Human Immunodeficiency Virus Type 1 Gag Assembly through Assembly Intermediates*

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Yuko Morikawa‡§, Toshiyuki Goto¶, and Fumitaka Momose‡

From the ‡Kitasato Institute of Life Sciences, Kitasato University, Shirokane 5-9-1, Minato-ku, Tokyo 108-8641 and the §College of Medical Technology, Kyoto University, Kawahara-cho 53, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan

Human immunodeficiency virus Gag protein self-assembles into spherical particles, and recent reports suggest the formation of assembly intermediates during the process. To understand the nature of such assembly intermediates along with the mechanism of Gag assembly, we employed expression in Escherichia coli and an in vitro assembly reaction. When E. coli expression was performed at 37 °C, Gag predominantly assembled to a high order of multimer, apparently equivalent to the virus-like particles obtained following Gag expression in eukaryotic cells, through the formation of low orders of multimer characterized with a discreet sedimentation value of 60 S. Electron microscopy confirmed the presence of spherical particles in the E. coli cells. In contrast, expression at 30 °C resulted in the production of only the 60 S form of Gag multimer, and crescent-shaped structures or small patches with double electron-dense layers were accumulated, but no complete particles. In vitro assembly reactions using purified Gag protein, when performed at 37 °C, also produced the high order of Gag multimers with some 60 S multimers, whereas the 30 °C reaction produced only the 60 S multimers. However, when the 60 S multimers were cross-linked so as not to allow conformational changes, in vitro assembly reactions at 37 °C did not produce any higher order of multimers. ATP depletion did not halt Gag assembly in the E. coli cells, and the addition of GroEL-GroES to in vitro reactions did not facilitate Gag assembly, indicating that conformational changes rather than protein refolding by chaperonins, induced at 37 °C, were solely responsible for the Gag assembly observed here. We suggest that Gag assembles to a capsid through the formation of the 60 S multimer, possibly a key intermediate of the assembly process, accompanied with conformational changes in Gag.

The major structural component of human immunodeficiency virus (HIV), Gag, is the sole protein required for viral particle budding, and expression of Gag protein alone in eukaryotic cells produces Gag virus-like particle (VLP), morphologically identical to the immature form of HIV particles (1–3). The process of Gag assembly is thought to consist of N-terminal myristoylation of Gag followed by relocation to the plasma membrane and multimerization of Gag to form VLP. Three discrete Gag regions responsible for virus particle production have been identified by genetic studies and termed the membrane-binding, the interacting, and the late domains. The membrane-binding domain is located at the N-terminal matrix/membrane (MA) of Gag and contains a bipartite membrane-binding signal (N-terminal myristoylation and a cluster of basic amino acids) that directs the association of Gag with membrane (1, 4, 5). The interacting domain is essential for Gag-Gag interactions and spans from the central capsid (CA) to nucleocapsid (NC) of Gag (6–9). The late domain, responsible for pinching off viral particles from the membrane, has been found in the C-terminal p6 domain of Gag (10, 11) but, in high level of expression systems, is often dispensable (1, 2).

An HIV particle is composed of ~2000 Gag molecules (12) that are arranged in a high order form. In fact, studies by high resolution electron microscopy have revealed that Gag molecules are arranged in a fullerene- or cage-like network consisting of hexagonal and trigonal units (13, 14), suggesting the ordered multimerization of Gag in a virus particle. However, little is known about the mechanisms involved in the formation of Gag capsid. The process of building Gag up into capsid remains to be elucidated and includes such questions as whether assembly consists of multiple sets of reactions involving discrete intermediates or a single sequential reaction and, in both cases, what the mechanism of Gag multimerization might be.

A number of electron microscopy studies to date have provided evidence for electron-dense Gag layers underneath the plasma membrane and nascent particles connected to the cell surface by a thin stalk (15), suggesting that Gag assemblies following membrane targeting and also suggesting that assembly intermediates, if any, would be similarly present on the membrane. Consistent with these observations, membrane-bound Gag complexes have been resolved on Optiprep density gradients (16). However, recent observations of Gag-expressing cells have revealed the occurrence of Gag complexes in the cytoplasm, suggesting Gag multimer formation prior to membrane relocation (17, 18). Similarly, data based on the detergent sensitivity of Gag complexes have suggested a detergent-resistant complex in the cytosol (19, 20), although these cytosolic Gag complexes may possibly be a dead end product (16, 21). These apparently conflicting results argue that the morphogenetic pathway of Gag assembly is not as clear cut as is commonly thought, but the data clearly show that some level of Gag multimer, plausibly assembly intermediates, occur during particle assembly. The definition of such intermediates would lead to a better understanding of the sequential nature of the Gag assembly reactions.

As a result of the lack of host N-myristoyltransferase, Gag expression in Escherichia coli confers neither N-myristoylation
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We have shown previously, using purified Gag protein lacking the C-terminal p6 domain, that the \textit{in vitro} assembly reaction is composed of two sequential steps: the formation of a 60 S complex, possibly an assembly intermediate, and complete assembly to 600 S, equivalent to the immature form of HIV capsids (26). Although the ideal for study of Gag assembly would be in higher eukaryotic cell expression systems, the level of Gag expression in such systems is often insufficient for biochemical and structural analyses, and neither expression nor assembly of Gag is synchronized. To examine the nature of assembly intermediates along with the mechanism of assembly, we have employed an inducible expression system of \textit{E. coli} and \textit{in vitro} assembly reaction. Our data show that the 60 S forms of Gag multimers have a defined structure and detergent sensitivity, suggesting that they may be genuine Gag assembly intermediates. The data also suggest that the Gag assembly process may be accomplished by conformational changes in Gag.

EXPERIMENTAL PROCEDURES

Materials—\textit{E. coli} expression vector pTrcHisA was purchased from Invitrogen, and metal chelate resin (HisBind Resin) was from Novagen. Sephadex G-25 (PD-10) and a high molecular weight calibration kit (Amersham Biosciences), 80 S ribosome, and 80 S ribosome for 4 h at 4 °C. Alternatively, the protein sample was applied onto a 20–70% (w/v) sucrose gradient and sedimented in an SW55 rotor at 150,000 × g for 4 h at 4 °C. After centrifugation, the fractions were incubated at either 37 or 30 °C for 3 h. In some experiments, assembly intermediates of Gag were incubated in the presence of 10% (v/v) glycerol (see the legend for Fig. 8) or incubated with GroEL-GroES (with a Gag-to-GroEL-GroES molar ratio of 1:1) in the presence of 1 mM ATP or ATP-β-S (see the legend for Fig. 10).

Cross-linking—For cross-linking, assembly intermediates of Gag were dialyzed against phosphate-buffered saline (pH 8.0) (for cross-linking with DTBP) or 0.2 M triethanolamine (pH 8.0) (for cross-linking with DTBP). DSP or DTBP was added to a final concentration of 1 mM, and the mixtures were incubated at room temperature for 30 min. After quenching with 50 mM Tris (pH 8.0), the materials were dialyzed in buffer A and subjected to \textit{in vitro} assembly reactions at 37 °C for 3 h.

Gradient Analysis—Protein was applied onto a 15–35% (w/v) sucrose gradient and sedimented in an SW55 rotor (Beckman Coulter) at 150,000 × g for 4 h at 4 °C. Alternatively, the protein sample was applied onto a 20–70% (w/v) sucrose gradient and sedimented in an SW55 rotor at 120,000 × g for 2 h at 4 °C. After centrifugation, the fractions were fractionated from the bottom to the top. A high molecular mass calibration kit (Amersham Biosciences), 80 S ribosome, and the immature form of HIV Gag VLPs purified from the supernatant of Gag-expressing S9 cells were used for molecular mass markers for sedimentation analyses.

Treatment with Detergent and Urea—Assembly intermediates of Gag were treated with 0.5% Triton X-100, 0.1 or 1.0% sodium deoxycholate, 0.1% SDS, or 6 M urea at room temperature for 15 min. Following treatment, the materials were centrifuged in an SW55 rotor at 220,000 × g for 4 h at 4 °C. As a control, the immature form of HIV Gag VLP purified from the supernatant of Gag-expressing S9 cells was similarly treated with the detergents or urea.

Protein Detection—Proteins were separated by SDS-PAGE and detected by Coomassie Brilliant Blue or silver staining or subjected to Western blotting using anti-HIV-1 CA monoclonal antibody.

Electron Microscopy—Electron microscopic examination was carried out by the standard procedures. Assembly intermediates were collected by centrifugation in an SW55 rotor at 220,000 × g for 4 h at 4 °C and fixed with 2% glutaraldehyde and subsequently with 1% osmium tetroxide. Ultrathin sections were stained with uranyl acetate and lead citrate.

RESULTS

Time Courses of HIV Gag Expression and Assembly in \textit{E. coli}.—The HIV-1 gag gene with the additional sequence encoding 6 histidine residues at the C terminus was cloned into pTrcHisA vector and used for Gag expression in \textit{E. coli} cells, as described previously (26). \textit{E. coli} was grown at 37 and 30 °C, and protein expression was induced by the addition of IPTG. To monitor Gag expression, whole cell lysates were prepared at 1-, 2-, and 4-hour time points following IPTG induction and subjected to SDS-PAGE followed by Coomassie Brilliant Blue staining (Fig. 1, upper) and Western blotting with anti-HIV-1 CA antibody (Fig. 1, lower). The levels of Gag expression were broadly equivalent with similar kinetics between the two temperatures (Fig. 1, whole cells). However, when the cell lysates were subjected to subcellular fractionation by centrifugation at 15,000 × g for 30 min, striking differences were observed between the two temperatures. In the 37 °C samples, Gag antigens were initially found in the supernatant but recovered in the pellets after 2 h of induction (Fig. 1, left). In contrast, when \textit{E. coli} was maintained at 30 °C, Gags were constantly recovered in the
supernatants throughout the period observed here (up to 4 h) (Fig. 1B). It has been reported for Mason-Pfizer monkey virus that Gag assembles to spherical particles in E. coli and forms inclusion bodies, which were precipitated by centrifugation under similar conditions (28).

To examine whether the temperature-dependent differences may represent the assembly state of the expressed Gag protein, sedimentation experiments using sucrose gradients were carried out. Whole cell lysates made at each time point were clarified by centrifugation at 1,000 × g for 3 min and then sedimented through 20–70% sucrose gradients at 120,000 × g for 2 h. Gag antigens spread within the gradients were detected by Western blotting. When the 37 °C samples were analyzed, a progression of Gag toward heavier gradient fractions was apparent with increasing incubation times (Fig. 2A, left). Gag antigens were initially found at the top of the gradient but, at 2 h after induction, sedimented to 50% sucrose fractions with a trace of Gag in the 25–30% sucrose fractions. By 4 h, the mobility shift to 50% sucrose fractions was largely complete. In contrast, when E. coli was maintained at 30 °C, Gag antigens were initially found at the top of the gradient and later shifted to the 25–30% sucrose fractions but not to the 50% sucrose fractions (Fig. 2A, right). These data show that at 37 °C, synthesized Gag forms a large complex from relatively small molecular weight complexes, whereas in contrast, at 30 °C, Gag remains in relatively small complexes.

Electron microscopy was carried out to examine whether the Gag complexes identified by gradient analysis had defined structures. Doughnut-like particles, typical of the immature form of Gag capsids, were observed in the E. coli cells maintained at 37 °C (Fig. 2B, left), confirming previous reports in which retroviral Gag protein (e.g. Mason-Pfizer monkey virus, Rous sarcoma virus, and HIV) assemble into VLPs inside E. coli cells (24, 27, 28). In contrast, no particles were seen in E. coli cells when maintained at 30 °C (Fig. 2B, right).

Sedimentation Profiles of Assembly Intermediates—To enrich the small complex form of Gag, E. coli cells were harvested at each time point after IPTG induction, and Gags were purified from the supernatants of the cell lysates by use of the Gag C-terminal polyhistidine tag. Following metal chelate chromatography, the eluted fractions were analyzed by SDS-PAGE followed by Coomassie Brilliant Blue staining (upper panels) and Western blotting with anti-HIV-1 CA antibody (lower panels). The lanes are as follows: M, prestained molecular mass markers; No, E. coli transformed with a parental expression vector pTrcHisA; 0–4, E. coli transformed with the HIV-1 gag gene-containing vector. 0 shows before induction, and 1, 2, and 4 show h after induction, respectively.

![Time courses of Gag expression in E. coli](image)

**Fig. 1.** Time courses of Gag expression in E. coli. E. coli was grown at 37 °C (left panels) and 30 °C (right panels). Following the addition of IPTG, cells were harvested at intervals. The cells were resuspended in 50 mM Tris (pH 8.0), 150 mM NaCl, 5 mM EDTA, and 1 mM dithiothreitol and disrupted by sonication. Subcellular fractionation was carried out by centrifugation at 15,000 × g for 30 min. Equal proportions of the whole cell lysates, the pellets, and the supernatants were analyzed by SDS-PAGE followed by Coomassie Brilliant Blue staining (upper panels) and Western blotting using anti-HIV-1 CA antibody (lower panels). The lanes are as follows: M, prestained molecular mass markers; No, E. coli transformed with a parental expression vector pTrcHisA; 0–4, E. coli transformed with the HIV-1 gag gene-containing vector. 0 shows before induction, and 1, 2, and 4 show h after induction, respectively.
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**FIG. 2.** Time courses of Gag assembly in E. coli. A, gradient analysis of the whole cell lysates of E. coli. E. coli was grown at 37 °C (left panels) and 30 °C (right panels) and, after IPTG induction, the cells were harvested at intervals. The whole cell lysates were prepared as described in the legend for Fig. 1 and then incubated with 10 μg/ml RNase A for 10 min. Following clarification by brief centrifugation at 1,000 × g for 3 min, the lysates were applied on 20–70% (w/v) sucrose gradients and centrifuged at 120,000 × g for 2 h at 4 °C. Gradient fractions from the bottom to the top (left to right) were analyzed by Western blotting using anti-HIV-1 CA antibody. Lane M shows prestained molecular mass markers. B, electron microscopy of E. coli. After 4 h of IPTG induction, E. coli cells were collected and subjected to electron microscopic analysis. Scale bars represent 100 nm.

Temperature-dependent. Monomeric Gag was purified following 1 h of IPTG induction and subjected to the in vitro assembly reaction described previously (26). The in vitro assembly products were analyzed on 20–70% sucrose gradients and compared with 80 S ribosomes and the immature form of HIV capsids sedimented in parallel. When the in vitro assembly reaction was carried out at 30 °C, Gag, whether derived from E. coli cultured at 37 or 30 °C, sedimented to a position corresponding to 60 S (Fig. 5, middle). In contrast, when the reaction was carried out at 37 °C, the sedimentation profiles for both Gags were shifted essentially to that of the immature form of Gag capsids, although a small fraction of Gag was still observed at 60 S (Fig. 5, bottom). These data indicate that, in our in vitro assembly system, the 37 °C reaction produces only 60 S Gag multimers and that higher order assembly requires incubation at 37 °C, paralleling the Gag assembly observed in E. coli cells. The data from the in vitro system also show that the levels of Gag assembly were dependent on the temperatures of the in vitro reaction but not on the temperatures at which the Gags were purified from E. coli.

**Morphology of Assembly Intermediates**—Electron microscopic examination was used to examine whether the 60 S Gag complex had a defined structure. E. coli-produced 60 S complex was purified by metal chelate chromatography from E. coli following 4 h of IPTG induction at 30 °C, and in vitro-assembled 60 S complex was prepared by in vitro reaction at 30 °C for 3 h using purified monomeric Gag protein. When observed by ultrathin section transmission microscopy, the E. coli-produced 60 S complex did not exhibit doughnut-like structures but rather exhibited crescent-shaped structures with double electron-dense layers. The structures very often resembled small patches of electron-dense materials observed under the plasma membrane of Gag-expressing eukaryotic cells (Fig. 6A). The in vitro-assembled 60 S complex showed a similar structure. The complex formed electron-dense structures with a gentle curvature, although the contours of the structures were not as sharp when compared with the 60 S complex produced from E. coli (Fig. 6B), suggesting that Gag-Gag interaction may be somewhat weaker following the in vitro assembly reaction. For comparison, we enriched the large Gag complex, corresponding to 600 S, which was observed in E. coli cells (Fig. 2, left) as described previously (28). As expected, the structure of the complex showed a hollow sphere surrounded by a double ring structure (Fig. 6C), typical of the immature form of Gag capsids. These data support the conjecture that the 60 S Gag complex observed in this work may represent a distinct assembly intermediate formed during the process of Gag assembly. The data also suggest that the formation of Gag capsids is not initiated by random accumulation of Gag proteins but rather by an ordered arrangement of Gag molecules following a defined assembly pathway.

**Detergent Resistance of Assembly Intermediates**—It has been reported that the immature forms of retroviral capsids remain intact following treatment with nonionic detergents such as Triton X-100 and Nonidet P-40 (34, 35) but are dissociated by SDS and urea. Accordingly, the detergent resistance of the 60 S Gag complexes observed here was examined to reveal the similarities with completely assembled Gag capsids. As the 60 S Gag complexes, whether E. coli-produced or in vitro-assembled, were wholly pelleted by centrifugation at 220,000 × g for 4 h (Fig. 7A), the centrifugation conditions were used for an analysis of the effect of detergent treatment. Irrespective of...
FIG. 4. Gradient analysis of Gag assembly intermediates produced in *E. coli*. Expression and purification of Gag were carried out as described in the legend for Fig. 3. The Gag protein purified after 1 or 4 h of induction was analyzed on 20–70% (w/v) sucrose gradients by centrifugation at 120,000 × g for 2 h (right panels) and on 15–35% (w/v) sucrose gradients by centrifugation at 150,000 × g for 4 h (left panels). Gradient fractions were subjected to SDS-PAGE followed by silver staining. Sedimentation markers are as follows: the immature form of HIV capsids (600 S) detected by Western blotting (left top panel); high molecular mass calibration markers consisting of thyroglobulin (669 kDa = 2 × 330 kDa), ferritin (440 kDa = 2 × 220 kDa), catalase (252 kDa = 4 × 60 kDa), lactate dehydrogenase (140 kDa = 4 × 36 kDa), and albumin (67 kDa) (Amersham Biosciences), stained with Coomassie Brilliant Blue (right top panel). Arrows on the top panels show a sedimented position of 80 S ribosomes. Lane *M* shows pre-stained molecular mass markers for SDS-PAGE.

their origin, the 60 S Gag complexes were not dissociated by treatment with 0.5% Triton X-100, mimicking the stability of the immature form of HIV capsids in the presence of the detergent. In contrast, when treated with 6 M urea or 0.1% SDS, the complexes dissociated completely, as was also observed with the immature form of Gag capsids. Differences were observed when the materials were treated with 0.1% sodium deoxycholate, however. The immature forms of Gag capsids were partially dissociated by the treatment, whereas in contrast, the 60 S forms of Gag complexes were completely dissociated (Fig. 7B). Treatment with 1% sodium deoxycholate led to complete dissociation of the immature form of Gag capsids (data not shown).

Mechanisms of Assembly—Our *in vitro* assembly reactions suggest that incubation at 37 °C was solely responsible for the formation of a high order of Gag multimer (Fig. 5). A simple interpretation of the data would be that Gag conformation might be altered or that Gag-Gag interactions might be stimulated at 37 °C. To test these possibilities, the 60 S Gag multimers were cross-linked, not to allow large conformational changes but possibly to allow multimer-multimer interactions in subsequent *in vitro* assembly reactions, and then subjected to sedimentation analysis on 20–70% sucrose gradients. When the 60 S Gag multimer was cross-linked with DSP, the *in vitro* reaction at 37 °C did not produce any higher order of multimers (Fig. 8, upper middle). A similar finding was observed when DTBP, an imidodester cross-linker potentially retaining the native conformation of protein, was used (Fig. 8, lower middle).

These data suggest that Gag conformational changes may be required for higher order assembly, although it cannot be ruled out that unwanted side chains introduced by the cross-linkers might inhibit multimer-multimer interactions. As glycerol is known to stabilize protein conformation, we also tested the effect of glycerol on Gag assembly. *In vitro* assembly reactions with the 60 S Gag multimers in the presence of 10% (v/v) glycerol, even when carried out at 37 °C, did not produce any higher order of multimers (Fig. 8, bottom), suggesting that stabilization of the multimers may have an inhibitory effect on the higher order of Gag multimerization. Alternatively, exposure to glycerol, a hydrogen donor, might lead to weakening the hydrogen bonds possibly formed by higher order assembly. It is unlikely that the addition of glycerol might cause an increase in the solution viscosity, leading to a reduction in protein mobility, because our *in vitro* reactions were carried out at 37 °C, a relatively high temperature.

Some recent studies have suggested that retroviral Gag assembly is energy-dependent (36–38). It is possible that the Gag assembly in *E. coli* cells observed in this study might occur in an energy-dependent manner but not simply in a temperature-dependent manner. To this end, NaN₃, which has been shown to deplete cellular ATP from *E. coli* (32, 33), was added at a range of 0–10 mM to the culture medium following IPTG addition, and *E. coli* was cultured at 37 °C for 4 h, conditions under which the majority of Gag normally assembles up to 600 S. Western blotting of whole cell lysates revealed near equivalent levels of Gag expression up to 5 mM NaN₃, suggesting little
**FIG. 5.** Gradient analysis of Gag assembly intermediates produced by *in vitro* assembly reaction. *E. coli* was cultured at 37 and 30 °C and, after 1 h of IPTG induction, Gag protein was purified by metal chelate chromatography as described in the legend for Fig. 3. Eluted fractions were desalted using Sephadex G-25 (PD-10) equilibrated with buffer A (20 mM Tris (pH 8.6 adjusted at room temperature), 100 mM NaCl, 0.2 mM EDTA, 5 mM MgCl₂, and 1 mM dithiothreitol). For *in vitro* assembly, the fractions were incubated at 30 °C (middle panels) or 37 °C (bottom panels) for 3 h. The products were applied on 20–70% (w/v) sucrose gradients and centrifuged at 120,000 × g for 2 h at 4 °C. Gradient fractions from the bottom to the top (left to right) were analyzed by SDS-PAGE followed by silver staining. *In vitro* assembly reaction was carried out using monomeric Gag protein purified from 37 °C-cultured *E. coli* (left panels) and using monomeric Gag protein purified from 30 °C-cultured *E. coli* (right panels). Arrows show sedimented positions of the immature form of HIV capsids (600 S) and 80 S ribosomes. Lane M shows prestained molecular mass markers for SDS-PAGE.

**FIG. 6.** Electron microscopy of Gag assembly intermediates. *E. coli*-produced 60 S complex was purified from *E. coli* following 4 h of induction at 30 °C (A), and *in vitro*-assembled 60 S complex was prepared by *in vitro* reaction at 30 °C for 3 h (B). For comparison, *E. coli*-produced 600 S complex, similar to the immature form of authentic Gag capsids, was purified as described previously (28) (C). The materials were observed by ultrathin section transmission. Scale bars represent 100 nm.

**FIG. 7.** Stability of Gag assembly intermediates. A, fractionation by centrifugation. Materials were centrifuged at 4 °C either at 120,000 × g for 1 h or at 220,000 × g for 4 h. The pellets (P) and supernatants (S) were analyzed by SDS-PAGE followed by Western blotting using anti-HIV-1 CA antibody. Pre shows materials before centrifugation. B, fractionation following detergent/urea treatment. Materials were treated with 0.5% Triton X-100, 0.1% sodium deoxycholate, 6 M urea, or 0.1% SDS and subjected to centrifugation at 220,000 × g for 4 h. Following fractionation into pellets and supernatants, Gag antigens were similarly detected by Western blotting. Materials are as follows: 1, the immature form of HIV capsids; 2, *E. coli*-produced 60 S complex; 3, *in vitro*-assembled 60 S complex. Lane M shows prestained molecular mass markers.
effect on Gag synthesis. In contrast, Gag expression was severely impaired when added at 7 mM. When cell lysates were subjected to subcellular fractionation by centrifugation as before, the majority of Gag antigens was recovered in the pellets in the 0–5 mM treatments (Fig. 9A). Consistent with this, when whole cell lysates were subjected to sedimentation analysis on 20–70% sucrose gradients, Gag multimerization to the 600 S form appeared unaffected up to 5 mM but was abolished at 7 mM when Gag synthesis was also impaired (Fig. 9B). Although we did not measure the intracellular levels of ATP, it is unlikely in this cell system that Gag assembly process is ATP-dependent.

We also tested whether the higher order assembly observed here, although temperature-dependent, could be facilitated with molecular chaperons. Equimolar GroEL-GroES was added to an in vitro assembly reaction with the 60 S Gag multimer, and the mixture was incubated in the presence of 1 mM ATP (or ATP-γS) at 37 °C for only 1 h, conditions under which Gag alone assembles to 150–350 S but not up to 600 S (26). Sedimentation analysis of the assembly products showed Gag distribution at a broad range of 150–350 S, essentially similar to that obtained by an in vitro assembly reaction with the 60 S Gag multimer alone (Fig. 10), indicating that GroEL-GroES did not facilitate Gag assembly. These data suggest that GroEL-GroES may not be capable of refolding of Gag or that the Gag molecule used here has already been folded into assembly-competent forms.

**DISCUSSION**

Retroviral Gag protein is initially synthesized in the cytosol but undergoes a process of assembly to form a viral particle. Particle assembly must require the ordered multimerization of Gags, and some studies suggest that the formation of assembly intermediates occurs during this process. Crystallographic studies have provided evidence of trimerization by MA (39, 40) and dimerization by CA (30, 41–44), and in vitro assembly studies have suggested Gag dimerization through NC-RNA interaction (45). Similar Gag oligomeric forms were observed in Gag-expressing eukaryotic cells (31). These data suggest that the minimum assembly units of Gag are a dimer and a trimer. In contrast, electron microscopic examination has provided clear evidence of only large-sized assembly intermediates, vis-

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**Fig. 8.** Effect of cross-linking and glycerol on higher order assembly. Gag assembly intermediates were incubated with DSP (upper middle panel) or DTBP (lower middle panel) at room temperature for 30 min. For the higher order of assembly, the materials were dialyzed in buffer A (as described in the legend for Fig. 5) and incubated at 37 °C for 3 h. Alternatively, Gag assembly intermediates were incubated at 37 °C for 3 h in the presence of 10% (v/v) glycerol (bottom panel). The products were analyzed on 20–70% (w/v) sucrose gradients by centrifugation at 120,000 × g for 2 h at 4 °C. Gradient fractions from the bottom to the top (left to right) were subjected to SDS-PAGE followed by silver staining. Arrows show sedimented positions of the immature form of HIV capsids (600 S) and 80 S ribosomes. Lane M shows prestained molecular mass markers for SDS-PAGE.

**Fig. 9.** Gag assembly in E. coli treated with NaN₃. A, subcellular fractionation of E. coli. Following the addition of IPTG and subsequently NaN₃, E. coli was grown at 37 °C for 4 h. Cell harvest and subcellular fractionation were carried out as described in the legend for Fig. 1. Equal proportions of the whole cell lysates, the pellets, and the supernatants were analyzed by SDS-PAGE followed by Western blotting using anti-HIV-1 CA antibody. The lanes are as follows: M, prestained molecular mass markers; Pre, before induction; 0, 3, 5, 7, and 10, 4 h after induction. 0, 3, 5, 7, and 10 show mM NaN₃, respectively. B, gradient analysis of the whole cell lysates of E. coli. The whole cell lysates prepared as described above were incubated with 10 μg/ml RNase A. Following clarification by brief centrifugation, the lysates were analyzed on 20–70% (w/v) sucrose gradients by centrifugation at 120,000 × g for 2 h at 4 °C. Gradient fractions from the bottom to the top (left to right) were analyzed by Western blotting using anti-HIV-1 CA antibody. Lane M shows prestained molecular mass markers.
Effective for C type retroviruses and lentiviruses such as HIV in Gag-expressing cells as electron-dense layers underneath the plasma membrane and membrane projections with nascent Gag capsules (15). These structures are likely to be assembly intermediates at a relatively late stage of the assembly process.

For putative assembly intermediates, we initially used E. coli expression systems and noted the appearance of 60 S Gag multimers that subsequently converted to completed 600 S capsids. Consistent with this finding, when the monomeric form of purified Gag was subjected to an in vitro assembly reaction, a similar level of Gag multimers formed that also subsequently shifted to 600 S, corresponding to completely assembled products. The data suggest that the 60 S form of Gag multimers is likely to be an assembly intermediate in these assembly systems. No other larger size classes of intermediate were detected in our studies. Although there have been a few reports on the biochemical features of such intermediates, one study has previously shown, by gradient analysis of in vitro translation products, discrete size classes of Gag intermediates at 10, 80, and 150 S, which were converted to a final completed capsid product with increasing reaction time (36). Our speculation is that the 80 S complex equates to the 60 S multimer observed in our work, as the S value assignments for both intermediates are very approximate estimates. It should be noted that in our study, the 60 S multimers accumulated when E. coli induction and in vitro assembly reactions were carried out at 30 °C but not at 37 °C (Figs. 2 and 5). When experiments were carried out at 37 °C, the 60 S multimers were only a transient form and rapidly converted to completely assembled capsids. This means that, in any studies at higher temperatures, Gag assembly occurs more rapidly and completely with the result that assembly intermediates may be more difficult to observe. Interestingly, in the in vitro translation experiments in which the formation of the 80 S forms of Gag complex was seen, assembly was carried out at 25 °C (36). Thus, incubation at lower temperatures might be the key factor in the observation of intermediates in both studies.

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For biochemical and structural studies of the Gag assembly intermediates, we purified the 60 S Gag complex and carried out experiments on detergent sensitivity and observation by electron microscopy. The stability profiles of the E. coli-produced and in vitro-assembled 60 S complexes were largely similar to those of the immature form of Gag capsids, suggesting somewhat parallel characteristics between partially assembled Gag products and complete Gag capsids. These suggestions were supported by our electron microscopic observations that both 60 S forms of Gag complexes had defined structures such as opposed electron-dense layers. The data suggest that the process of assembly involves Gag being arranged in order from the beginning of the assembly process rather than being rearranged following random accumulation.

However, the instability of the 60 S Gag multimer in 0.1% sodium deoxycholate and the requirement of incubation at 37 °C for higher order assembly suggest that there may be some structural differences between the 60 S form of Gag multimers and the complete form of Gag capsids. To understand the higher order of Gag assembly, we used cross-linkers so as not to allow Gag conformational changes and found that, once cross-linked, the 60 S Gag multimers did not shift to any higher order forms. These data suggest, although do not prove, that Gag assembly may be accompanied by the conformational changes in Gag induced at 37 °C. A similar in vitro assembly study with Rous sarcoma virus Gag protein has suggested that acidic pH triggers conformational changes in Gag, leading to a high order of Gag multimerization such as VLP (46).

Some recent studies have suggested the involvement of ATP-dependent pathways in retroviral Gag assembly (36–38), but the stage at which ATP acts has not been determined. One study in which ATP was depleted from COS-1 cells expressing HIV Gag showed that the blockage occurs at the stage at which the VLPs are pinched off from membrane but not at any earlier stages such as Gag membrane binding and multimerization (37). However, when a similar experiment was carried out with Mason-Pfizer monkey virus Gag, a prototype for capsid formation prior to membrane relocation, the blockage was observed at both stages of capsid assembly and transport (38). Another study in which ATP was depleted from an in vitro translation system for the synthesis of HIV Gag showed the blockage at the stage of Gag multimerization, suggesting the involvement of a chaperonin-like host factor (36, 47). These somewhat conflicting data need to be reconciled by further investigation. In our study, neither ATP nor GroEL-GroES, a prokaryotic chaperonin, was required for Gag multimerization, although we cannot rule out the possibility that eukaryotic chaperonins may support Gag multimerization in higher eukaryotic cells. The association of retroviral Gags with eukaryotic chaperonins have recently been reported (47, 48), but it remains to be elucidated whether or not Gags are folded into assembly-competent forms by the chaperonins.

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Yuko Morikawa, Toshiyuki Goto and Fumitaka Momose

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