The VP2/VP3 Minor Capsid Protein of Simian Virus 40 Promotes the \textit{in Vitro} Assembly of the Major Capsid Protein VP1 into Particles*

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The SV40 capsid is composed primarily of 72 pentamers of the VP1 major capsid protein. Although the capsid also contains the minor capsid protein VP2 and its amino-terminally truncated form VP3, their roles in capsid assembly remain unknown. An \textit{in vitro} assembly system was used to investigate the role of VP2 in the assembly of recombinant VP1 pentamers. Under physiological salt and pH conditions, VP1 alone remained dissociated, and at pH 5.0, it assembled into tubular structures. A stoichiometric amount of VP2 allowed the assembly of VP1 pentamers into spherical particles in a pH range of 7.0 to 4.0. Electron microscopy observation, sucrose gradient sedimentation analysis, and antibody accessibility tests showed that VP2 is incorporated into VP1 particles. The functional domains of VP2 important for VP1 binding and for enhancing VP1 assembly were further explored with a series of VP2 deletion mutants. VP3 also enhanced VP1 assembly, and a region common to VP2 and VP3 (amino acids 119–272) was required to promote VP1 pentamer assembly. These results are relevant for controlling recombinant capsid formation \textit{in vitro}, which is potentially useful for the \textit{in vitro} development of SV40 virus vectors.

Viral capsids are highly organized protein complexes that assemble and disassemble depending on environmental conditions during the viral life cycle. An elucidation of the mechanisms of assembly and disassembly of viral capsids may greatly help in understanding these highly organized protein complexes.

SV40 is a small, nonenveloped DNA tumor virus that belongs to the family Polyomaviridae. Its capsid is formed by 72 copies of pentamers of the VP1 major capsid protein (360 molecules in total) and 72 molecules of the VP2 or VP3 minor capsid proteins. VP1 pentamers are arranged in a \textit{T} = 7d icosahedral lattice, and the carboxyl-terminal arm of VP1 mediates inter-pentameric contacts that hold the capsid together (1–4). VP3 is an amino-terminally truncated form of VP2. One molecule of either minor capsid protein binds to the center of a VP1 pentamer through their common carboxyl-terminal region inside the virion (5–7).

However, the precise molecular mechanism for virion assembly is not known.

We and others have shown that the VP1 proteins of SV40 (8–12) and of closely related viruses such as JC virus (13, 14), murine polyomavirus (8, 15–19), and papillomavirus (20, 21) form virus-like particles (VLPs)\textsuperscript{2} when expressed in insect Sf9 cells from baculoviral vectors. The properties of VP1-VLPs are very similar to those of wild type virions in that they dissociate into pentamers following treatment with a calcium-chelating agent (EGTA) under reducing conditions \textit{in vitro} (22–24). Previously, we prepared highly purified SV40 VP1 pentamers (9, 25, 26), which assembled into morphologically heterogeneous particles under various nonphysiological conditions (26). Such particles include \textit{T} = 7d (triangulation number 7 dextro) spherical particles, small \textit{T} = 1 (triangulation number 1) particle composed of 12 pentamers, and long tubular structures of 40–45 nm in diameter (26). However, under physiological salt and pH conditions, pentamers do not assemble into particles \textit{in vitro} (26), suggesting that component(s) that promote proper assembly are lacking.

The purpose of the present study was to determine the precise role of the minor coat protein VP2 in the assembly of VP1 pentamers. We found that VP2 promotes VP1 assembly into particles under physiological salt and pH conditions, although these particles are somewhat irregular. We also identified the functional domains of VP2 that promote VP1 assembly. These results provide information on the mechanism of SV40 virus assembly and suggest a new approach to construct SV40 viral vectors \textit{in vitro}.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—A bacterial expression plasmid, pET-HF-VP2, containing amino-terminal fusions of VP2 with the His (HHHHHHH) and FLAG (DYKDDDDK) tags was constructed as follows. BamHI and EcoRI restriction sites were introduced into the 5’ and 3’ ends, respectively, of a DNA fragment encoding VP2 by PCR amplification using SV40 genomic DNA as a template. This fragment was ligated to an SpeI-BamHI fragment encoding the FLAG peptide, and the resulting fragment was inserted into SpeI-EcoRI-digested pBluescript to make pBS-F-VP2. The SpeI-EcoRI fragment encoding FLAG-tagged VP2 was inserted into the BamHI site of pET14b after 5’ overhangs were blunt-ended with the Klenow fragment.

All HF-VP2 deletion mutants were generated from pET-HF-VP2. Sequences of primers and linkers used for PCR and construction will be

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\*This work was supported by a grant-in-aid for scientific research, the 21st Century COE Program of the Ministry of Education, Culture, Sports, Science, and Technology in Japan, and a grant for research and development projects in cooperation with academic institutions from the New Energy and Industrial Technology Development Organization. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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2. The abbreviations used are: VLPs, virus-like particles; BSA, bovine serum albumin; HF-VP2, His-FLAG-tagged VP2.
given upon request. All mutants were confirmed by dyeoxy nucleotide sequencing analysis.

The construction of recombinant VP1 displaying the FLAG tag on the capsid surface will be described elsewhere. In brief, a FLAG tag sequence with glycine spacers (GGG-DYKDDDDK-GGG) was inserted between amino acids 273 and 274 of VP1.

Expression and Purification of Capsid Proteins—The construction of the SV40 VP1-expressing baculovirus and expression of the VP1 protein in Spodoptera frugiperda (Sf9) cells have been described (9, 25, 26). Briefly, VP1 protein assembled into particles (VP1-VLPs) in Sf9 cells was purified in two successive fractionations by cesium chloride density gradient centrifugation. The purified particles were dissociated into pentamers by incubation in a buffer (20 mM Tris-HCl (pH 7.9), 100 mM NaCl, 0.1% Nonidet P-40, 25 mM EGTA, and 30 mM dithiothreitol) at 37 °C for 1 h. The pentamers were further purified by size-exclusion chromatography (Superdex-200, Amersham Biosciences) to enrich for completely dissociated VP1 pentamers.

His-FLAG-tagged VP2 protein and deletion mutants were produced in the BL21 (RII) strain of Escherichia coli (Novagen) harboring pET-HF-VP2 or corresponding plasmids after isopropyl-β-D-thiogalactopyranoside induction. The cells were washed with phosphate-buffered saline and resuspended in binding buffer (20 mM Tris-HCl (pH 7.9), 10% glycerol, 500 mM KCl, 0.2 mM EDTA, 0.1% Nonidet P-40, 0.5 mM dithiothreitol, 10 mM imidazole, 2 mM phenylmethylsulfonyl fluoride, and 1 μg/ml each of chymostatin, aprotinin, leupeptin, antipain, and pepstatin). The suspension was sonicated on ice and cleared by centrifugation at 14,000 × g for 20 min. Recombinant proteins in the supernatant were purified with nickel-nitrilotriacetic acid-agarose (Qiagen) as recommended by the manufacturer, except that they were washed with binding buffer containing 20 mM imidazole instead of 10 mM imidazole. The eluate was further dialyzed against binding buffer lacking imidazole and containing 100 mM KCl.

In Vitro Assembly and Electron Microscopic Observation—The purified recombinant VP1 pentamer fraction (0.44 μM as pentamer and 2.2 μM as monomer), alone or mixed with recombinant HF-VP2 proteins (0.44 μM or as otherwise described), was placed into mini-dialysis units (molecular weight cut-off, 3,500; Pierce) and dialyzed at 22°C for 16 h against a buffer containing 150 mM NaCl and 2 mM CaCl2 at various pH values (pH 7.0–10.0 with 20 mM Tris-HCl or pH 4.0–6.0 without Tris). VP1 pentamer assembly was observed by electron microscopy as described previously (9).

Sucrose Gradient Sedimentation Analysis—Twenty microliters of in vitro assembly reactions (~600–1,000 ng of VP1 protein) or 800 ng of control VP1-VLPs were loaded onto a 0.6-ml preformed 20–40% sucrose gradient in 20 mM Tris-HCl (pH 7.9) in a 5 x 41-mm open-top tube (Beckman) and centrifuged at 50,000 rpm for 1 h at 4 °C in an SW 55Ti rotor with appropriate adapters. After centrifugation, fractions (55 μl each) were collected from the top of the tube. The bottom of the tube was washed with 55 μl of sonication buffer (20 mM Tris-HCl (pH 7.9), 1% sodium deoxycholate) and used as the pellet fraction. Aliquots of each fraction (30%) were separated by SDS-PAGE and immunoblotted with an anti-SV40 VP1 polyclonal antibody (courtesy of M. Ikeda and I. Tamai, MBL, Nagoya, Japan) and a mouse monoclonal anti-FLAG M2 antibody (Sigma) using the ECL detection system (Amersham Biosciences).

Native Agarose Gel Electrophoresis—Twenty microliters of purified pentamers (20 ng), HF-VP2 proteins (20 ng), assembled samples (200 ng), VP1-VLPs (60 ng), or VLP-FLAG (60 ng) was mixed with 5 μl of loading buffer (250 mM Tris acetate (pH 8.1), 25% glycerol, 0.125% bromphenol blue). When indicated, the samples were preincubated with 49 and 490 ng or 4.9 μg of mouse monoclonal anti-FLAG M2 antibody (Sigma). The samples were loaded onto a 0.8% agarose gel, run in 50 mM Tris acetate (pH 8.1) for 2 h at 4 °C, and transferred onto a polyvinylidene fluoride membrane in 50 mM NaOH by a standard capillary transfer method (9). VP1, HF-VP2, and the anti–FLAG antibody were detected by immunoblotting with anti-VP1, anti-FLAG, and anti-mouse IgG antibodies, respectively.

In Vitro Binding Assay for VP1 and HF-VP2 Proteins—To examine the abilities of the HF-VP2 and their derivatives to bind VP1 pentamers, purified VP1 pentamer or bovine serum albumin (BSA) was cross-linked to N-hydroxysuccinimide-activated Sepharose 4 Fast Flow (Amersham Biosciences) following the manufacturer’s instruction. Approximately 170 ng of VP1 or BSA was immobilized onto 1 μl of beads. The immobilized beads (40 μl) were mixed with 6.48 μg of recombinant HF-VP2 protein or derivatives in 0.2 ml of binding assay buffer (20 mM Tris-HCl (pH 7.9), 150 mM NaCl, 10% glycerol, 0.1% Nonidet P-40), incubated for 1 h at room temperature, washed with binding assay buffer, and eluted with 40 μl of SDS loading dye (125 mM Tris-HCl (pH6.8), 25% glycerol, 4.1% SDS, 284 mM 2-mercaptoethanol, 0.05% bromphenol blue). Aliquots of the input and eluted fractions were subjected to SDS-PAGE, transferred onto a polyvinylidene fluoride membrane, and detected by Western analysis using mouse anti-FLAG antibody as the primary antibody followed by ECL detection.

RESULTS

Minor Capsid Protein VP2 Promotes Assembly of VP1 into Virus-like Particles—We have established a system to evaluate conditions for the assembly of highly purified recombinant VP1 pentamers into particles by dialyzing a VP1-containing solution against various buffers, with subsequent EM observation (9, 25, 26). As we showed previously, VP1 pentamers do not assemble into particles under physiological salt and pH conditions (150 mM NaCl, pH 7.0) (Fig. 1A, top panel). The minor capsid proteins are integral aspects of virion structure (5–7), although their role in virion assembly has yet to be established. To examine their effects on the in vitro assembly of VP1 pentamers, we used recombinant VP1 protein tagged with the His and FLAG motifs at its amino terminus (HF-VP2). Addition of HF-VP2 to VP1 pentamers resulted in particle formation under physiological salt and pH conditions (Fig. 1A, middle panel). We compared the morphology of these particles to that of recombinant VP1 particles assembled in vivo (Fig. 1, A, bottom panel, and B). As shown previously (27–30), the in vivo preparation contained two types of particles as follows: large virus-like particles (VP1-VLPs) of ~45 nm in diameter (Fig. 1A, bottom panel, arrows), and T = 1 tiny particle of ~20 nm (Fig. 1A, bottom panel, arrowheads). In contrast, particles assembled from VP1 and HF-VP2 in vitro were more heterogeneous. They included T = 1 tiny particle (Fig. 1A, middle panel, arrowheads) and somewhat irregularly shaped larger particles (Fig. 1A, middle panel, arrows). The mean diameter of the larger particles was smaller than that of VP1-VLPs, ranging from 25 to 40 nm (Fig. 1B, upper panel). It should be noted that, in contrast to VP1-VLPs that were internally stained by uranium acetate and appeared to be occupied, suggesting that the VP2 protein is enclosed within these particles (compare arrows in the middle and bottom panels of Fig. 1A).

We then analyzed assembly reactions by sucrose gradient sedimentation following by Western analysis using anti-VP1 and anti-FLAG antibodies. VP1 alone remained in the top fraction (Fig. 1C, panel 1),
Assembly of VP1 pentamers in the presence or absence of HF-VP2. Purified recombinant VP1 pentamers with or without recombinant HF-VP2 were dialyzed at 22 °C for 16 h against a buffer containing 150 mM NaCl, 2 mM CaCl₂, pH 7.0. A, electron micrographs of the assemblies after dialysis. Samples were visualized by negative staining. Top panel, VP1 alone. Middle panel, VP1 plus HF-VP2. Bottom panel, purified VP1 assemblies produced in insect Sf9 cells. Scale bars are 50 or 500 nm (insets). Large and T = 1 tiny particle are
indicating that VP1 pentamers do not assemble. HF-VP2 alone was detected in fractions 1–4 (Fig. 1C, panel 4). In contrast, the addition of HF-VP2 led to a dramatic change in the VP1 sedimentation profile, and VP1 and HF-VP2 co-migrated (Fig. 1C, panels 2 and 3), suggesting that VP2 stimulates the assembly of VP1 pentamers by directly interacting with VP1. Also, VP1 and HF-VP2 were distributed more broadly throughout the sedimentation gradient than were VP1-VLPs assembled in vivo (Fig. 1C, panels 2 and 5), which is consistent with EM observations. These results indicate that HF-VP2 allows VP1 pentamers to form imperfectly shaped particles.

Effects of Solvent pH on Assembly of VP1 Pentamers and HF-VP2—We next examined the assembly of VP1 pentamers at various pH values in the presence or absence of HF-VP2. VP1 alone did not assemble at pH 4.0 or 6.0 (Fig. 2A, upper panels). Under these conditions, the addition of HF-VP2 resulted in particle formation (Fig. 2A, lower panels).

At pH 5.0, VP1 pentamers assembled into extremely long tubular structures as reported previously (Fig. 2A, upper panel, pH 5.0, open arrowheads) (9). The diameter of these tubes was ~40–45 nm, and they were apparently infinitely long although disrupted during preparation. Sucrose gradient sedimentation also indicated large structure formation, as most VP1 protein was found in the bottom fraction (Fig. 2B, panel 1). Even under this condition, the addition of HF-VP2 resulted in the formation of particles that are morphologically similar to those formed at pH 7.0 (Fig. 2A, see also Fig. 1A). Sucrose gradient sedimentation also supports this conclusion (Fig. 2B, panels 2 and 3).

At pH 8.0, 9.0, and 10.0, VP1 pentamers alone assembled into spherical structures (Fig. 2A). Close examination by EM indicated that these particles are not completely closed (Fig. 2A, upper panels, arrows). Upon sucrose gradient sedimentation, some VP1 protein was found to be incorporated into VP1-VLPs (fractions 7–10) but most was in the top fraction (Fig. 2B, panel 4). The addition of HF-VP2 did not change the extent of large particle formation and did not alter particle shape (Fig. 2A, lower panels, arrows), but it did result in the appearance of T = 1 tiny particle (Fig. 2A, lower panels, arrowheads). Sucrose sedimentation (Fig. 2B, panels 5 and 6) showed that HF-VP2 was associated with both T = 1 tiny particle (fractions 3–5) and large particles (fractions 7–10) at pH 9.0. From these results, we conclude that HF-VP2 enhances 25–40 nm particle formation by VP1 pentamers at pH values below 7.0.

Molar Ratio of VP1 Pentamers and HF-VP2 in the Assemblies—In the above experiments, we mixed VP1 pentamers and HF-VP2 at a 1:1 ratio, because this is the ratio of VP1 pentamers to minor capsid proteins in wild type SV40 virions. We asked if HF-VP2 enhances VP1 assembly by acting as a chaperone or by acting as a structural component that must be present at stoichiometrically appropriate levels. To address this issue, we varied the level of HF-VP2 and maintained a constant level of VP1 pentamers.

When HF-VP2 and VP1 pentamers were mixed at a 12:72 molar ratio, most VP1 assembled into tubular structures, and only a small number of large particles were formed (Fig. 3A, top panel). The formation of tubular structures is indicative of insufficient HF-VP2. At a 22:72 molar ratio, large particles increased in number, although some tubular structures were still observed (Fig. 3A, 2nd panel). When equimolar amounts of HF-VP2 and VP1 pentamers (72:72) were mixed, most of the assemblies were large particles, with a few tubes (Fig. 3A, 3rd panel). A further
increase in HF-VP2 decreased the formation of large particles and tubular structures and instead increased particle aggregation (Fig. 3A, bottom panels). Sucrose sedimentation analyses were consistent with these EM observations (Fig. 3B). It is of note that excess amounts of aggregated HF-VP2 protein were detected in fraction 12 (Fig. 3B, panels 8 and 10), whereas HF-VP2 alone was detected only in the top fractions (see Fig. 1C, panel 4). These results suggest that VP2 self-association is promoted in the presence of VP1 pentamers. Overall, the results show that large particle formation of VP1 pentamers occurs most efficiently in the presence of HF-VP2 at a 1:1 molar ratio under these conditions. These results suggest that HF-VP2 enhances VP1 assembly not by a chaperone-like activity but as a structural component.

Enclosure of HF-VP2 into the VP1 Capsid—The results described above suggested that the enhancement of VP1 assembly by HF-VP2 involves the direct interaction of VP2 with VP1. To determine whether VP2 is in the assembled VP1 capsid, we used native agarose gel electrophoresis and subsequent immunoblot analysis. As shown in Fig. 4A, purified VP1 pentamers and reconstituted VLP particles could be separated by native agarose gel electrophoresis (Fig. 4A, lanes 1 and 4). VP1 structures assembled at pH 5.0 (tubular structures) were detected at the top of the gel (Fig. 4A, lane 2). On the other hand, VP1 reassembled in the presence of HF-VP2 at pH 5.0 migrated to a position similar to that of VP1-VLPs (Fig. 4A, lane 3, upper panel). HF-VP2 protein was detected at the same position as VP1 (Fig. 4A, lane 3, bottom panel). These results are consistent with those obtained by sucrose gradient sedimentation analysis (Fig. 1C).

We then examined if HF-VP2 is incorporated within VP1 particles. If HF-VP2 is externally associated with VP1 particles, preincubation of particles with the anti-FLAG antibody should change their electrophoretic mobility. However, preincubation with the anti-FLAG antibody did not affect the mobility of the band that corresponds to VP1 plus HF-VP2 particles (Fig. 4B, lanes 3 and 4), and the band did not contain anti-FLAG antibody (Fig. 4B, lane 5). In contrast, preincubation of HF-VP2 protein alone with the anti-FLAG antibody resulted in the appearance of a new band that contains both HF-VP2 and anti-FLAG antibody (Fig. 4B, lanes 6–8). As another control, we used recombinant FLAG-VP1 capsids, which display the FLAG epitope on the surface. When capsids were mixed with anti-FLAG antibody, their mobility was shifted in a dose-dependent manner (Fig. 4C). Taken together, these results suggest that the HF-VP2 FLAG epitope and thus HF-VP2 itself are inside the particles. This interpretation is consistent with EM observations indicating that VP1-HF-VP2 particles are filled (Fig. 1A).

Functional Domains in HF-VP2 that Promote VP1 Assembly—The results described above suggest that, upon promoting VP1 particle assembly, HF-VP2 associates with and is enclosed in the particles. Previous structural and biochemical studies have proposed that VP2 proteins form complexes with VP1, via amino acid 273–309 of polyomavirus VP2 (5, 7) and via the carboxyl-terminal 40 residues of SV40 (6) (Fig. 5A). However, the functional domains involved in promoting VP1 particle formation are not known. To address this issue, HF-VP2 deletion mutants were generated and examined for their ability to promote VP1 particle assembly (Fig. 5B). Equimolar amounts of HF-VP2 fragments were mixed with purified VP1 pentamers and dialyzed at pH 5.0 to allow VP1 assembly formation. The samples were observed by EM (Fig. 5C). It was expected that the appearance of tubular structures reflects an inability of HF-VP2 to promote VP1 particle assembly and that the absence of assemblies suggests that the HF-VP2 fragment inhibits the assembly of VP1.

Previous studies have shown that the sequence-independent DNA-binding domain (31) and the nuclear localization signal (32, 33) are located at the carboxyl-terminal region of VP2. Deletion of these regions (HF-VP2-(1–342), HF-VP2-(1–332), and HF-VP2-(1–312)) did not interfere with the ability of truncated VP2 proteins to promote VP1 particle assembly (Fig. 5C). Unexpectedly, a mutant with a more extensive deletion of the previously identified VP1-binding region (5–7) (HF-
VP2-(1–272)) retained promoting activity (Fig. 5C). Therefore, this region does not play a role in promoting VP1 assembly in vitro. The deletion mutants promoted the formation of mixtures of $T = 1$ tiny particle (20 nm in diameter) and large particles (25–40 nm) as HF-VP2, but the ratios of these two types of particles were different depending on the HF-VP2 mutant (Fig. 5D). Further deletion of up to 200 amino acid residues from the carboxyl terminus (HF-VP2-(1–152)) resulted in the preferential formation of smaller particles, which appeared to be $T = 1$ tiny particle (Fig. 5C and D).

Fragments with a deletion of up to 234 amino acid residues from the carboxyl terminus (HF-VP2-(1–118), equivalent to the VP2 unique region) did not promote assembly (Fig. 5C), and most VP1 remained as pentamers, indicating that these truncated HF-VP2 proteins retained the ability to interact with VP1 (see below).

A mutant with a deletion of the VP2 unique region (HF-VP2-(119–352), equivalent to VP3) could promote VP1 particle assembly (Fig. 5, C and E). Size distribution analysis (Fig. 5D) and sucrose gradient sedimentation analysis (Fig. 5F) of the particles showed that the assemblies were mixtures of large particles and $T = 1$ tiny particle, although the mean diameter of the large particles was larger than that of HF-VP2 particles. Deletion of the amino-terminal 58 amino acids (HF-VP2-(59–352)) destroyed its ability to promote VP1 large particle formation but facilitated the assembly of $T = 1$ tiny particle at a low frequency (Fig. 5, C and D). Therefore, the region unique to VP2 (residues 1–118)
is dispensable for VP1 particle formation but affects VP1 particle morphology.

Deletion of up to 195 or 273 amino acid residues (HF-VP2-(196–352) and HF-VP2-(274–352)) from the amino terminus did not promote VP1 particle assembly, and most VP1 pentamers assembled into tubular structures (Fig. 5C). These results are consistent with the observation that the carboxyl-terminal region (273–352) is dispensable for promoting VP1 particle assembly.

An antibody accessibility test, consisting of native agarose gel electrophoresis and subsequent immunoblot analysis, showed that the FLAG epitopes of all the HF-VP2 deletion mutants (except for HF-VP2-(1–118)) that could promote particle assembly were inside the particles (Fig. 5G and data not shown).

Taken together, VP2 region 119–272 is necessary for directing particle formation by VP1 pentamers, and the VP2 unique region (1–118) and previously identified VP1 interaction domain (273–352) are dispensable. Unfortunately, however, we could not obtain a VP2-(119–272) fragment and could not determine whether it alone promotes VP1 particle assembly.

**Definition of Regions in HF-VP2 that Interact with VP1**—One of the unexpected results described above is that the previously proposed VP1-binding region (5–7) is dispensable for promoting VP1 particle formation. To explore the relation between VP1 particle formation and VP1 binding, we re-evaluated the VP1-binding region of VP2. For this purpose, the same HF-VP2 fragments were examined for their ability to bind recombinant VP1 pentamers, which were covalently immobilized to agarose beads. BSA-coupled agarose beads served as a control.

As shown in Fig. 6, full-length HF-VP2 and the amino-terminally truncated fragment (HF-VP2-(119–352) equivalent to VP3), which can promote VP1 particle assembly, bound to the VP1 pentamer. The carboxyl-terminal truncated fragments (HF-VP2-(1–312) and HF-VP2-(1–272)), which lack the previously identified VP1-binding domain, also bound to the VP1 pentamer, although the binding appeared to be weak.
FIGURE 5—continued

D. 

Number of particles

- 0-5
- 10-15
- 20-25
- 30-35
- 40-45
- >50

pH5

VP1

E.

pH5

VP1/

HF-VP2

pH5

VP1/

HF-VP3

F.

Top 1 2 4 6 8 12 P Bottom

VP1/

HF-VP3

α-VP1

α-Flag

HF-VP3

α-Flag

T=1 tiny particle VLPs

G. 

Anti-Flag Ab

Tube or aggregate

Particle

Pentamer

α-VP1

α-Flag

α-Ab

FIGURE 5—continued
SV40 Capsid Assembly by VP1 and VP2/VP3

compared with that with full-length HF-VP2 and HF-VP2-(119–352) (equivalent to VP3). In contrast, the amino-terminally truncated fragments (HF-VP2-(196–352) and HF-VP2-(274–352)), which contain the previously proposed VP1-binding region (5–7) but which are ineffective for VP1 assembly, did not bind the VP1 pentamer in our assay. Unfortunately, again, we could not determine whether the VP2/VP3 common region lacking only the previously proposed VP1-binding region (5–7) (for example, HF-VP2-(119–272)) binds to the VP1 pentamer, because we could not obtain this recombinant HF-VP2 protein. We also found that two VP2 fragments shown to be inhibitory for promoting VP1 assembly (HF-VP2-(59–352) and HF-VP2-(1–152)) bound to the VP1 pentamer. Another inhibitory fragment (HF-VP2-(1–118), VP2 unique region) alone could bind the VP1 pentamer.

Therefore, two VP2 domains, one in the VP2 unique region (1–118) and the other in the VP2/VP3 common region (119–352), bind the VP1 pentamer independently, and this ability is related to VP1 particle assembly, although binding is not sufficient for promoting VP1 particle assembly.

DISCUSSION

The VP1 pentamer is a building unit of the SV40 virion. It is theoretically impossible to construct T = 7d icosahedral lattice from pentavalent units. X-ray crystallographic studies of the SV40 virion have revealed that the carboxyl-terminal arms of VP1 that emerge from each pentamer form three different types of inter-pentameric interactions (α-α′, α′-β′, and γ). These VP1 pentamer interactions construct this geometrically difficult structure (2–4). However, the molecular mechanism that ensures that these interactions are correctly made in vivo is not known.

In this study, we analyzed assemblies of VP1 pentamers alone or together with VP2 in vitro and found that they form different types of particles under different pH conditions. For example, VP1 pentamers alone assembled into long tubular structures in vitro at pH 5.0. It is plausible that the structure of the carboxyl-terminal arm of VP1 is fixed in a particular conformation that allows tubular structures to form under this specific condition. This idea is supported by our previous finding that VP1 carrying amino acid substitutions in the carboxyl-terminal arm (E329Q, E330Q, and D345N) preferentially assembles into tubular structure in S9 cells (9). At pH 7.0, VP1 pentamers did not form particles (26). Sucrose gradient sedimentation and EM observation suggested that VP1 pentamers do not interact with each other and remain as pentamers. However, the addition of HF-VP2 led them to form spherical particles. The stimulatory effect of HF-VP2 was maximal when the HF-VP2:VP1 pentamer ratio was 1:1, and the relationship is not that of a chaperone. Therefore, one HF-VP2 molecule seems to interact with one VP1 pentamer to promote pentamer-pentamer interaction. Furthermore, HF-VP2 also seems to induce a conformation of VP1 arms that is conducive to spherical particle formation, because it mediates a drastic change in VP1 pentamers, causing them to form spherical particles but not long tubular particles at pH 5.0 (26). We also demonstrated here that the HF-VP2 protein is incorporated into the particles, as is the case for native virions. VP2-VP2 interactions inside the particle may also participate in forming the spherical structure. At pH 8.0 and above, VP1 pentamers alone can form particles, although the efficiency is low and particle morphology is irregular. However, under these pH conditions, HF-VP2 does not stimulate large particles assembly, although HF-VP2 appears to interact with VP1 pentamers. Under alkaline conditions, HF-VP2 cannot promote VP1 assembly but can instead promote T = 1 tiny particle formation.

We also showed that full-length HF-VP2, the amino-terminally truncated fragment 119–352 (equivalent to VP3), and the carboxyl-terminally truncated fragments 1–312 and 1–272 can promote VP1 particle assembly and bind to the VP1 pentamer. Therefore, the 119–272 region seems to be essential for enhancing VP1 pentamer assembly. Most unexpectedly, the amino-terminally truncated fragments, 196–352 and 274–352, containing the VP1-interaction domain previously identified by in vitro binding analysis and x-ray crystallography (5–7), did not interact with VP1 pentamers in our assay and did not enhance VP1 pentamer assembly. Therefore, the interaction of this domain with VP1 may be too weak to be detected in our assay system, and it may have another role in the virus life cycle, such as nuclear import of VP2/VP3 (32, 33), in genomic DNA packaging (31, 34), and virion disassembly.

We also showed that the carboxyl-terminally truncated fragment 1–152 and amino-terminally truncated fragment 59–352 only allowed formation of T = 1 tiny particle. In other words, these mutants bind to VP1 pentamers but induce the structural change of the VP1 arm ready to accumulate only into T = 1 tiny particle. The ability to induce VP1 structural change for the 25–40 nm large particle formation seems to be lost for the 1–152 fragment or masked for the 59–352 fragment. It should be noted that the simultaneous addition of two overlapping VP2 fragments, 1–118 and 59–352, either of which alone cannot promote VP1 particle assembly, successfully promoted VP1 particle formation, although at low efficiency. Therefore, although the VP2/VP3 common region (119–352) is sufficient for stimulating VP1 assembly in vitro, the VP2 unique region (1–118) can bind to VP1 pentamers and thus may have unknown role(s) in virion assembly in vivo.

However, we are still far from a complete understanding of the mechanisms of virion assembly. The particles formed in vitro with VP1 pentamers and HF-VP2 are morphologically irregular, indicating that some other component(s) is required to form completely spherical virions. It has been suggested that proper virus assembly requires chaperones in vivo (35, 36). Furthermore, the SV40 minichromosome is also

\[\text{FIGURE 6. Binding of HF-VP2 mutants to VP1 pentamers. Purified HF-VP2 deletion mutants expressed in E. coli were incubated with agarose covalently immobilized with purified VP1 pentamers (lanes 3 and 4) or BSA (lanes 1 and 2). After extensive washing, HF-VP2 proteins were eluted with SDS sample buffer. One percent of input (I) and 40% of eluted fractions (E) were separated by SDS-PAGE, and HF-VP2 mutant proteins were visualized with the anti-FLAG antibody.}\]
included in the intact virion. We are now trying to incorporate DNA into particles in vitro. Controlling the assembly and disassembly of SV40 capsids by minor capsid proteins and buffer conditions in vitro is also of great utility for developing useful vehicles for gene therapy.

Acknowledgment—We thank Yuki Yamaguchi for helpful discussions.

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