Simian virus 40 VP1 capsid protein forms polymorphic assemblies \textit{in vitro}

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The simian virus 40 (SV40) capsid is composed of 72 pentamers of VP1, the major protein of SV40. These pentamers are arranged in a $T=7d$ icosahedral surface lattice, which is maintained by three types of appropriately arranged, non-equivalent interactions between the pentamers. However, it remains unclear how these interactions are achieved. In this study, the \textit{in vitro} assembly of recombinant VP1 was analysed. Electron microscopy observations revealed that these recombinant VP1 proteins assembled into structurally polymorphic particles depending on environmental conditions. VP1 pentamers assembled efficiently into virus-like particles (VLPs) when high concentrations of ammonium sulfate were present. However, in the presence of 1 M NaCl and 2 mM CaCl$_2$ at neutral pH, VP1 pentamers formed not only VLPs but also produced tiny $T=1$ icosahedral particles and tubular structures. The exclusion of CaCl$_2$ resulted in the exclusive formation of tiny particles. In contrast, in the presence of 150 mM NaCl at pH 5, the VP1 pentamers produced only extraordinarily long tubular structures. VP1 is thus quite unique in that it can assemble into such diverse structures. These observations provide clues that will help elucidate the mechanisms underlying SV40 capsid formation.

INTRODUCTION

Simian virus 40 (SV40), a member of the family \textit{Polyomaviridae}, is a small, non-enveloped tumorigenic virus. Its capsid is about 40–45 nm in diameter and is formed by 72 pentamers composed of its major capsid protein, VP1. These pentamers are arranged in a $T=7d$ icosahedral lattice. The SV40 capsid is potentially useful as a capsule for drug or gene delivery because it efficiently infects a wide range of human cells, including haematopoietic stem cells (Strayer, 1999). However, the mechanism by which the capsid is formed from its VP1 pentamer is poorly understood and this hinders our ability to manipulate capsid re-assembly \textit{in vitro} for therapeutic purposes.

The structure of polyomaviruses had long been an enigma because it is geometrically impossible to construct a $T=7d$ icosahedral lattice from pentavalent units. X-ray crystallography studies of the SV40 virion have since revealed that all of the VP1 pentamers in the capsid have identical conformations, except for their carboxy-terminal arms (Stehle \textit{et al.}, 1996; Liddington \textit{et al.}, 1991). However, it remains unclear what determines the binding specificity of the VP1 arms so that the pentamers are correctly arranged.

SV40 capsids, as well as those of closely related murine polyomavirus (MPV), dissociate into VP1 pentamers following treatment with the calcium-chelating agent EGTA under reducing conditions \textit{in vitro} (Brady \textit{et al.}, 1977, 1978; Colomar \textit{et al.}, 1993). Consistent with these observations, crystallography analyses of the SV40 virion have identified a disulfide linkage between $\beta-\beta'$ pentamers and two calcium ions for each VP1 molecule that is involved in interpentamer interactions (Stehle \textit{et al.}, 1996; Liddington \textit{et al.}, 1991). Furthermore, dissociated MVP virions (Brady \textit{et al.}, 1979) or recombinant MPV VP1 proteins expressed in \textit{Escherichia coli} (Salunke \textit{et al.}, 1986, 1989) will re-assemble into virus-like particles (VLPs) \textit{in vitro} when the reducing agent is removed and calcium ions are added. Curiously, however, the dissociated SV40 virion will assemble only into incomplete particles under similar conditions (Colomar \textit{et al.}, 1993). At present, it is still not clear which \textit{in vitro} conditions will promote the assembly of SV40 VP1.

As the first step in determining the molecular mechanisms...
involved in SV40 capsid assembly, we showed previously that recombinant SV40 VP1 proteins produced by a baculovirus expression system assemble in the nucleus of insect cells into VLPs that are morphologically indistinguishable from wild-type SV40 capsids (Ishizu et al., 2001; Kosukegawa et al., 1996). Previous studies have also shown that VP1s of MPV (Montross et al., 1991; Forstova et al., 1993; Gillock et al., 1997) and human JC virus (Chang et al., 1997) are able to self-assemble into VLPs when expressed in insect cells. These VLPs could be dissociated efficiently into VP1 pentamers following treatment with both DTT and EGTA, suggesting that the structural properties of the recombinant VLPs are very similar to those of wild-type polyomavirus particles (Ishizu et al., 2001; Kosukegawa et al., 1996; Chang et al., 1997).

In this report, we analysed the self-assembly properties of highly purified recombinant VP1 pentamers in vitro by dialysis against a variety of buffers and by electron microscopy. We found that different conditions induced the pentamers to assemble into a variety of structurally polyhedral particles, including wild-type-like VLPs, tiny $T=1$ icosahedral particles and, markedly long tubular structures.

**METHODS**

**Expression and purification of recombinant VP1 pentamers.** Construction of the SV40 VP1-expressing baculovirus and expression of the VP1 protein in Spodoptera frugiperda (Sf9) cells have been described previously (Ishizu et al., 2001; Kosukegawa et al., 1996).

Purification of VP1 VLPs was performed as described previously (Ishizu et al., 2001) with minor modifications. That is, 5 ml of infected Sf9 cell lysates were loaded gently onto 6 ml preformed 20, 30, 40 and 50 % (w/v) caesium chloride density step gradients made with 20 mM Tris/HCl (pH 7.9) and centrifuged at 30 000 r.p.m. for 2-5 h at 4°C in an SW41Ti rotor (Beckman). After centrifugation, the white band in the middle of the gradient that contained the VLPs was collected. This solution was then added to a 37 % (w/v) caesium chloride solution made with 20 mM Tris/HCl (pH 7.9) and centrifuged at 50 000 r.p.m. for 20 h at 4°C in an SW55Ti rotor (Beckman). The VP1-containing band was again collected. Nonidet P-40 was added to a final concentration of 0-1 % and caesium chloride was removed by dialysis against 20 mM Tris/HCl (pH 7-9) and 0-1 % Nonidet P-40 at 4°C overnight with two buffer changes. After dialysis, samples were collected and centrifuged at 15 000 g for 5 min at 4°C. Supernatants containing the purified VLPs were then collected.

To prepare the VP1 pentamers, EGTA and DTT were added to the purified VLP preparation at final concentrations of 25 and 30 mM, respectively. Samples were incubated for 1 h at 37°C and separated on a Superdex 200 gel filtration chromatography column (Pharmacia) with 20 mM Tris/HCl (pH 7-9), 150 mM NaCl, 5 mM EGTA and 5 mM DTT at 4°C. Peak fractions corresponding to molecules approximately 200 kDa in size were collected and stored at -80°C.

**Re-assembly of VP1 pentamers in vitro.** VLPs were re-assembled in vitro from VP1s by dialysing 150 l of the purified pentamer preparation (60 µg VP1 protein ml$^{-1}$) for 24 h against the various reconstitution buffers we tested.

pH values in the 1 M NaCl assembly buffer were adjusted by 20 mM sodium acetate (pH 5-0 and 5-5), MES/NaOH (pH 6-0 and 6-5), Tris/HCl (pH 7-2 and 7-9), CHES/NaOH (pH 9-0) or CAPS/NaOH (pH 10-0 and 11-0). pH values in the 150 mM NaCl assembly buffer (pH 4-0–7-0) were adjusted with HCl only or with 20 mM Tris/HCl.

To provide divalent ions, CaCl$_2$, MgCl$_2$, CdCl$_2$, MnCl$_2$, BaCl$_2$, SrCl$_2$, ZnSO$_4$, CoCl$_2$, CuSO$_4$ and NiSO$_4$ were added to the dialysis buffer at final concentrations of 2 mM.

After dialysis, samples were collected and stored at 4°C until electron microscopy was carried out.

**Electron microscopy.** 5 µl of each sample was absorbed onto glow-discharged, carbon-coated copper grids. Grids were washed with water, stained with 2 % uranyl acetate and air-dried. Specimens were examined with an H-7500 electron microscope (Hitachi) at 80 kV.

**RESULTS**

**Preparation of recombinant SV40 VP1 pentamers.**

In our previous work, we constructed a recombinant baculovirus expressing SV40 VP1 from which VP1 proteins were produced efficiently and assembled into VLPs within baculovirus-infected Sf9 cells (Ishizu et al., 2001; Kosukegawa et al., 1996). These VLPs can be purified efficiently by caesium chloride density gradient ultracentrifugation and electron microscopy shows that these preparations contain spherical particles of approximately 45 nm in diameter and are indistinguishable from wild-type SV40 (Fig. 1a).

When this VLP preparation was subjected to size-exclusion chromatography in the presence of 150 mM NaCl (pH 7-9), VLPs were detected in the void volume fractions (Fig. 1b, top panel). When VLPs were incubated at 37°C with EGTA and DTT together with 150 mM NaCl (pH 7-9) and then subjected to size-exclusion chromatography, the VP1 proteins dissociated and most of the VP1 proteins were detected in fractions 8 and 9 (Fig. 1b, middle panel), which correspond to the molecular mass of the VP1 pentamer (approximately 200 kDa). This confirms our previous observations (Ishizu et al., 2001; Kosukegawa et al., 1996). Electron microscopy of fractions 8 and 9 revealed that the VLPs had indeed dissociated into pentamers (Fig. 1c). We also found that the recombinant VLPs dissociated only partially when 1 M NaCl (rather than 150 mM) was present during incubation and gel filtration (Fig. 1b, bottom panel). This agrees with a previous report that showed that a high salt concentration stabilizes the wild-type MPV capsid (Brady et al., 1977). Thus, the dissociation behaviour of the recombinant VLPs is quite similar to that of the wild-type virion.

Using this system, we could obtain VP1 pentamers (over 95 % pure, 60 µg ml$^{-1}$) (Fig. 1d). We then assessed the effect of various reconstitution buffers on the in vitro re-assembly of VP1 pentamers by dialysing the pentamers against these buffers followed by electron microscopy observation and gel filtration analysis.
VP1 pentamers formed heterogeneous particles in vitro

We assessed two basic types of buffers for their ability to promote the in vitro assembly of VP1s into VLPs, namely, a buffer with 2 M ammonium sulfate and a buffer with 1 M NaCl. Both buffers contained 2 mM CaCl₂ and had a pH of 7·2. Previous studies have shown that recombinant VP1 pentamers of MPV expressed in E. coli will assemble into VLPs when high concentrations of ammonium sulfate are present (Salunke et al., 1986, 1989). Therefore, we first tested whether SV40 VP1 would also re-assemble under similar conditions (2 M ammonium sulfate and 2 mM CaCl₂, pH 7·2) at 4 °C. Purified VP1 pentamers were dialysed against the buffer and observed by electron microscopy. V, VLP; I, intermediate particle; Ti, tiny particle. We found that VP1 assembled into spherical particles (Fig. 2a) and gel filtration analysis showed that the VP1 protein was detected in the void volume but not in the pentamer fractions (data not shown), indicating that VP1 pentamers had efficiently assembled into particles. However, electron microscopy revealed that while most of the re-assembled particles were similar to the
VLPs prepared from Sf9 cells (see Figs 1a and 2a), some particles were relatively small in size (see below).

Next, we tested the effect of the buffer with physiological pH (pH 7.2) and salt concentrations (150 mM NaCl) that lacked the reducing agent but contained 2 mM CaCl₂. However, assembly was not observed (see below), which is contrary to observations reported previously in that recombinant MPV VP1 assembles into VLPs under these conditions (Salunke et al., 1986, 1989). We then tested the ability of VP1 to assemble in a buffer containing 1 M NaCl and 2 mM CaCl₂ at pH 7.2 and at room temperature. As we noted above, 1 M NaCl severely inhibits the dissociation of VLPs (Fig. 1b, bottom panel) and, thus, these conditions may promote the assembly of VLPs. Pentamers did assemble into VLP-like particles but particles with different sizes and morphologies were also observed (Fig. 2b).

For convenience, we divided the particles observed in the ammonium sulfate buffer (2 M ammonium sulfate and 2 mM CaCl₂, pH 7.2, at 4 °C) and the NaCl buffer (1 M NaCl and 2 mM CaCl₂, pH 7.2, at room temperature) into four groups according to particle diameter and shape (Fig. 2c). The first group consists of VLPs (Fig. 2a, b, V) that are similar in size (approximately 40 nm in diameter) and shape to the original VLP and whose pentamer configurations resemble those in wild-type SV40 capsids. The second group consists of ‘intermediate particles’ (Fig. 2a, b, I) and includes particles that are relatively small in size (25–35 nm in diameter). These particles could be T = 3- or T = 4-like icosahedral particles (Anderer et al., 1967; Koch et al., 1967) or, alternatively, octahedral particles consisting of 24 pentamers (Salunke et al., 1989), but we could not distinguish between these forms even under a higher magnification (Fig. 2c). The intermediate particles may also be composed of a mixture of all of these types of particles. The third group consists of ‘tiny particles’ (Fig. 2a, b, Ti) that were much smaller and homogeneously in size (approximately 20 nm in diameter). These particles are star-shaped (Fig. 2c), which suggests that they are T = 1 icosahedral particles consisting of 12 VP1 pentamers (Anderer et al., 1967; Koch et al., 1967; Salunke et al., 1989). The fourth group found in the NaCl condition contains the ‘tubular structures’ (Fig. 2b, c, Tu) (Baker et al., 1983). These tubular structures are approximately 40–45 nm in width and vary in length. The average length under these conditions is 120 nm but the longest exceeded 500 nm (data not shown).

Collectively, we found that SV40 VP1 can assemble into various types of particles and that the in vitro conditions greatly affect this. To elucidate further the influence of environmental parameters on VP1 pentamer assembly in vitro, we analysed the effect of altering particular conditions.

**Manipulation of VP1 pentamer assembly in vitro**

We first modified the ammonium sulfate assembly conditions by adding EGTA and DTT, removing CaCl₂ or altering the concentration of ammonium sulfate. After each treatment, we counted the different types of particles and assessed whether their relative proportions in the particle population had changed (Fig. 3a). Particles were formed even when CaCl₂ was excluded. Although the VLPs purified from Sf9 cells will dissociate into pentamers when DTT and

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**Fig. 3.** Proportions of the four types of particles assembled under various in vitro conditions. Four people independently counted the numbers of particles on several electron microscopic views of each condition and the averages (%) are shown. About 200–1000 particles were counted for each condition. Some experimental conditions performed only once are presented without SE. (a) 2 M ammonium sulfate and 2 mM CaCl₂ (pH 7.2) at 4 °C with and without EDTA and DTT, and 2 M ammonium sulfate (pH 7.2) at 4 °C lacking CaCl₂. (b) 1 M NaCl and 2 mM CaCl₂ (pH 7.2) at room temperature, or 1 M NaCl (pH 7.2) at 4 °C with various CaCl₂ concentrations. (c) 1 M NaCl and 2 mM CaCl₂ at room temperature at different pHs. V, VLP; Ti, tiny particles; I, intermediate particles; Tu, tubular structures.
EGTA are present (Fig. 1b, c), the particle population in the ammonium sulfate buffer was unchanged by the presence of both DTT and EGTA. Thus, neither calcium ion-dependent interactions nor inter-pentameric disulfide linkages are necessary for assembly when high concentrations of ammonium sulfate are present. Also, varying the concentrations of ammonium sulfate between 1 and 3 M did not significantly affect assembly efficiency and particle heterogeneity (data not shown).

We then modified the NaCl assembly conditions. When we dialysed the dissociated VP1s against the NaCl buffer (1 M NaCl and 2 mM CaCl2, pH 7·2) at 4 °C rather than at room temperature, we found that tiny particles were rarely formed (Fig. 3b). We also noticed that tubular particles formed at 4 °C were shorter than those formed at room temperature (data not shown).

Next, we modified assembly conditions by reducing the CaCl2 concentration, altering the pH and decreasing the NaCl levels. When the calcium concentration decreased from 2 to 0·1 mM, the proportion of tiny particles gradually increased (Fig. 3b) and, at calcium concentrations less than 0·01 mM, VP1 pentamers assembled exclusively into tiny particles (Figs 3b and 4a). Thus, under conditions of 1 M NaCl (pH 7·2) at 4 °C, the inter-pentamer interactions that generate VLPs, intermediate and tubular particles require the binding of calcium ions to VP1 pentamers. In contrast, the formation of tiny particles does not require calcium ions. When we replaced the calcium ions with cadmium and manganese ions, the selective formation of tiny particles due to the absence of calcium ions was partially abrogated (Fig. 4b, c). The addition of other divalent ions such as magnesium, zinc, barium, strontium, copper(II), cobalt(II) and nickel(II) ions had no effect (data not shown). Of these divalent ions, only the ionic radii of cadmium (0·97 Å) and manganese (0·80 Å) ions are similar to that of the calcium ion (0·99 Å). This supports the notion that divalent ions of appropriate ionic radii must bind to the calcium-binding pockets of VP1 before this protein can assemble into larger particles.

Then, we analysed the effects of pH on VP1 assembly under conditions of 1 M NaCl and 2 mM CaCl2 (Fig. 4c). In this experiment, dialysis was performed at room temperature. Both the shape and proportion of the four types of particles were similar at near-neutral pHs (pH 6·0–9·0). At pH 5·5, however, VP1 pentamers assembled preferentially into VLPs and intermediate particles, but more than 90% of VP1 proteins were precipitated after a brief centrifugation, suggesting that most VP1 proteins were aggregated (data not shown). At even more acidic conditions (pH 5·0), particles were not observed at all and almost all VP1 proteins aggregated (data not shown). In contrast, at pH 10·0, VLPs and tiny particles were formed preferentially but, at a higher pH (11·0), almost all VP1 proteins remained as pentamers (data not shown).

We also tried to assemble the VP1 pentamers in lower salt concentrations because previous reports have shown that MVP VP1s assemble efficiently into VLPs when the reducing agent is removed and when CaCl2 is added to a buffer with a physiological pH and salt concentration (Salunke et al., 1986, 1989). However, in the presence of 150 or 300 mM NaCl (and 2 mM CaCl2, pH 7·2, at 4 °C), VP1s aggregated and formed no homogeneous particles (data not shown). Furthermore, in the presence of 500 mM NaCl and 2 mM CaCl2, pH 7·2, at 4 °C, only incomplete particle-like structures were formed (data not shown).

We then changed the pH of the buffer containing physiological NaCl (150 mM) levels. VP1 pentamers were aggregated at pH 7·0 and 6·0 (data not shown) but at pH 5·0 (and 150 mM NaCl and 2 mM CaCl2 at room temperature) we found that extraordinarily long tubular structures were formed selectively (Fig. 4d). Even when
CaCl$_2$ was absent, these tubular structures were formed, although they were shorter (data not shown). At pH 4-0, VP1 remained in the pentamer structure (data not shown).

**DISCUSSION**

Here we analysed the buffer conditions needed to promote the *in vitro* assembly of highly purified recombinant VP1 pentamers. We found that the conditions needed to dissociate recombinant SV40 VLPs obtained from infected Sf9 cells are similar to those facilitating wild-type SV40 and MPV capsid disassembly in that both DTT and EGTA must be present (Brady *et al.*, 1977, 1978; Colomar *et al.*, 1993). Furthermore, SV40 VLP dissociation, like MPV capsid disassembly, is inhibited by high salt concentrations (Brady *et al.*, 1977). In contrast, the conditions that promote SV40 and MPV assembly seem to be different. For example, unlike MPV VP1 (*Salunke et al.*, 1986, 1989), SV40 VP1 did not assemble into VLPs under conditions of physiological salt and pH. However, we must be careful to compare these studies because the re-assembly procedures and the VP1 expression systems (*E. coli* and baculovirus Sf9 cells) are different.

We found that, depending on the *in vitro* conditions, SV40 VP1 could assemble into morphologically divergent types of particles, namely, wild-type-like VLPs, and intermediate, tiny and tubular particles. Intermediate particles are 25–35 nm in diameter and may be either octahedral lattices, which have been observed previously in re-assembled MPV VP1s (*Salunke et al.*, 1989), or $T=3$- and $T=4$-like icosahedral particles, which have been observed in wild-type SV40 preparations (*Anderer et al.*, 1967; Koch *et al.*, 1967). Tiny particles are 20 nm in diameter and may be $T=1$ icosahedral particles composed of 12 pentamers. Previous studies have also found $T=1$ icosahedral tiny particles in caesium chloride-purified wild-type SV40 preparations (7–8 %) (*Anderer et al.*, 1967; Koch *et al.*, 1967). Tubular structures are 40–45 nm wide and have also been observed in wild-type MPV preparations but at low frequency (*Baker et al.*, 1983). Thus, the VP1 proteins of polyomaviruses have the potential to assemble into a number of divergent structures. That VP1 as a single polypeptide chain can form such highly polymorphic structures makes it quite an unique protein.

It is plausible that the carboxy-terminal arms of the VP1 proteins that emerge from the pentamer endow this property. In order to build a geometrically complex $T=7d$ icosahedral lattice from pentamer units, the carboxy-terminal arms need to be highly flexible and capable of forming three types of non-equivalent interactions ($\alpha'\beta'$, $\beta'\gamma'$ and $\gamma'\alpha'$) (*Yan et al.*, 1996; Stehle *et al.*, 1996; Liddington *et al.*, 1991; Rayment *et al.*, 1982). We speculate that the arms could be fixed into particular conformations by specific buffer conditions and that these then lead to the accumulation of specific kinds of particles. Supporting this notion is that we could identify the specific conditions that yielded the different particles.

For example, we showed that VP1 pentamers assembled exclusively into tiny particles in a 1 M NaCl buffer with a near-neutral pH that lacks CaCl$_2$ (Fig. 4a). The high salt concentration seems to be required to block the non-specific electrostatic attraction and repulsion between the pentamers because in lower (150–500 mM) NaCl concentrations, the pentamers form incomplete aggregates. Assembly in the presence of 1 M NaCl also required a neutral pH because the VP1 proteins aggregated at lower pH and did not interact with each other at higher pH, probably because of the electric repulsion between negative charges. CaCl$_2$ has to be present in the 1 M NaCl (pH 7–2) buffer to generate VLPs and intermediate and tubular particles. Clearly, calcium ions are needed to promote the interactions of the carboxy-terminal arms that produce such structures. In the absence of CaCl$_2$, the arms appear to form only a particular conformation that allows the pentamers to organize into only tiny particles. This notion is supported by our observation that two divalent ions (cadmium and manganese) with ionic radii similar to that of the calcium ion can substitute for the calcium ion in helping the VP1s to form larger particles (Fig. 4b, c). Furthermore, we have reported previously that some mutant VP1 proteins, whose calcium ion-mediated interactions are stronger than those of wild-type VP1, form fewer tiny particles (5–25 %) than wild-type VP1 (35 %) in insect cells (*Ishizu et al.*, 2001). Thus, the *in vitro* relationship between calcium binding and tiny particle formation also seems to be true *in vivo*.

In contrast to the 1 M NaCl and neutral pH conditions, however, the formation of VLPs in high concentrations of ammonium sulfate did not require CaCl$_2$. Furthermore, the formation of tubular structures in 150 mM NaCl at pH 5–0 also did not need CaCl$_2$. Thus, we are still far from understanding completely the conditions that promote the various steps of VP1 pentamer association that lead to the formation of VLPs or other types of particles. In addition, we were not able to reconstitute VLPs from VP1 pentamers under physiological conditions (150 mM NaCl, pH 7–2) and, thus, additional factor(s), such as a cellular chaperon protein, may be required. Nevertheless, our observations provide a lot of information about the self-assembly properties of SV40 VP1 pentamers. Moreover, this information now allows us to manipulate VP1 pentamers so that they will selectively assemble into tiny particles (1 M NaCl, pH 7–2), long tubular structures (150 mM NaCl and 2 mM CaCl$_2$, pH 5–0) or VLPs plus tiny particles (1 M NaCl and 2 mM CaCl$_2$, pH 10–0).

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