Two determinants in the capsid of a persistent type 3 poliovirus exert different effects on mutant virus uncoating

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Mutant polioviruses (PV) have been previously found to be capable of establishing persistent infections in HEp-2c cells. Together, two amino acid substitutions in the viral capsid of a type 3 poliovirus (PV-3), at positions VP2₁₃ and VP₁₂₉₀, are sufficient to confer the persistent phenotype to a normally lytic virus. When susceptible cells are infected, the double mutant T₇L₊₂L₁₃₁N₂₉₀ undergoes unique conformational changes in the capsid, modifying its sedimentation coefficient from 160S to 147S. In the present study, we have further investigated mutant PV decapsidation and, in particular, the effect of each determinant independently. Our results indicate that the novel 147S form was also generated by a mutant carrying only the determinant 1N₂₉₀. This form was not produced as a result of inherent capsid instability and it was generated only upon specific PV→host cell interactions. The second viral determinant, 2L₁₃, also modified receptor-induced conformational changes, although differently from 1N₂₉₀.

Poliovirus (PV), the prototype member of the family Picornaviridae, is composed of a small single-stranded positive-sense RNA enclosed in an icosahedral capsid which consists of 60 copies of each of the four viral structural proteins (VP₁, VP₂, VP₃, VP₄). To initiate infection, PV attaches to the cell by way of a cell surface molecule known as the PV receptor (PVR) which is a member of the immunoglobulin superfamily (Koike et al., 1990; Mendelsohn et al., 1989; Olson et al., 1993; Wimmer et al., 1994). This adsorption step occurs even at 0°C. At 37°C, the PVR induces specific conformational changes in the viral capsid (Gomez Yafal et al., 1993; Kaplan et al., 1990). The virion, which sediments with a coefficient of 160S in sucrose gradients, is altered, externalizing the normally internal N terminus of VP₁ and losing the internal protein, VP₄, to give a 135S intermediate (Flore et al., 1990; Fricks & Hogle, 1990). Some of the 135S particles detach from the cells and are eluted in the cell supernatant, while the remaining cell-associated particles could permit decapsidation of the PV genome. However, the existence of an alternative, larger and highly transient uncoating intermediate has recently been proposed (Dove & Racaniello, 1997). Subsequent steps in the virus cycle result in the synthesis of progeny virions and lysis of the infected cell, typically within 8 h (Wimmer et al., 1993).

Despite this normally very lytic nature of PV, we have shown that most PV strains can establish persistent infections in IMR-32 neuroblastoma cells and that mutant PV selected during these infections are capable of persistently infecting non-neural HEp-2c cells, unlike their corresponding parental strains (Colbère-Garapin et al., 1989; Pelletier et al., 1991). Recently, we identified viral determinants of persistence in HEp-2c cells: two on the capsid surface for the type 1 PV strain, Sabin 1 (Pelletier et al., 1998a); and one on the inside, Leu₁₃ (2L₁₃), and one on the surface Asn₂₉₀ (1N₂₉₀) for the type 3 PV (PV-3) strain, T₇-Leon (T₇L) (Duncan et al., 1998). Both PV-3 determinants are required for efficient persistence; their localization on the three-dimensional structure of the capsid indicated that the internal determinant 2L₁₃ is located near a region critical to capsid stability (Filman et al., 1989), while the surface determinant 1N₂₉₀ is located in neutralization antigenic site 3a (Minor et al., 1986), outside of the putative footprint of the PVR (Colston & Racaniello, 1994; Harber et al., 1995; Olson et al., 1993).

Previously, we observed several important differences in the early steps of the virus cycle between the parental lytic virus T₇-Leon and the persistent double mutant T₇L₊₂L₁₃₁N₂₉₀. Firstly, the persistent mutant exhibits increased adsorption and decreased elution of attached particles, suggesting an increased affinity for the PVR (Duncan et al., 1998). Secondly, the persistent mutant, with a sedimentation coefficient of 160S, generated a novel 147S form upon adsorption onto PVR-expressing cells, even at 0°C.

In the current study, we have further investigated the novel receptor-mediated conformational changes and we have analysed the independent effects of each of the two determinants.
Fig. 1. PVR-induced particle alteration in HEp-2c cells. In each case, infections with [35S]methionine-labelled viruses were first synchronized by a 2.5 h adsorption period at 0 °C. After 0 or 90 min of uncoating at 37 °C, cell-associated and extracellular viral particles were analysed separately by sucrose gradient centrifugation. (A) The uncoating profiles observed for the lytic T7-Leon and persistent T7L+2L13N290 viruses after 90 min of additional incubation at 37 °C. (B) The profiles observed for each of the two poorly persisting single mutants, T7L+2L13 and T7L+1N290, immediately after 2.5 h adsorption at 0 °C (left-hand panels) or after an additional 90 min incubation at 37 °C (right-hand panels). Purified 160S and 80S particles were used as markers in parallel gradients.
We first determined the nature of the particle(s) which elute at 37 °C in the case of the persistent mutant T7L + 2L_{13}1N_{290}, since when virus and cells are incubated at 37 °C for 90 min after adsorption, over 35% of the initially bound viral particles are present in the extracellular medium (Duncan et al., 1998). In order to know whether 147S or 135S particles are eluted, we first adsorbed [35S]methionine-radiolabelled virus onto human HEp-2c cells at 0 °C and then removed unattached virus by repeated washes before switching the infection to 37 °C for 90 min. Cell supernatants and cell lysates were then analysed by sucrose gradient centrifugation, as previously described (Duncan et al., 1998). In the case of T7-Leon (Fig. 1A, top panel), the sedimentation profile revealed, as expected, the 160S virion, the 135S intermediate and the 80S empty capsid, all detectable both in cell-associated and extracellular fractions. In contrast, in the case of the persistent T7L + 2L_{13}1N_{290} virus (Fig. 1A, bottom panel), only the novel 147S form and the 80S empty capsids were visible in the cell-associated fractions. Surprisingly, however, the 135S particle was detectable in the supernatant, although the ratio of 135S particles to 160S and 80S particles appeared greatly reduced as compared to that observed for T7-Leon. Most importantly, the 147S form did not appear to be present in the extracellular fractions, suggesting that it is found solely in association with the cell.

Although both of the point mutants T7L + 2L_{13} and T7L + 1N_{290} are only capable of establishing persistent infections in HEp-2c cells with very low efficiency, we previously observed that they did have modified cell tropisms in comparison with the lytic parental virus T7-Leon, as evidenced by reduced plaque size in HEp-2c cells and an increased difference in viral titres on IMR-32 cells versus HEp-2c cells (Duncan et al., 1998). We therefore chose to investigate their individual contributions to the atypical receptor-mediated conformational changes observed for the double mutant T7L + 2L_{13}1N_{290}.

After adsorption for 2.5 h on ice, the two single mutants had distinctly different profiles when associated with the cell (Fig. 1B, left-hand panels). In fact, the single mutant T7L + 2L_{13} appeared to form a peak sedimenting at the 160S position, although the peak was always slightly asymmetrical, with a longer slope towards the 147S position (Fig. 1B, top left-hand panel). Conversely, the second single mutant, T7L + 1N_{290}, formed a peak sedimenting at the 147S position. When the decapsidation profiles were observed after a further 90 min incubation period at 37 °C, the differences between the two viruses were even more marked. In the cell-associated fraction, the mutant T7L + 2L_{13} (Fig. 1B, top right-hand panel), formed a main peak at the 160S position of virions, again with a longer slope towards the top of the gradient. Very few 135S particles were eluted with this mutant, as observed for the double mutant T7L + 2L_{13}1N_{290}. The second single mutant, T7L + 1N_{290} (Fig. 1B, bottom right-hand panel), formed a main peak of cell-associated particles at the 147S position, like the double persistent mutant, but had an elution profile similar to that of the parental lytic virus T7-Leon (Fig. 1A, top panel). Taken together, these results all suggest that each of the two viral determinants, 2L_{13} and 1N_{290}, exert different effects on the viral capsid during receptor-mediated uncoating.

It has been previously demonstrated that the 160S to 135S receptor-mediated conformational changes only occur at temperatures greater than 32 °C (Gomez Yafal et al., 1993). However, in the case of the persistent PV T7L + 2L_{13}1N_{290}, the generation of the 147S particle is observed even at 0 °C (Duncan et al., 1998), suggesting inherent capsid instability. We therefore investigated the susceptibility of the viral capsid to heat inactivation by incubating viruses for various times at 48 °C before determining the titre by an endpoint micro-method. As can be seen in Fig. 2, the persistent mutant T7L + 2L_{13}1N_{290} was in fact extremely stable at high temperature, having over a thousandfold higher titre than the wild-type virus after 15 min incubation at 48 °C. Interestingly, the two single point-mutants were even slightly more stable than the double mutant. Altogether, these results showed that the mutant PV-3 capsids were in fact extremely heat-stable in PBS, indicating that the atypical uncoating profiles are not due to capsid instability.

Recent studies have demonstrated that it is possible to induce the 160S to 135S conformational changes in vitro, in the absence of the PVR, by incubating virions at 50 °C in a hypotonic calcium-containing buffer (Curry et al., 1996). We decided to see if the novel 147S form could also be produced by our mutant viruses under these conditions. The results
shown in Fig. 3 clearly indicate that while the peaks corresponding to the native 160S virion, the 135S uncoating intermediate and the 80S empty capsid were all detectable in the case of T7-Leon and the persistent double mutant, neither the double mutant nor either of the single mutants were able to produce detectable amounts of the 147S form in a cell-free system. Interestingly, the relative amount of 135S compared to 160S particles produced in this assay appeared to correspond to that observed for the eluted particles upon infection at 37 °C (Figs 1 and 3): T7L + 2L13N290 and T7L + 2L13 produced very little or no detectable 135S particles, respectively, while T7L + 1N290 produced a quantity of 135S particles comparable to that produced for the wild-type virus T7-Leon. In the event that the 135S forms were more fragile for the mutant viruses T7L + 2L13N290 and T7L + 2L13, additional experiments were performed using shorter incubation times and temperatures lower than 50 °C. However, it was never possible to detect a greater amount of these particles (data not shown).

Taken together, the results presented here indicate that the two single mutants differ from each other in the amount of the 147S form produced upon interaction with the PVR (very low if at all for T7L + 2L13 and a major peak for T7L + 1N290) and the amount of eluted 135S particles (very low for T7L + 2L13 and a significant peak for T7L + 1N290). These results suggest that the ability of PV-3 to efficiently establish persistent infections in HEp-2c cells is linked to these two aspects of PV–PVR interactions: novel receptor-mediated conformational changes in the viral capsid giving a 147S form and a reduction in the quantity of 135S particles. As discussed elsewhere (Pelletier et al., 1998b), these modifications allow persistence only at low m.o.i. (10−4 TCID50 per cell), where even a slight inefficiency during uncoating and/or penetration could allow an infected cell to survive. At higher multiplicities, the sheer number of virions infecting each cell would compensate for their inefficiency during the early steps, always resulting in a lytic cycle.

As to the nature of the 147S form, our results to date, presented here and elsewhere (Duncan et al., 1998) suggest that virus–receptor interactions are absolutely required for its formation. Since the 147S form appears to result from virions having an increased affinity for the PVR (Duncan et al., 1998) coupled with remarkable capsid stability, one cannot exclude the possibility that this form corresponds to a very stable association between a viral particle and a cellular molecule, most likely the PVR, which would block PV entry. However, increasing the SDS concentration in cell extracts up to fourfold did not prevent the detection of the 147S form (data not shown). The results presented here are rather in agreement with two of our previously presented hypotheses (Duncan et al., 1998; Pelletier et al., 1998b), i.e. the 147S form could be either a stable, abortive, uncoating intermediate which forms immediately upon contact between certain mutant virions and the PVR, or a normally occurring but usually highly transitory uncoating intermediate, which is defective in the case of certain mutant viruses.

In conclusion, we report here the independent effects of each of two viral determinants of persistence, 2L13 and 1N290, on virus uncoating at the molecular level, which appear to be

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**Fig. 3.** Conformational changes induced in the viral capsid of the lytic T7-Leon, persistent T7L + 2L13N290, and poorly persisting T7L + 2L13 and T7L + 1N290 viruses, in a cell-free system, in the complete absence of the PVR. [35S]Methionine-labelled viruses were incubated for 3 min at 50 °C in a hypotonic buffer containing calcium, as previously described (Curry et al., 1996). The resulting particles were then analyzed by sucrose gradient centrifugation. Purified 160S and 80S particles were used as markers in parallel gradients.
responsible for the production of a reduced number of 135S particles and for the generation of a novel 147S form, respectively.

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