Cys^9, Cys^{104} and Cys^{207} of simian virus 40 Vp1 are essential for infectious virion formation in CV-1 cells

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Simian virus 40 (SV40) belongs to the papovavirus family of DNA tumour viruses. In simian cells, SV40 initiates a permissive infection leading to virion progeny. The viral structural proteins Vp1, Vp2 and Vp3 are synthesized in the cytoplasm of the permissive host cell and are transported to the nucleus for assembly (Tooze & Acheson, 1980; Lin et al., 1984). Pentamers of Vp1 form the capsomere, while small quantities of Vp2 and Vp3 associate internally with the capsid (Rayment et al., 1982; Baker et al., 1988; Liddington et al., 1991; Stehle et al., 1996). Several virus dissociation and assembly studies have implicated disulfide bonds between Vp1 molecules in the stabilization of the viral capsid (Brady et al., 1977, 1978, 1980; Ng & Bina, 1981; Kosukegawa et al., 1996). Liddington et al. (1991) visualized the structure of SV40 by X-ray crystallography at a resolution of 3.8 Å and showed three of the seven Vp1 cysteine residues in the SV40 capsid to be unavailable during mercury labelling, suggesting that they may be involved in covalent disulfide bonds. These are cysteines at positions 9, 104 and 207. More recently, the SV40 structure was refined to 3.1 Å and inter-pentamer disulfides were detected between Vp1 Cys^{104} residues (Stehle et al., 1996). Most recently, we have reported the involvement of the same three cysteines in disulfide-linked stabilization of inter-pentamer Vp1 complexes in cell-free lysates (Jao et al., 1999). Here, we report that the same three cysteines are involved in the formation of infectious SV40 virions in permissive simian cells. Single-, double- and triple-mutant Vp1 at Cys^9, Cys^{104} and Cys^{207} continued to localize to the nuclei of transfected CV-1 cells and to bind DNA, but showed a range of abilities to form plaques. Only mutants containing the Cys^9 → Ser change showed defects in plaque formation. Single mutants at Cys^9 formed small plaques; mutants at Cys^9, Cys^{104}, Cys^{207} and Cys^9, Cys^{104}, Cys^{207} formed no plaques. All three isolated revertants contained back-mutations at the Vp1 Cys^9 codon. These results further confirm the involvement of the three Vp1 cysteines in protein–protein interactions during virus assembly. Cys^9 is critical for production of wild-type infectious virions, whereas Cys^{104} and Cys^{207} play secondary roles.

Structural studies have implicated Cys^9, Cys^{104} and Cys^{207} of simian virus 40 (SV40) Vp1 in disulfide bond formation. Recently, we have shown the three cysteines to be essential for disulfide linkage of Vp1 complexes in vitro. Here, the role of the three cysteines was explored during the course of SV40 infection. Single-, double- and triple-mutant Vp1 at Cys^9, Cys^{104} and Cys^{207} continued to localize to the nuclei of transfected CV-1 cells and to bind DNA, but showed a range of abilities to form plaques. Only mutants containing the Cys^9 → Ser change showed defects in plaque formation. Single mutants at Cys^9 formed small plaques; mutants at Cys^9, Cys^{104}, Cys^{207} and Cys^9, Cys^{104}, Cys^{207} formed no plaques. All three isolated revertants contained back-mutations at the Vp1 Cys^9 codon. These results further confirm the involvement of the three Vp1 cysteines in protein–protein interactions during virus assembly. Cys^9 is critical for production of wild-type infectious virions, whereas Cys^{104} and Cys^{207} play secondary roles.

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Simian CV-1 cells were transfected with pSV40 plasmid encoding the entire SV40 genome (Clever et al., 1993). Transfected plasmids included wild-type Vp1 codons or single, double and triple mutations at Vp1 Cys^9, Cys^{104} and Cys^{207} codons, introduced via site-directed mutagenesis (Transformer kit, Clontech). Transfected cells were harvested 40 h post-transfection (p.t.) and were subjected to indirect double-labelled immunofluorescence studies using MABs against large T-Ag (CalBiochem) and polyclonal antibodies against Vp1 (anti-GST–Vp1, a kind gift of A. Oppenheim, Hebrew University–Hadassah Medical School, Jerusalem, Israel). Each mutant sample showed wild-type patterns of expression and nuclear localization of Vp1 (Fig. 1). Anti-T-Ag staining was used to allow better assessment of variations in transfection efficiencies and frequencies of nuclear localization of Vp1 among different mutants. Both wild-type and mutant plasmids...
led to transfection efficiencies of 10–15% and resulted in nuclear staining of Vp1 in over 60% of transfected cells (data not shown).

In vitro DNA-binding assays were performed to assess the binding of wild-type and Cys → Ser mutant Vp1 expressed in rabbit reticulocyte lysates to immobilized SV40 DNA. All mutants tested showed SV40 DNA binding comparable to that of wild-type (Fig. 2a). Thus, nuclear localization patterns and DNA binding of single, double and triple Vp1 mutants at Cys9, Cys104 and Cys207 were indistinguishable from those of wild-type.

Since all mutants showed normal nuclear accumulation and DNA binding, later events of virus assembly and infectivity of the mutants were investigated by tracking CPE and by plaque assays (Fig. 2b; Table 1). While classic CPE was detected by 7 days p.t. in cells transfected with wild-type SV40 DNA, no CPE was detectable by 14 days p.t. in cells transfected with SV40Vp1.3X DNA (the triple mutant). These results indicate that one or more of the cysteines at aa 9, 104 and 207 is essential for the onset of CPE and for production of infectious virions in permissive cells.

When SV40 single mutants at each of the three cysteine codons of Vp1 were used in DNA transfections, both CPE and plaques were detected (Fig. 2b; Table 1). Single mutants consistently showed delays in the onset of CPE relative to wild-type, with delays of 48 h for SV40Vp1.C104S and 24 h for SV40Vp1.C9S and SV40Vp1.C207S; all three single mutants, however, exhibited titres equivalent to wild-type. When cells were transfected with SV40 DNA containing double mutations at Vp1 Cys9.C104S, Cys9.C207S or Cys104.Cys207, only SV40Vp1.C104S.C207S-transfected cells showed CPE and plaques. Virions were isolated from transfected plates that exhibited CPE and were subjected to three rounds of plaque purification. Virion isolation was either by standard caesium chloride banding or via a novel small-scale virion isolation method described by Orlando et al. (2000). DNA from plaque-purified mutants was subjected to PCR amplification of the appropriate cysteine codon region and the amplified fragment was sequenced. In each case, plaque-purified single mutants at each of Vp1 Cys9, Cys104 and Cys207 codons and the double mutant at Cys104.Cys207 were confirmed and continued to form plaques (Fig. 2b). While all
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Fig. 2. (a) The Vp1 Cys → Ser mutants studied do not exhibit DNA binding defects. [35S]Methionine-labelled products of wild-type- and mutant pSp6Vp1-coupled transcription/translation (Jao et al., 1999) were incubated with SV40 DNA immobilized on PVDF membranes. DNA binding is expressed as the percentage of input c.p.m. retained on the membrane. Values represent means of two independent experiments. (b) Various mutants at Cys⁹, Cys¹⁰⁴ and/or Cys²⁰⁷ show defects in CPE and plaque formation. CV-1 cells were transfected with linear SV40 DNA (row A) or infected with plaque-purified virions (row B) as indicated and were stained with crystal violet 12 days after transfection/infection.

Table 1. Summary of infection phenotypes of mutants

<table>
<thead>
<tr>
<th>Input DNA</th>
<th>Onset of CPE (days p.t.)</th>
<th>Mean plaque diameter (mm)</th>
<th>Titre (p.f.u./plaque)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>WT</td>
<td>7</td>
<td>2.0 ± 0.3</td>
<td>2.0 × 10⁴</td>
</tr>
<tr>
<td>SV40Vp1.C9S</td>
<td>8</td>
<td>1.2 ± 0.2</td>
<td>2.0 × 10⁴</td>
</tr>
<tr>
<td>SV40Vp1.C104S</td>
<td>9</td>
<td>2.1 ± 0.4</td>
<td>3.0 × 10⁴</td>
</tr>
<tr>
<td>SV40Vp1.C207S</td>
<td>8</td>
<td>2.2 ± 0.3</td>
<td>1.5 × 10⁴</td>
</tr>
<tr>
<td>SV40Vp1.C9S.C104S</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>SV40Vp1.C9S.C207S</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>SV40Vp1.C104S.C207S</td>
<td>8</td>
<td>2.0 ± 0.3</td>
<td>6.0 × 10⁴</td>
</tr>
<tr>
<td>SV40Vp1.3X</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

three single mutants and the SV40Vp1.C104S.C207S double mutant led to plaques by 12 days post-infection, the mean size of SV40Vp1.C9S-induced plaques was consistently smaller (1.2 mm diameter) than those induced by wild-type SV40, SV40Vp1.C104S, SV40Vp1.C207S or SV40Vp1.C104S.C207S (> 2.0 mm diameter). Thus, only mutants containing a Vp1 Cys⁹ → Ser change showed defects in plaque formation; the single mutant at Vp1 Cys⁹ led to smaller plaques while both the double mutants at Cys⁹.Cys¹⁰⁴ and Cys⁹.Cys²⁰⁷ led to no plaques.

Cells transfected with SV40Vp1.C9S.C104S, SV40Vp1.C9S.C207S and SV40Vp1.3X showed no sign of CPE by 14 days p.t. Several samples were monitored for extended periods of up to 24 days p.t. Three late-onset CPE clusters were detected in three separate transfections. Late-onset plaques were picked and virions were isolated. Both input DNA and DNA from isolated virions were subjected to PCR amplification and sequencing. All three revertants contained back-mutations to the Cys⁹ codon; isolated revertant virions showed wild-type infection kinetics (data not shown).

Both SV40 structural studies by others (Liddington et al., 1991; Stehle et al., 1996) and in vitro SV40 Vp1 assembly studies by us (Jao et al., 1999) have implicated Cys⁹, Cys¹⁰⁴ and Cys²⁰⁷ of Vp1 in disulfide linkage of post-pentameric complexes. Here, by generating a series of single, double and triple
mutations at Cys\(^9\), Cys\(^{104}\) and/or Cys\(^{207}\) codons of Vp1 and their introduction into permissive cells in the context of an otherwise wild-type SV40 genome, we have assayed the role of the three cysteines in the course of permissive infection in vivo. We have presented evidence for the involvement of SV40 Vp1 Cys\(^9\), Cys\(^{104}\) and Cys\(^{207}\) residues in the onset of CPE and in the production of infectious virions in permissive CV-1 cells. The present study indicates that the same three cysteines implicated in stabilization of disulfide-linked Vp1 post-pentameric complexes in vitro are also essential for infectious virion formation in vivo.

Our results also show that none of the three cysteines, individually or in double or triple combinations, is involved in the correct nuclear localization or DNA-binding of Vp1.

All Vp1 mutants defective in plaque formation localized successfully to the nucleus of the host cell and bound DNA, but showed no CPE. This suggests that the Vp1 mutants are defective in protein–protein interactions involved in stable formation of virions, rather than in their ability to enter the nucleus and bind DNA or in the ability of assembled virions to exit the infected cell or infect a new cell successfully. In fact, we have been unsuccessful in isolating virus-like particles from any of the mutants defective in plaque formation (data not shown). A defect in the early steps of assembly is consistent with our in vitro results, where mutants defective at the same three residues continued to form pentamers but failed to form disulfide-linked post-pentameric complexes in cell-free lysates (Jao et al., 1999). Most recently, Cys\(^9\) and Cys\(^{104}\) of SV40 Vp1 have been implicated in disulfide-bonded formation of virus-like particles in insect cells (Ishizu et al., 2001).

Due to the overlapping nature of the Vp2/Vp3 and Vp1 genes, Cys\(^9\) → Ser mutagenesis also resulted in the conservative Leu\(^{206}\) → Phe codon change in Vp2/Vp3. Leu\(^{206}\) is the last residue in the proposed nuclear localization signal of Vp3, but is not involved in DNA binding or in Vp1–Vp3 interactions (Gharakhanian et al., 1988; Clever & Kasamatsu, 1991). The nuclear localization of Vp3 was studied in pSV40Vp1.C9S transfections using anti-GST–Vp3 antibodies (kind gift of A. Oppenheim) and was indistinguishable from that of wild-type Vp3 (S.J. Orlando and E. Gharakhanian, unpublished results).

Several virus dissociation and assembly studies have implicated disulfide bonds in the stabilization of the viral capsid (Brady et al., 1977, 1978, 1980; Ng & Bina, 1981; Kosukegawa et al., 1996). The conservative nature of the Cys → Ser mutations, along with the fact that isolated revertants invariably involved back-mutations of Ser → Cys codons, suggests strongly that Cys\(^9\), Cys\(^{104}\) and Cys\(^{207}\) exert their role in formation of infectious virions via disulfide bond formation. Both the current in vivo results and our recent in vitro results (Jao et al., 1999) support a cumulative effect in the role of the cysteines such that all single mutants continue to form infectious virions. However, while all double mutants continued to form at least some post-pentameric complexes in vitro, only the double-mutant Vp1.C104S.C207S continued to lead to CPE and plaque formation in vivo. This suggests that assembly in cells may require further stabilization and may involve more stringent requirements than that observed in cell-free lysates. Furthermore, since only mutants containing Cys\(^9\) → Ser mutations in our studies showed defects in plaque formation and since all isolated revertants contained a back-mutation at that site, Cys\(^9\) may be the most crucial of the three cysteines for disulfide-linked stabilization, with Cys\(^{104}\) or Cys\(^{207}\) having secondary roles. Li et al. (2000) have recently suggested the significance of Cys\(^{207}\) of Vp1 in efficient calcium binding and in formation of infectious SV40 virions.

While our results suggest disulfide bonding at Cys\(^9\) to be crucial to infectious virion formation, the only disulfide linkage detected in structural studies of SV40 has been between two Cys\(^{104}\) residues (Stehle et al., 1996). In all structural studies of SV40, however, the N-terminal 13 amino acids of Vp1 have been disordered, such that structural information was not available for that region (Liddington et al., 1991; Stehle et al., 1996; Yan et al., 1996). Thus, the specific positioning of Cys\(^9\) and its interactions have not been established. In the current structural model, the C terminus of Vp1 is the main mechanism of contact between pentamers. C-terminal arms extend from every Vp1 in the pentamer and insert into binding sites on adjacent pentamers such that the acceptor monomer forms a ‘clamp’ to fix the position of the invading arm. Based on available structural information on Vp1 and our results, the most likely role of Cys\(^9\) may be in the stabilization of the N-terminal ‘clamp’ and its interactions with the C terminus of the invading Vp1.

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References


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