Retroviral Gag protein is sufficient to produce Gag virus-like particles when expressed in higher eukaryotic cells. Here we describe the in vitro assembly reaction of human immunodeficiency virus Gag protein, which consists of two sequential steps showing the optimal conditions for each reaction. Following expression and purification, Gag protein lacking only the C-terminal p6 domain was present as a monomer (50 kDa) by velocity sedimentation analysis. Initial assembly of the Gag protein to 60 S intermediates occurred by dialysis at 4 °C in low salt at neutral to alkaline pH. However, higher order of assembly required incubation at 37 °C and was facilitated by the addition of Mg2+. Prolonged incubation under these conditions produced complete assembly (600 S), equivalent to Gag virus-like particles obtained from Gag-expressing cells. Neither form assembled by treatment with nonionic detergent, suggesting that correct assembly might occur in vitro. Electron microscopic observation confirmed that the 600 S assembly products were spherical particles similar to authentic immature human immunodeficiency virus particles. The latter assembly stage but not the former was accelerated by the addition of RNA although not inhibited by RNaseA treatment. These results suggest that Gag protein alone assembles in vitro, but that additional RNA facilitates the assembly reaction.

The main structural component of human immunodeficiency virus (HIV) particle, Gag, is encoded by the gag gene and is the sole protein required for formation of Gag virus-like particles (VLPs), analogous to the immature form of authentic HIV. Accordingly, expression of Gag protein alone by recombinant viruses or by transfection of expression plasmids leads to simultaneous assembly and budding of Gag VLPs from the cell surface (1–4). This process is thought to consist of several steps: N-terminal myristoylation of Gag protein followed by targeting to the plasma membrane and self-assembly of Gag protein underneath the plasma membrane to form Gag VLPs and budding (1, 5, 6). Although the N-terminal myristoylation is essential for Gag targeting to the plasma membrane (1, 7, 8), assembly of the Gag protein itself appears not to require the myristoylation, since nonmyristoylated Gag protein co-assembles with myristoylated Gag protein and is found in budded Gag VLPs (4, 9, 10). Furthermore, expression of Gag protein in Escherichia coli, which lacks N-myristoyltransferase activity (11), yields Gag VLP-like structures inside the cells (12) despite the lack of Gag myristoylation.

HIV Gag protein consists of four distinct domains, the N-terminal matrix (MA, p17), the central capsid (CA, p24), the nucleocapsid (NC, p7), and the C-terminal p6 domain (13), each of which is produced by processing of Gag protein during or soon after virus particle budding (14, 15). HIV particles just after budding are spherical but, concomitant with Gag processing, are transformed to particles containing conical cores. The Gag regions responsible for virus particle assembly have been extensively studied by amino acid deletion and substitution experiments, and evidence has accumulated to suggest that the C-terminal third of the CA domain, including the p2 peptide, which is located at CA/NC junction, is essential (16–19). In contrast, most of NC and the entire p6 domain are dispensable for Gag VLP formation (1–3, 20), although the NC domain contains a crucial determinant for packaging of viral genomic RNA (21–25). Data on the requirement of the MA domain for assembly have been apparently conflicting. Recent studies have shown that deletion of the entire globular domain of MA does not abolish virus particle formation (5, 26, 27), yet the globular domain plays a key role for trimerization of MA as well as MA-CA, suggesting the contribution of MA to authentic Gag assembly (28, 29).

In contrast to these in vivo analyses, in vitro assembly of retroviral Gag protein was originally reported for Mason-Pfizer monkey virus, showing a spherical structure following renaturation of partially purified Gag protein (30), but the optimal conditions for the assembly reaction were not studied. Recently, the in vitro assembly giving rise to long tubular rather than spherical structures has been reported using the individual Gag domains such as CA and CA-NC (31, 32). Morphological conversion of the assembly products from tubes to spheres was observed when several amino acids of MA were fused onto the N terminus of the CA domain (12, 33), but unfortunately this construct was devoid of the globular domain of MA and the p2 region. More recently, the in vitro assembly of Gag protein including these domains has been carried out, showing spherical particles (34). However, the conditions for the in vitro assembly reaction appeared not to be optimized, as the in vitro assembled particles were much smaller than authentic HIV particles. To understand the authentic Gag assembly reaction, it is necessary to establish an efficient in vitro assembly reaction with Gag protein including all the necessary domains and determine the requirements for the assembly reaction to produce spherical particles that more closely mimic authentic HIV. Here, using Gag protein lacking only the C-terminal p6 domain, we describe an in vitro assembly reaction that is composed of two sequential steps: formation of 60 S assembly...
intermediate and complete assembly to 600 S equivalent to authentic Gag VLPs.

**EXPERIMENTAL PROCEDURES**

**Materials**—E. coli expression vector pTrcHisA was purchased from Invitrogen and metal chelate resin (HisBind Resin) from Novagen. A high molecular weight calibration kit was purchased from Amersham Pharmacia Biotech, and prestained protein molecular weight markers (low range) were from Bio-Rad. Calf liver RNA and anti-polyhistidine mouse monoclonal antibody were obtained from Sigma. Anti-HIV-1 CA mouse monoclonal antibody was provided by Dr. H. Holmes (Medical Research Council AIDS reagent repository, National Institute for Biological Standards and Control, Hertford, UK), and 80 S ribosome was kindly supplied by Dr. K. Mizumoto (Kitasato University, Japan). Other reagents, unless otherwise specified, were commercially available of analytical grade.

**DNA Construction**—The HIV-1 gag gene encoding the Gag region essential for virus particle formation (MA-CA-p2-NC) with the sequence encoding additional six histidine residues at the C terminus was amplified by polymerase chain reaction with 5'-CCGCCATGGGTGC-3' and 5'-CCGGAAAATTCAGTGATGTGA-TGATTTAAGCTGCTCCAGT-3' (underlines in the sequence encode six histidine residues). The polymerase chain reaction fragment was digested with NcoI and EcoRI and cloned into E. coli expression vector pTrcHisA (Invitrogen).

**Protein Expression and Purification**—An overnight culture of transformed E. coli cells was inoculated at 1:20 and grown for 2 h at 37 °C. After 1 h of induction with 1 mM isopropyl-β-D-thiogalactopyranoside, the E. coli cells were immediately chilled and harvested by centrifugation at 4 °C at 8,000 × g for 15 min. The cells were resuspended in binding buffer (20 mM Tris (pH 7.9), 150 mM NaCl, and 10 mM imidazole), sonicated at 4 °C for 5 min, and then lysed by addition of Nonidet P-40 to a final concentration of 0.2%. After centrifugation at 4 °C at 15,000 × g for 30 min, the supernatant was subjected to metal chelate chromatography (Novagen). After washes with 25 volumes of binding buffer and with 20 volumes of wash buffer (20 mM Tris (pH 7.9), 150 mM NaCl, and 60 mM imidazole), bound protein was eluted with 5 volumes of elution buffer (20 mM Tris (pH 7.9), 150 mM NaCl, and 1 M imidazole).

**In Vitro Assembly Reaction**—Following chromatography, eluted protein solution was initially adjusted with EDTA to a final concentration of 2 mM (to chelate Ni²⁺) and then dialyzed overnight at 4 °C against 20 mM Tris (pH 8.6 adjusted at room temperature), 100 mM NaCl, 0.2 mM EDTA, and 1 mM dithiothreitol (DTT) unless otherwise indicated. In some experiments, calf liver RNA or RNaseA (Sigma) was added before dialysis. For higher order or complete assembly, the dialyzed protein was incubated in the presence of 5 mM MgCl₂ at 37 °C for 1 or 3 h unless otherwise indicated (see text and legends for Figs. 3, 4, and 5).

**RESULTS**

**Purification of HIV VLP**—HIV Gag VLP was purified from a culture media of Spodoptera frugiperda (Sf9) cells infected with a recombinant baculovirus containing HIV-1 gag gene, as described previously (10). Briefly, the Gag VLP was pelleted through a 30% (w/v) sucrose gradient and then purified by centrifugation in a 20–60% (w/v) sucrose gradient spun at 4 °C at 147,000 × g for 2 h. For higher order assembly, protein sample (multimerized Gag protein) and Gag VLP were applied onto 20–70% (w/v) sucrose gradients in phosphate-buffered saline and sedimented at 4 °C at 120,000 × g for 2 h. After centrifugation, the gradients were fractionated by 200 µl from the bottom to the top. A high molecular weight calibration kit (Amersham Pharmacia Biotech) and 80 S ribosome were used for molecular weight markers for sedimentation analysis.

**Protein Detection**—Protein sample was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) on 14% acrylamide. After electrophoresis, protein in a gel was either directly detected by Coomassie Brilliant Blue or silver staining. The procedure for microscopic examination was described previously (36). In vitro assembly products were collected through a 30% (w/v) sucrose cushion and fixed with 2% glutaraldehyde in 50 mM cacodylate buffer (pH 7.2) at 4 °C for 2 h. After post-fixation with 1% osmium tetroxide in 50 mM cacodylate buffer (pH 7.2) at 4 °C for 1 h, the pellets were embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with an electron microscope (Hitachi H-800).

**Electron Microscopic Examination**—The procedure for microscopic examination was described previously (36). In vitro assembly products were collected through a 30% (w/v) sucrose cushion and fixed with 2% glutaraldehyde in 50 mM cacodylate buffer (pH 7.2) at 4 °C for 2 h. After post-fixation with 1% osmium tetroxide in 50 mM cacodylate buffer (pH 7.2) at 4 °C for 1 h, the pellets were embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with an electron microscope (Hitachi H-800).
Protein interaction is stimulated in the presence of Mg$^{2+}$ by dialysis under these conditions. In general, protein-somes. This indicates that monomeric Gag protein assembles to a calculated S value of 60 S when compared with 80 S ribo-

incubated at 30 °C with or without MgCl$_2$, the sedimentation velocity sedimentation analysis on a 20–70% (w/v) sucrose gradient, most of the Gag protein sedimented faster than the Gag protein before dialysis (compare Fig. 2, A and B) and had a calculated S value of 60 S when compared with 80 S ribo-
somes. This indicates that monomeric Gag protein assembles to 60 S by dialysis under these conditions. In general, protein-protein interaction is stimulated in the presence of Mg$^{2+}$, yet further assembly of Gag to greater than 60 S did not occur by dialysis in the presence of 5 mM MgCl$_2$ at 4 °C (Fig. 2C). Assembly to the 60 S occurred in dialysis buffer of neutral to alkaline pH at low salt concentration but failed at acidic pH or at high salt concentration (Table I). The optimized conditions for the reaction were similar to those under which HIV CA-

2mM EDTA and dialyzed overnight at 4 °C against 20 mM Tris (pH 8.6 adjusted at room temperature), 100 mM NaCl, 0.2 mM EDTA, and 1 mM DTT to remove any excess concentration of imidazole. When the dialyzed Gag protein was subjected to velocity sedimentation analysis on a 20–70% (w/v) sucrose gradient, most of the Gag protein sedimented faster than the Gag protein before dialysis (compare Fig. 2, A and B) and had a calculated S value of 60 S when compared with 80 S ribo-
somes. This indicates that monomeric Gag protein assembles to 60 S by dialysis under these conditions. In general, protein-protein interaction is stimulated in the presence of Mg$^{2+}$, yet further assembly of Gag to greater than 60 S did not occur by dialysis in the presence of 5 mM MgCl$_2$ at 4 °C (Fig. 2C). Assembly to the 60 S occurred in dialysis buffer of neutral to alkaline pH at low salt concentration but failed at acidic pH or at high salt concentration (Table I). The optimized conditions for the reaction were similar to those under which HIV CA-

impaired Gag protein was dialyzed in the presence of 10 mM RNaseA; E, dialyzed in the presence of calf liver RNA at a concentration of 3% of the Gag protein. Lane M, prestained molecular mass markers (BioRad).

**Fig. 2.** Initial assembly of Gag protein by dialysis at 4 °C. Purified Gag protein was dialyzed against 20 mM Tris (pH 8.6 adjusted at room temperature), 100 mM NaCl, 0.2 mM EDTA, and 1 mM DTT and subjected to velocity sedimentation analysis on 20–70% (w/v) sucrose gradients at 4 °C at 120,000 × g for 2 h. The gradient fractions from the bottom to the top (left to right) were analyzed by SDS-PAGE and then subjected either to silver staining (panels A, B, and C) or to Western blotting using anti-HIV-1 CA monoclonal antibody (panels D and E). Panels: A, before dialysis; B, post dialysis; C, dialyzed in the presence of 5 mM MgCl$_2$; D, dialyzed in the presence of 10 μg/ml RNaseA; E, dialyzed in the presence of calf liver RNA at a concentration of 3% of the Gag protein. Lane M, prestained molecular mass markers (BioRad).

**Fig. 2.** Initial assembly of Gag protein by dialysis at 4 °C. Purified Gag protein was dialyzed against 20 mM Tris (pH 8.6 adjusted at room temperature), 100 mM NaCl, 0.2 mM EDTA, and 1 mM DTT and subjected to velocity sedimentation analysis on 20–70% (w/v) sucrose gradients at 4 °C at 120,000 × g for 2 h. The gradient fractions from the bottom to the top (left to right) were analyzed by SDS-PAGE and then subjected either to silver staining (panels A, B, and C) or to Western blotting using anti-HIV-1 CA monoclonal antibody (panels D and E). Panels: A, before dialysis; B, post dialysis; C, dialyzed in the presence of 5 mM MgCl$_2$; D, dialyzed in the presence of 10 μg/ml RNaseA; E, dialyzed in the presence of calf liver RNA at a concentration of 3% of the Gag protein. Lane M, prestained molecular mass markers (BioRad).

Still appeared insufficient for complete assembly. However, when the incubation time for the reaction was prolonged to 3 h, the sedimentation profile was shifted to that of Gag VLP, suggesting that Gag assembly might proceed to completion within 3 h under these conditions, although a small fraction of the Gag protein was still observed at 60 S (Fig. 4A). The proportion of Gag remaining at 60 S in the complete reaction was similar to that observed in the partial assembly reaction, suggesting that the Gag molecules that fail to assemble to 150–350 S by 1 h reaction never participate in the higher order of assembly, presumably due to denaturation during dialysis. Gag VLP and immature authentic HIV particles are not dissociated
by treatment with nonionic detergents such as Triton X-100 and Nonidet P-40 (37, 38). Accordingly, the detergent treatment was applied to the in vitro assembly product of 600 S as a general measure to examine whether the correct assembly of Gag protein occurred in vitro. The in vitro assembly product of 600 S was not dissociated by treatment with 0.5% Triton X-100 (Fig. 4B), similar to the stability of Gag VLP in the presence of 0.5% Triton X-100 similarly (panel C). Sedimentation conditions on 20–70% (w/v) sucrose gradients were described in the legend for Fig. 2. The gradient fractions from the bottom to the top (left to right) were analyzed by SDS-PAGE and subjected either to silver staining (panels A and B) or to Western blotting using anti-HIV-1 CA monoclonal antibody (panel C). Lane M, prestained molecular mass markers (BioRad).

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**Fig. 3. Requirements for higher order of assembly of Gag protein.** Dialyzed Gag protein was incubated for 1 h with combinations of incubation temperatures and concentrations of MgCl₂. Sedimentation on 20–70% (w/v) sucrose gradients were carried out as described in the legend for Fig. 2. The gradient fractions from the bottom to the top (left to right) were analyzed by SDS-PAGE followed by silver staining. **Panels:** A, incubated at 37 °C in the presence of 5 mM MgCl₂; B, incubated at 37 °C in the absence of MgCl₂; C, incubated at 30 °C in the presence of 5 mM MgCl₂. Lane M, prestained molecular mass markers (BioRad).

**Fig. 4. Complete assembly of Gag protein and detergent treatment after the assembly reaction.** Dialyzed Gag protein was incubated in the presence of 5 mM MgCl₂ at 37 °C for 3 h (panel A) and then treated with 0.5% Triton X-100 at 4 °C for 30 min (panel B). Gag VLPs purified from the supernatant of Gag-expressing Sf9 cells were treated with 0.5% Triton X-100 similarly (panel C). Sedimentation conditions on 20–70% (w/v) sucrose gradients were described in the legend for Fig. 2. The gradient fractions from the bottom to the top (left to right) were analyzed by SDS-PAGE and subjected either to silver staining (panels A and B) or to Western blotting using anti-HIV-1 CA monoclonal antibody (panel C). Lane M, prestained molecular mass markers (BioRad).
Gag protein alone assembles to 600 S in vitro but that the addition of RNA accelerates the higher order of assembly. Microscopic Examination of Complete Assembly Product—Electron microscopic examination was carried out to confirm the defined structure of the in vitro assembly product of 600 S. Almost spherical (Fig. 6A) but often faceted particles (Fig. 6B) were observed by ultrathin section transmission electron microscopy. The particles were hollow surrounded by double-ring structures, with an average diameter of 80 nm. When compared with immature HIV Gag VLPs (Fig. 6C), these features suggest the structure of the in vitro assembly products is similar to that of authentic immature HIV.

DISCUSSION

In vitro assembly of HIV Gag protein was initially observed when a CA-p2-NC protein fragment was dialyzed at 4 °C under low salt conditions at pH 8, although the assembly efficiency was very low (31). Recent studies on in vitro assembly have been carried out using CA-p2-NC, CA, and CA fused with several amino acids of or entire MA (12, 32–34). In these studies, the conditions used for assembly varied, since protein-protein interaction depends on salt concentration, pH, and temperature, which themselves influence protein conformation. We described the in vitro assembly of nearly full-length HIV Gag protein (MA-CA-p2-NC), devoid of only the C-terminal p6 domain, showing the optimal condition for formation of a spherical particle with a double-ring structure, similar to authentic immature HIV particles. In parallel, the assembly efficiency of the Gag protein was semiquantitated by velocity sedimentation analysis and estimated that approximately 77% of the total Gag protein finally assembled to 600 S under the optimized condition. The assembly reaction appeared to be composed of two steps, both of which proceeded at low ionic strength at neutral to alkaline pH but failed at high salt or at acidic pH (Table 1). The optimal salt concentration differed from that in the recent studies in which CA-driving assembly was observed (at 0.5–1 M salt), although the optimal pH range (neutral to alkaline) was consistent with those studies (32). It is plausible that the presence of the entire MA domain on the Gag protein used in our experiment resulted in the preference for the low salt condition, since a previous report has shown that MA-driving trimerization was sensitive to salt concentration (29).

Electron microscopic analysis of previous CA-driving assembly reactions has revealed that both CA and CA-p2-NC formed tubular or conical structures in vitro, which represent conical cores of mature HIV particles (32, 33). The formation of spherical structures were observed when the CA domain was extended at the N terminus by a small portion of MA (12, 33), although the in vitro assembly of this construct is also presumably driven by the CA domain, as it occurred at high ionic strength. In contrast, our Gag assembly reactions appeared not to be CA-driven as they occurred under low salt condition, but produced a spherical particle. A similar finding has been reported by Campbell and Rein (34). From these data, we speculate that whichever domain of Gag could trigger for Gag assembly, a final shape of Gag assembly products depends on whether the MA domain (or even a small portion of MA) is present within Gag constructs used for assembly reactions. This interpretation is supported by a recent proposal that creation of the intermolecular salt bridge at the C terminus of MA domain occurs after cleavage of the MA/CA junction and redirects Gag assembly from spheres to cones (33).

It is well known that protein-protein interaction is stimulated by factors such as temperature and Mg$^{2+}$ ion concentration. In our experiment, the initial assembly to 60 S intermediates occurred only by dialysis at 4 °C, but the higher order of assembly to 600 S required incubation at 37 °C in the presence of Mg$^{2+}$. This indicates that the higher Gag assembly state requires more stimulating factors. However, it is possible that a higher concentration of Gag protein for assembly reaction could compensate for these requirements, as it is reported that those factors had little effect on the yield of assembly product when a high concentration of CA protein was used (32).

Retroviral genomic or even non-cognate RNA is incorporated into assembling Gag VLPs via the zinc finger motifs located in the NC domain. Recent studies on in vitro assembly reactions with CA-p2-NC suggest that RNA serves as a scaffold that effectively concentrates the protein in the microenvironment (31), although the protein has also been reported to assemble in the absence of RNA but only at a high concentration of salt or protein (32). Recent in vitro assembly reaction with the nearly full-length Gag protein, which was carried out at room temperature, was completely dependent on additional RNA (34), but in contrast, RNA was not absolutely required for our in vitro assembly reaction at 37 °C (Fig. 5). Since the Gag assembly from 60 S to 600 S was not inhibited by RNaseA treatment but was accelerated by addition of RNA, we suggest that RNA requirement for Gag assembly reaction is reduced by incuba-
tion at higher temperature. We believe that the *in vitro* assembly of the nearly full-length Gag protein described here with RNA to form a spherical particle requires an understanding of the physiological situation of authentic Gag assembly *in vivo*, because Gag VLPs are produced from various eukaryotic cells including insect cells cultured at 27–28 °C; in the latter cell case, RNA may be used as an essential scaffold.

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