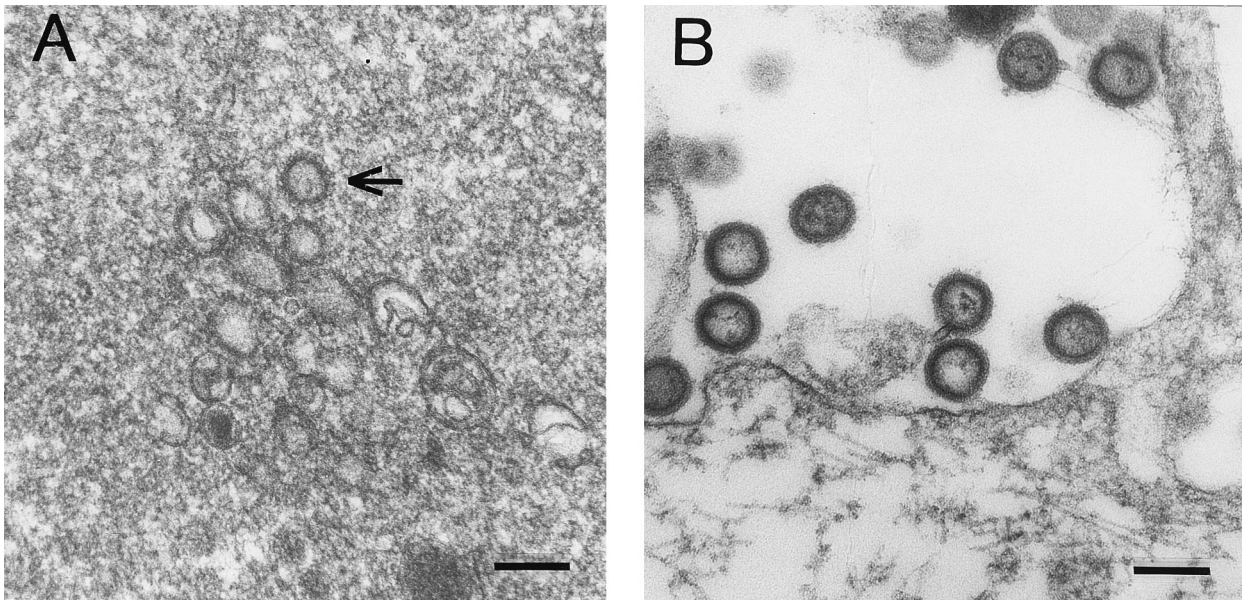

FIG. 7. Transmission electron microscopy of immature capsids. (A) Structures produced by reticulocyte lysate programmed with p55G1. The arrow indicates a complete capsid-like form approximately 90 nm in diameter. (B) Retrovirus-like particles budding from plasma membrane of BSC-40 cells following transfection with p55G1. Magnification, ×62,750; bars, 159 nm.

tibility to proteolytic digestion was demonstrated for the capsids in the absence of detergent, indicating that these structures cannot be completely enveloped. We conclude from these data and from the electron microscopy results that these structures may represent incompletely enveloped immature capsids. It is postulated that Gag proteins produced in reticulocyte lysates can derive a lipid coat from membranes which are present in the lysate itself (as shown in Fig. 6A) but are unable to complete envelopment and thus remain sensitive to proteolytic digestion. Alternatively, the in vitro-translated HIV immature capsids themselves may have a density of 1.15 g/ml in the absence of association with lipid.

The matrix protein region of the Gag polyprotein has been shown to be essential to the formation of HIV particles at the plasma membrane of cells (25, 28). The matrix protein has also been postulated to contribute to multimerization of the $Pr55^{Gag}$ molecule, together with domains within CA and NC (18). However, the present study has demonstrated that HIV Gag proteins produced in a cell-free system are capable of immature capsid formation in either the presence (p55G1) or absence (p39G1) of most of this region. These data are consistent with data from mammalian cell systems in which matrix-deleted HIV Gag proteins have been shown to form immature intracellular particles (8, 25). Thus, this study provides additional evidence that a contribution of the matrix protein region to Gag-Gag multimerization is not required for the formation of the immature capsid.

Reticulocyte lysate translation systems are complex, containing significant amounts of RNA, DNA, and ribosomes as well as other cellular macromolecules. It is possible that lysate components in addition to Gag proteins and membranes may have contributed in this study to successful capsid formation. RNA has been shown to act as a scaffolding in the formation of cylindrical multimers of Gag protein from Rous sarcoma virus and from HIV when high concentrations of purified protein and optimal pH and ionic strength are employed (2). A role for RNA in HIV capsid formation in reticulocyte lysates, despite the apparent stability of these capsids to RNase digestion, has not been ruled out. A goal of future studies in this area will be to fully define the individual components required for the formation of HIV capsids.

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ADDENDUM

While the manuscript was under review, Sakalian et al. (24) published data demonstrating the formation of capsids by M-PMV Gag protein produced in reticulocyte lysates. Their findings are in agreement with the findings of this study regarding the lower-equilibrium-density peak of HIV Gag proteins compared with that of M-PMV capsid-like structures and regarding the utility of reticulocyte lysate reactions for studying retroviral capsid formation in vitro. However, these investigators were unable to visualize capsid structures by electron microscopy when HIV $Pr55^{Gag}$ was expressed in their reticulocyte lysates. We cannot fully explain this discrepancy. However, it is likely that either differences in Gag protein translation levels in our experiments or differences in the methods used in pelleting and preparing samples account for the variation in detection of in vitro-translated HIV capsids by electron microscopy. The level of efficiency of production or stability of the capsid-like structures produced in vitro by M-PMV Gag may be higher than that of the HIV Gag capsids, making detection of the M-PMV products more apparent. Future studies are planned to address these questions.

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