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New Insights into Poliovirus Uncoating

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Running title: poliovirus/CD155-IgG2a interactions

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ABSTRACT

A Sabin 1 strain poliovirus (PV) mutant, S1(2Y-1I), carrying a Tyr at amino acid position $VP2_{142}$ and an Ile at position $VP1_{160}$, can establish persistent infections in HEp-2c cells. This mutant forms atypical 147S particles upon interaction at 0°C with either cells expressing PV receptor (PVR) CD155, or PVR-IgG2a, a chimeric molecule consisting of an extracellular moiety of PVR and the hinge and Fc portion of a mouse IgG2a. Upon interaction with PVR at 37°C, S1(2Y-1I), like the parental strain, forms both 135S A particles and 80S empty capsids. At 0°C, surprisingly, at a concentration equal to or greater than 5 nM, PVR-IgG2a induced both the extrusion of VP4 from the capsid of S1(2Y-1I) and the formation of 80S particles. The same transitions were observed at 0°C with the parental strain Sabin 1 at 40 nM PVR-IgG2a. Thus, the formation of 80S particles and VP4 extrusion, considered as one of the steps of PV uncoating, can be temperature-independent at high PVR concentration. This implies that structural changes of the PV capsid occurred following adsorption at low temperature.

INTRODUCTION

Poliovirus (PV), the causative agent of poliomyelitis, is the prototype member of the *Picornaviridae* family. The virion consists of a small single-strand positive-sense RNA enclosed in a non-enveloped icosahedral capsid made up of 60 copies of each of the four structural proteins, VP1 to VP4. The three-dimensional structure of the viral particle has been elucidated by crystallographic studies (Hogle, Chow, and Filman, 1985). The capsid consists of 12 pentamers, each composed of five protomers and the virion thus has a total of 60 PV receptor (PVR) binding-sites (Arita et al., 1998; Belnap et al., 2000; He et al., 2000). PVR (CD155) is a member of the immunoglobulin superfamily and its natural functions are unknown (Koike et al., 1990; Mendelsohn, Wimmer, and Racaniello, 1989), although it has recently been shown specifically to bind vitronectin (Lange et al., 2001) by the extracellular domains, and Tctex-1, a light chain of the dynein motor complex, by the cytoplasmic domain (Mueller et al., 2002; Ohka and Nomoto, 2001). The first step of infection starts with the binding of PV to PVR on the surface of susceptible cells. This adsorption step can occur *in vitro* at 0°C. Dove and Racaniello reported results suggesting that the PV capsid must undergo structural changes to bind to PVR (Dove and Racaniello, 2000). At temperatures higher than 32°C, the PVR induces changes in viral conformation, which modify the sedimentation coefficient of the virion from 160S to generate A particles, at 135S, and empty capsids, at 80S (Flore et al., 1990; Fricks and Hogle, 1990). There has been some question about the role of 135S A particles in infection, because the efficiency of A particle-mediated infection is low and A particles do not accumulate when virus is grown at 26°C (Curry, Chow, and Hogle, 1996; Dove and Racaniello, 1997). Recently, it has been shown that the efficiency of infection of A particles can be increased; production of A particles is rate limiting at 26°C and therefore they would not be expected to accumulate (Huang, Hogle, and Chow, 2000). Both A particles and empty capsids have lost VP4, but only the latter has lost

the viral genome. Therefore, it is generally believed that the formation of 80S empty capsids results from viral uncoating. PVR-IgG2a (or CD155-IgG2a), a chimeric molecule consisting of an extracellular moiety of PVR and the hinge and Fc portion of a mouse IgG2a, together with Fc receptor-expressing cells, were used to confirm the importance of specific PV-PVR interactions in viral uncoating (Arita, Horie, and Nomoto, 1999). It has also been shown that PVR acts as a catalyst, increasing the rate of capsid transitions by lowering the activation barrier (Tsang et al., 2001).

In studies of PV persistence in HEp-2c cells, we demonstrated the accumulation of an atypical particle, sedimenting at 147±3 S, upon adsorption of a type 3 PV (PV3) mutant onto PVR-expressing cells at 0°C (Duncan, Pelletier, and Colbère-Garapin, 1998). The 147S particle was not produced as a result of inherent capsid instability and it was generated only upon specific PV-host cell interactions (Duncan and Colbère-Garapin, 1999). Persistent Sabin 1 (S1) mutants have also been studied and two determinants of persistence were identified, mapping to amino acid residues $VP1_{160}$ (Val-> Ile) (1I) and $VP2_{142}$ (His-> Tyr) (2Y) (Pelletier, Duncan, and Colbère-Garapin, 1998). One (2Y) is in the E-F loop of VP2, in the canyon, and directly interacts with the PVR (Belnap et al., 2000; He et al., 2000). The other is in β strand E, near the E-F loop, at the interface between protomers, near residues of the PVR-binding site, and above the hydrophobic pocket which is believed to contain a sphingosine molecule in the mature virion (Belnap et al., 2000; Colston and Racaniello, 1995; Filman et al., 1989; He et al., 2000). This determinant may alter the plasticity of the viral capsid of persistent mutants. The 1I and 2Y mutations independently modify the adsorption, elution and uncoating transitions of the mutants, but no 147S forms have been detected following interaction of these single point mutants with PVR (Pelletier, Duncan, and Colbère-Garapin, 1998). To investigate further the uncoating process of persistent mutants, we studied PVR-induced transitions of the corresponding double mutant S1(2Y-1I) after interaction with either PVR-expressing cells or soluble PVR-IgG2a. Surprisingly, at some PVR-IgG2a

concentrations, the soluble receptor alone was capable of producing 147S and 80S particles at 0°C. The extrusion of VP4 from the capsid indicated that the structure of the PV capsid changed following adsorption at low temperature.

RESULTS

PV receptor-induced conformational changes after adsorption onto HEp-2c cells

To determine the cumulative effect of the two determinants 1I and 2Y on receptor-induced conformational changes, we studied the kinetics of transition from the 160S to the 135S A particles for the S1(2Y-1I) double mutant. Radiolabeled virions were prepared in HEp-2c cells with [^{35}S]methionine and purified as previously described (Pelletier et al., 1998). HEp-2c cell infections were synchronized by a 2.5 h period of adsorption at 0°C, and then conformational transitions were started by incubation at 37°C for 3 or 10 min. Cell-associated viral particles were separated by centrifugation in sucrose gradients (15-30% w/v) as described (Pelletier et al., 1998). The sedimentation profile of the parental virus S1 showed, as expected, one peak corresponding to the 160S virions. This peak decreased upon incubation at 37°C, and a peak of 135S A particles appeared concomitantly (Fig. 1, top). In contrast, the sedimentation profile of the S1(2Y-1I) mutant was atypical. At the end of the adsorption period at 0°C, there was a peak corresponding to the 147S particles previously observed for the PV-3 mutants (Duncan and Colbère-Garapin, 1999; Duncan, Pelletier, and Colbère-Garapin, 1998). Incubation at 37°C caused a decrease of the 147S peak, with a simultaneous shift to a position corresponding to the 135S A particles, suggesting that the 147S particles underwent a conformational transition to A particles (Fig. 1, bottom). As expected, these A particles were unstable in cells: the peak of cell-associated particles decreased substantially upon incubation of cells at 37°C. When the fraction corresponding to

the top of the 147S peak was analyzed again in a 15-30% sucrose gradient, only half of the remaining radioactivity was found associated with particles still sedimenting at 147S (data not shown). This suggests that the 147S particles of S1(2Y-1I) are unstable. The conformational transition to 135S A particles at 37°C was complete for the S1(2Y-1I) mutant but only partial for S1 (Fig. 1, bottom). This suggests that the flexibility of the capsid and viral uncoating were enhanced by the S1(2Y-1I) mutations.

Characterization of 147S particles

We first analyzed the protein composition of 147S particles to determine whether or not they contained VP4. The 147S particles were obtained by incubating HEp-2c cells with radiolabeled S1(2Y-1I) for 2.5 h at 0°C. After sedimentation in sucrose gradients, an aliquot of each fraction was counted to verify the profile of the peak (Fig. 2, A). Then, fractions (8 to 15) including most of the 147S particles were analyzed by SDS-PAGE in parallel to virions of 160S and eluted A particles of 135S for both the S1 and S1(2Y-1I) viruses. The radioactivity of each of the four capsid protein bands was quantified using a STORM 820 apparatus (Molecular Dynamics®). As expected, the VP4 protein was detected in the 160S particles and not in the A particles of both the S1 strain and the S1(2Y-1I) mutant (Fig. 2, B). The VP4 protein was present in each of the 8 fractions of the peak corresponding to the 147S particles of the S1(2Y-1I) mutant (Fig. 2, B), and the proportions of each of the four capsid proteins were similar for the 147S particles and the control 160S particles (Fig. 2, C).

We determined the density of 147S particles by CsCl gradient centrifugation. The cells were incubated with the radiolabeled S1 and S1(2Y-1I) virions for 2.5 h at 0°C, and then half of each sample was analyzed in sucrose gradients and the other half in CsCl gradients (Fig. 3). The interaction between the PVR and the S1(2Y-1I) mutant induced the expected modification of the sedimentation coefficient of the particle (147S), but the density was similar for both 147S and 160S particles, strongly suggesting that the low sedimentation

coefficient (147S) of the S1(2Y-1I) particles was not a result of the loss of the viral genome. This was confirmed by testing the infectivity of 147S particles (data not shown).

Interactions of viruses with the chimeric receptor PVR-IgG2a

To see whether the 147S particle is induced by the specific virus-receptor interaction alone, without any contribution from other cellular factors, we studied the transition kinetics of S1 and S1(2Y-1I) using PVR-IgG2a. ^{35}S-labeled virions (2 X 10^{10} particles) were incubated for 2.5 h at 0°C with various concentrations of PVR-IgG2a (0 to 20 nM, in a volume of 200 μl) and the sedimentation profiles in sucrose gradients determined (Fig. 4A and B).

The parental virus S1 appeared to form a peak sedimenting at the 160S position when the PVR-IgG2a concentration was less than or equal to 5 nM (Fig. 3B). Surprisingly, at higher PVR-IgG2a concentrations (10 or 20 nM), the peak was shifted towards the top of the gradient, near the 147S position (Fig. 4B, left hand panels). Thus, the parental strain S1 seemed to form 147S particles in these cell-free conditions, although they have never been observed upon adsorption onto cells.

We verified that S1(2Y-1I) sedimented at 160S like S1, after incubation for 2.5 h at 0°C in the absence of PVR-IgG2a (Fig. 4A). Even at the lowest PVR-IgG2a concentration (1.25 nM) at 0°C, the peak of S1(2Y-1I) particles was shifted to the 147S position. This receptor concentration corresponds to 0.125 molecules per binding site and 7.5 molecules per virion, which would be the equivalent of 15 PVR domains per virion, assuming both heads of the chimera can bind, but these numbers are likely to be upper estimates for the actual occupancy. When a concentration of 5 nM PVR-IgG2a (i.e. 0.5 molecules per binding site) was used with S1(2Y-1I), this peak was again slightly shifted toward the top of the gradient, at 135S, and surprisingly, a peak at the 80S position of empty capsids appeared (Fig. 4B, right hand panels). In addition, at even higher PVR-IgG2a concentrations (20 nM, i.e. 2 molecules per binding site), the transition of S1(2Y-1I) to 80S particles was almost complete. Although

such sedimentation profiles are normal at 37°C where uncoating occurs, it was unexpected under these experimental conditions, i.e. at 0°C. These results suggested that uncoating could occur at 0°C in the presence of high concentrations of PVR-IgG2a. We tested whether this phenomenon was observed with the S1 strain at a very high PVR-IgG2a concentration (40 nM, i.e. 4 molecules per binding site). The 80S peak was observed (Fig. 4C), but the PVR-IgG2a concentration required to obtain a sedimentation profile similar to that of S1(2Y-1I) was 8-fold higher in the case of S1 (Fig. 4B and C).

When the adsorption period at 0°C was followed by an incubation of 7 min at 37°C, soluble PVR-IgG2a, like PVR-expressing cells, induced uncoating transitions (Fig. 4D). S1 transitions were almost complete at PVR-IgG2a concentrations of 5 nM or above (Fig. 4D). For S1(2Y-1I) at 37°C, the transition kinetics were similar for all PVR-IgG2a concentrations and the transition was in all cases more efficient than that observed for the S1 strain (Fig. 4D). These findings agree with those obtained with HEp-2c cells (Fig. 1).

Digestion of the 147S particles with V8 protease

As 147S and 160S particles seem to only differ by their sedimentation coefficient but not their composition, we looked for externalization of the N-terminus of VP1, as observed during the transition from 160S virions to A particles at 37°C (Fricks and Hogle, 1990). We studied the susceptibility of the 147S particles to the Glu-c V8 endoproteinase. The first four Glu residues of VP1 in A particles are accessible to this protease, such that it generates four digestion fragments (Curry, Chow, and Hogle, 1996). The concentrations of PVR-IgG2a which induced transitions similar to those obtained after interaction of the viruses with HEp-2c cells were chosen (5 nM and 1.25 nM). Radiolabeled viruses were incubated for 2.5 h at 0°C with (or without) PVR-IgG2a, then incubated for 7 min at 0°C or 37°C. An aliquot of each sample was layered onto sucrose gradients to verify the position of the peaks. In parallel, samples were exposed to various concentrations (0, 5 or 10 μg per ml) of V8 for 1h

at 4°C. As expected, 135S A particles from both S1 and S1(2Y-1I) viruses were digested by the enzyme: the VP1 protein migrated as a smear between the VP2 and VP3 proteins which remained intact (Fig. 5). VP1 proteins in 160S particles used as controls were not digested. The 147S particles from the S1(2Y-1I) mutant seemed to be resistant to V8 digestion, suggesting that the N terminus of the VP1 protein was not externalized during the 160S to 147S conversion at 0°C. Therefore, 147S particles have several properties in common with 160S particles. A modification of the sedimentation coefficient of atypical particles could be due to either a modification of the conformation of the particle, such as an expansion, or the attachment of PVR-IgG2a molecules, or both.

Are 147S particles saturated with hPVR-IgG2a molecules?

To address the possibility that the 147S particles could be the result of an excess of bound PVR-IgG2a, we quantified this molecule in the gradient by slot blot assay, as described in Materials and Methods. In parallel to S1 and S1(2Y-1I), Coxsackievirus B1 (CVB1), which does not interact with hPVR, was used as a negative control. No chimeric receptor cosedimented with CVB1 (Fig. 6). For all three viruses, the majority of PVR-IgG2a molecules were located at the same position between fractions 30 and 35 (Fig. 6). A small number of PVR-IgG2a molecules cosedimented with the 147S particles of S1 and S1(2Y-1I) (Fig. 6). The total number of (bound and free) PVR-IgG2a molecules present in the fractions of peaks was calculated. There was an average of about 3 PVR-IgG2a molecules per viral particle for the 160S particles of S1, 9 for the 147S particles of S1 and 3 for the 147S particles of S1(2Y-1I). Therefore, these particles were far from being saturated with receptor and similar amounts of receptor cosedimented with S1 160S virions and S1(2Y-1I) 147S particles. However, the possibility that the attachment of a few PVR-IgG2a molecules contributed to delaying the sedimentation of 147S particles was not excluded.

PVR-IgG2a-mediated VP4 extrusion from particles at 0°C

The PVR-IgG2a-mediated formation of 135S and 80S particles at 0°C was unexpected. We therefore looked for the presence of VP4 in the various particles by SDS-PAGE (Table 1). The particles obtained after incubation of S1 with 5 nM PVR-IgG2a at 0°C were at the 160S position and contained the VP4 protein, as expected. When this adsorption period was followed by 7 min incubation at 37 °C, the particles sedimented at 135S and did not contain VP4 and therefore appeared to be A particles. S1 particles sedimenting at 147S and 135S positions after interaction with the receptor at 0°C still contained VP4. This indicates that these 135S particles differed from A particles. In contrast, VP4 was not detected in S1 80S particles which formed at 0°C and 40 nM PVR-IgG2a (Table 1).

Similar results were obtained with the S1(2Y-1I) mutant but at lower concentrations of PVR-IgG2a (Table 1). At 0°C and 1.25 nM PVR-IgG2a, 147S particles formed, still containing VP4. After incubation at 37°C, A and 80S particles formed, both having lost VP4, even at the lowest concentration of PVR-IgG2a. Particles formed at 0°C and 20 nM PVR-IgG2a and sedimenting at 135S still contained VP4, but the 80S particles which formed simultaneously did not contain VP4 (Table 1).

DISCUSSION

Like some type 3 persistent PV mutants (Duncan and Colbère-Garapin, 1999; Duncan, Pelletier, and Colbère-Garapin, 1998), the type 1 persistent mutant S1(2Y-1I) was able to form 147S particles following virus adsorption onto HEp-2c cells at 0°C. However, unlike the type 3 mutants, these particles were efficiently converted into A particles upon incubation at 37°C, suggesting that the 147S particle may be an intermediate in the formation of A particles, and possibly in the uncoating process of this mutant (Fig. 1). This conversion was complete for the mutant in 10 min whereas it was only partial for S1, suggesting that the mutations in S1(2Y-1I) facilitated the conformational transitions of the capsid. The VP4 protein was present in 147S particles, in the same proportion as in the native virion, indicating that the modification of the sedimentation coefficient of viral particles following adsorption onto cells at 0°C was not due to a change in the protein composition. Consistent with the presence of the viral genome in 147S particles, these particles had the same density as virions as assessed by CsCl density gradient ultracentrifugation and were infectious (data not shown). 147S particles were similarly formed with soluble chimeric PVR-IgG2a molecules and with PVR-expressing cells and are thus specifically induced by the PVR. They were resistant to V8 protease digestion, suggesting that the N-terminus of VP1 in 147S particles is not externalized.

Remarkably, high PVR-IgG2a concentrations induced the formation of 80S particles and VP4 extrusion at 0°C with both virus strains. The simultaneous presence of the two mutations of S1(2Y-1I) facilitated the required transitions, at 0°C like at 37°C. The transition of S1(2Y-1I) to 80S particles at 0°C was almost complete for as few as two PVR-IgG2a molecules per binding site. Even at low temperature, it is thus probable that the mutations increased capsid flexibility, thereby lowering the energy activation barrier of the reaction catalyzed by PVR. The formation of 80S particles implies that conformational transitions of

the capsid occurred. Therefore, the progressive shift of VP4-containing particles toward the top of the gradient, at 147S and up to 135S, may correspond to progressive conformational transitions of the capsid. However, it is possible that the binding of some PVR-IgG2a molecules to viral particles contributed to delay their sedimentation, as proposed in the case of human rhinovirus 3 upon interaction with its receptor, ICAM-1 at 34°C (Casasnovas and Springer, 1994; Greve et al., 1991; Hoover-Litty and Greve, 1993).

At the cell surface, the binding of the virion to PVR may recruit further adjacent PVR molecules (Racaniello, 1996). Possibly, PVR induces a progressive conformational change in the capsid forming several successive transient intermediates as observed with increasing PVR-IgG2a concentration. Constrained multivalent receptor attachment, applied by the linkage to the Fc fragment in the chimeric receptor, or by the cell membrane, may facilitate the formation of 80S particles and RNA release. The transition intermediates may be unstable, particularly at 37°C. The presence of the PVR as clusters on cells (Mannweiler et al., 1990) may favor the conformational changes of the capsid. This is consistent with the broad 147S peak observed with the mutant after adsorption onto cells at 0°C: several particle conformations may be present simultaneously at the cell surface.

In conclusion, the formation of 80S particles, which is considered to be one of the final steps of PV uncoating, can be temperature-independent at high PVR-IgG2a concentration. The persistent PV mutants display highly modified PV-PVR interactions, and are precious tools for investigating the early steps of PV infection.

MATERIALS AND METHODS

Cells, viruses and recombinant PVR

HEp-2c cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% newborn calf serum (NCS). We used the PV Sabin 1 strain LSc 2ab (S1) (Sabin and Boulger, 1973) and the mutant virus S1(2Y-1I) (Pelletier, Duncan, and Colbère-Garapin, 1998) carrying two amino acid substitutions selected during persistent infection of human neuroblastoma IMR-32 cells. S1 viral stocks were prepared on HEp-2c cells and those of S1(2Y-1I) on IMR-32 cells (to maintain the selective pressure for the mutations). Infectivity was titered by an endpoint micromethod on HEp-2c cells in 96-well plates. The infectious titers are expressed as infectious doses 50 (ID_{50}) per milliliter, and correspond to the $\log_{10}$ of the highest virus dilution for which cytopathic effects and cell lysis were observed in 50% of wells 7 days after infection.

PVR-IgG2a is a chimeric molecule in which the extracellular moiety of human PVR is joined to the hinge and Fc region of a mouse IgG2a molecule (Arita, Horie, and Nomoto, 1999). Crude PVR-IgG2a was prepared from culture supernatants of recombinant baculovirus-infected Sf9 cells and purified on a protein A-Sepharose 4B column (Pharmacia Biotech) as described (Arita, Horie, and Nomoto, 1999). The concentration of PVR-IgG2a, considered as a dimer like an IgG, was determined by measuring the absorbance at 280 nm. PVR-IgG2a was stored at –80°C in phosphate buffered saline (PBS) (10 mM phosphate buffer pH 7, 137 mM NaCl, and 2.6 mM KCl) supplemented with 1% bovine serum albumin (BSA).

Preparation of purified radiolabeled virions

Radiolabeled virions were prepared in HEp-2c cells with [^{35}S]methionine (1400 Ci/mmol; ICN) and purified by CsCl density gradient centrifugation as previously described

(Blondel et al., 1983; Pelletier et al., 1991). The specific activities of S1 and S1(2Y-1I) varied between 10^{-4} and 5 x 10^{-4} cpm/ ID_{50}.

For experiments with hPVR-IgG2a, radiolabeled virions were prepared as recommended by Arita and coll (Arita et al., 1998). Briefly, HEp-2c cells in monolayer culture (2 X 10^7 cells) were infected with PV at an MOI of 10 ID_{50}/cell. Two and a half hour postinfection, the medium was changed to methionine- and cysteine-free medium containing 4 mCi of [^{35}S]methionine and [^{35}S]cysteine (TRAN^{35}S-LABEL™; ICN). Cells were collected 7h postinfection and virus was purified from cytoplasmic extracts of the infected cells on sucrose gradient (15-30% w/v) then by CsCl density gradient centrifugation as previously described (Blondel et al., 1983; Pelletier, Duncan, and Colbère-Garapin, 1998). Purified virus was desalted by gel filtration on a PD-10 column (Pharmacia Biotech) equilibrated with PBS, and then stored at –80°C. The concentration of virus was determined by measuring absorbance at 260 nm, with 1.0 optical density unit being considered to be equivalent to 9.4 X 10^{12} viral particles (Rueckert, 1976). Under these conditions, the specific activities of S1, S1(2Y-1I) and CVB1 were between 0.7 and 1.5 X 10^{-5} cpm/viral particle.

Analysis of PVR-induced transitions in sucrose gradients

HEp-2c cells in suspension (10^7 cells) were infected with ^{35}S-methionine-labeled virions at a MOI of 50 ID_{50}/cell in 2 ml of DMEM-2% fetal calf serum (FCS) on ice for 2.5 h. Cells were then washed twice in DMEM-FCS on ice. Conformational alterations were started by the addition of 3 ml of DMEM-FCS preheated to 37°C and cells were incubated for various times (0, 3, and 10 min) at 37°C. Cells were then washed successively with ice-cold DMEM-FCS and PBS. Cell pellets were resuspended in 500 µl of 140 mM NaCl, 50mM Tris-HCl pH 8, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), and incubated on ice for 20 min (cell lysis step). Samples were centrifuged for 5 min at 10,000 rpm at 4°C and the supernatant was layered onto 15 to 30% (w/v) sucrose gradients prepared in PBS. Gradients

were centrifuged in a Kontron TST41.14 rotor for 2 h at 40,000 rpm at 4°C and fractionated. The amount of ^{35}S label of all or a part of each fraction was counted in scintillation vials.

For experiments with PVR-IgG2a, microtubes were first coated overnight with BSA, using a 1% BSA solution in PBS (PBS-BSA). Radiolabeled virions (2 X 10^{10} particles) were incubated for 2.5 h at 0°C in 200 μl of PBS-BSA in the presence of various concentrations of PVR-IgG2a (0 to 40 nM). Then, the samples were incubated for 0 or 7 min at 37°C, treated with 1% NP40 and 0.1% SDS, as in the cell lysis step, and layered onto 15 to 30% (w/v) sucrose gradients as described above.

Virions (160S) and 80S particles, purified by CsCl gradient centrifugation, and eluted 135S particles (see below), were layered onto parallel sucrose gradients and used as sedimentation markers.

Preparation of eluted 135S particles

To obtain eluted 135S particles, HEp-2c cells in suspension (5 X 10^7 cells) were first infected with radiolabeled S1 or S1(2Y-1I) virus at an MOI of 50 ID_{50}/cell in 5 ml of DMEM-FCS on ice for 2.5 h. Cells were then washed twice in DMEM-FCS on ice. Alterations were started by the addition of 800 μl of DMEM-FCS preheated to 37°C and allowed to continue for 15 min at 37°C. Cells were then pelleted by centrifugation for 5 min at 1000 rpm at 4°C, and the supernatants containing eluted A particles were layered onto sucrose gradients, which were centrifuged and fractionated as described above.

Analysis of viral proteins by polyacrylamide gel electrophoresis

Fractions from sucrose gradients were incubated for 6 min at 90°C in the presence of 1% SDS and loaded onto 0.1% SDS-14% polyacrylamide gels for electrophoresis (SDS-PAGE). Purified radiolabeled S1 and S1(2Y-1I) virions and the corresponding eluted 135S particles were analyzed on sucrose gradients. The 2 or 3 fractions containing the majority of

the 160S and 135S particles were used as 160S and 135S markers, respectively. After SDS-PAGE, the radioactivity of each of the four capsid protein bands was quantified using a STORM 820 apparatus (Molecular Dynamics®) and is expressed as a percentage of the total radioactivity with the Image Quant software.

V8 protease digestion

Radiolabeled S1 and S1(2Y-1I) (2 X 10^{10} particles) were incubated for 2.5 h at 0°C in 200 μl of PBS-BSA with, or without, PVR-IgG2a (at concentrations of 1.25 and 5 X 10^{-9} M, respectively; see Results). Then, samples were incubated for 0 or 7 min at 37°C. Aliquots of 10 μl of these samples in PBS were treated with various V8 protease concentrations (0, 5 and 10 μg/ml) for 1h at 4°C in a final volume of 100 μl. Samples were then analyzed by 0.1%SDS-12.5% PAGE, as described above.

Quantification of PVR-IgG2a in sucrose gradients.

Radiolabeled S1, S1(2Y-1I) and CVB1 viruses were incubated with PVR-IgG2a (at the indicated concentration) for 2.5 h at 0°C and samples were analyzed in sucrose gradients (15-30%) as described above. The radioactivity in 50 μl of each fraction was counted, and 100 μl was used in a slot blot assay to detect PVR-IgG2a with an anti-mouse IgG Fc fragment conjugated with peroxidase (Sigma). The bound peroxidase activity was revealed with the ECL+ Plus Western blotting detection system (Amersham Pharmacia Biotech, UK) and quantified with a MultiImager Fluor-S™ and the Quantity one software (Bio-Rad), using a range of known concentrations of PVR-IgG2a. Then, in the three fractions corresponding to the top of peaks, the number of PVR-IgG2a molecules per radiolabeled particle was calculated, based on the number of cpm and the specific activity of the radiolabeled virus.

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REFERENCES

Arita, M., Horie, H., and Nomoto, A. (1999). Interaction of poliovirus with its receptor affords a high level of infectivity to the virion in poliovirus infections mediated by the Fc receptor. *Journal of Virology* **73,** 1066-74.

Arita, M., Koike, S., Aoki, J., Horie, H., and Nomoto, A. (1998). Interaction of poliovirus with its purified receptor and conformational alteration in the virion. *Journal of Virology* **72,** 3578-3586.

Belnap, D. M., McDermott, B. M., Filman, D. J., Cheng, N., Trus, B. L., Zuccola, H. J., Racaniello, V. R., Hogle, J. M., and Steven, A. C. (2000). Three-dimensional structure of poliovirus receptor bound to poliovirus. *Proc. Natl. Acad. Sci., USA* **97,** 73-78.

Blondel, B., Akacem, O., Crainic, R., Couillin, P., and Horodniceanu, F. (1983). Detection by monoclonal antibodies of an antigenic determinant critical for poliovirus neutralization present on VP1 and on heat inactivated virions. *Virology* **126,** 707-710.

Casasnovas, J. M., and Springer, T. A. (1994). Pathway of rhinovirus disruption by soluble intercellular adhesion molecule 1 (ICAM-1): an intermediate in which ICAM-1 is bound and RNA is released. *Journal of Virology* **68,** 5882-9.

Colston, E. M., and Racaniello, V. R. (1995). Poliovirus variants selected on mutant receptor-expressing cells identify capsid residues that expand receptor recognition. *J. Virol.* **69,** 4823-4829.

Curry, S., Chow, M., and Hogle, J. M. (1996). The poliovirus 135S particle is infectious. *J. Virol.* **70,** 7125-7131.

Dove, A. W., and Racaniello, V. R. (1997). Cold-adapted poliovirus mutants bypass a postentry replication block. *Journal of Virology* **71,** 4728-4735.

Dove, A. W., and Racaniello, V. R. (2000). An antiviral compound that blocks structural transitions of poliovirus prevents receptor binding at low temperatures. *J. Virol.* **74,** 3929-3931.

Duncan, G., and Colbère-Garapin, F. (1999). Two determinants in the capsid of a persistent type 3 poliovirus exert different effects on mutant virus uncoating. *Journal of General Virology* **80,** 2601-2605.

Duncan, G., Pelletier, I., and Colbère-Garapin, F. (1998). Two amino acid substitutions in the type 3 poliovirus capsid contribute to the establishment of persistent infection in HEp-2c cells by modifying virus-receptor interactions. *Virology* **241,** 14-29.

Filman, D. J., Syed, R., Chow, M., Macadam, A. J., Minor, P. D., and Hogle, J. M. (1989). Structural factors that control conformational transitions and serotype specificity in type 3 poliovirus. *EMBO J.* **8,** 1567-1579.

Flore, O., Fricks, C. E., Filman, D. J., and Hogle, J. M. (1990). Conformational changes in poliovirus assembly and cell entry. *Sem. Virol.* **1,** 429-438.

Fricks, C. E., and Hogle, J. M. (1990). Cell-induced conformational change in poliovirus: externalization of the amino terminus of VP1 is responsible for liposome binding. *J. Virol.* **64,** 1934-1945.

Greve, J. M., Forte, C. P., Marlor, C. W., Meyer, A. M., Hoover-Litty, H., Wunderlich, D., and McClelland, A. (1991). Mechanisms of receptor-mediated rhinovirus

neutralization defined by two soluble forms of ICAM-1. *Journal of Virology* **65,** 6015-6023.

He, Y., Bowman, V. D., Mueller, S., Bator, C. M., Bella, J., Peng, X., Baker, T. S., Wimmer, E., Kuhn, R. J., and Rossmann, M. G. (2000). Interaction of the poliovirus receptor with poliovirus. *Proc. Natl. Acad. Sci., USA* **97,** 79-84.

Hogle, J. M., Chow, M., and Filman, D. J. (1985). Three dimensional structure of poliovirus at 2.9 Å resolution. *Science* **229,** 1358-1365.

Hoover-Litty, H., and Greve, J. M. (1993). Formation of rhinovirus-soluble ICAM-1 complexes and conformational changes in the virion. *Journal of Virology* **67,** 390-397.

Huang, Y., Hogle, J. M., and Chow, M. (2000). Is the 135S poliovirus particle an intermediate during cell entry? *Journal of Virology* **74,** 8757-8761.

Koike, S., Horie, H., Ise, I., Okitsu, A., Yoshida, M., Iizuka, N., Takeuchi, K., Takegami, T., and Nomoto, A. (1990). The poliovirus receptor protein is produced both as membrane-bound and secreted forms. *EMBO J.* **9,** 3217-3224.

Lange, R., Peng, X. Z., Wimmer, E., Lipp, M., and Bernhardt, G. (2001). The poliovirus receptor CD155 mediates cell-to-matrix contacts by specifically binding to vitronectin. *Virology* **285,** 218-227, doi:10.1006/viro.2001.0943.

Mannweiler, K., Nobis, P., Hohenberg, H., and Bohn, W. (1990). Immunoelectron microscopy on the topographical distribution of the poliovirus receptor. *Journal of General Virology* **71,** 2737-2740.

Mendelsohn, C. L., Wimmer, E., and Racaniello, V. R. (1989). Cellular receptor for poliovirus : molecular cloning, nucleotide sequence and expression of a new member of the immunoglobulin superfamily. *Cell* **56,** 855-865.

Mueller, S., Cao, X., Welker, R., and Wimmer, E. (2002). Interaction of the poliovirus receptor CD155 with the dynein light chain Tctex-1 and its implication for poliovirus pathogenesis. *J. Biol. Chem.* **277,** 7897-7904.

Ohka, S., and Nomoto, A. (2001). Recent insights into poliovirus pathogenesis. *Trends Microbiol*. **9,** 501-506.

Pelletier, I., Couderc, T., Borzakian, S., Wyckoff, E., Crainic, R., Ehrenfeld, E., and Colbère-Garapin, F. (1991). Characterization of persistent poliovirus mutants selected in human neuroblastoma cells. *Virology* **180,** 729-737.

Pelletier, I., Duncan, G., and Colbère-Garapin, F. (1998). One amino acid change on the capsid surface of poliovirus sabin 1 allows the establishment of persistent infections in HEp-2c cell cultures. *Virology* **241,** 1-13.

Pelletier, I., Duncan, G., Pavio, N., and Colbère-Garapin, F. (1998). Molecular mechanisms of poliovirus persistence: key role of capsid determinants during the establishment phase. *Cellular & Molecular Life Sciences* **54,** 1385-1402.

Racaniello, V. R. (1996). The poliovirus receptor - a hook, or an unzipper. *Structure* **4,** 769-773.

Rueckert, R. R. (1976). On the structure and morphogenesis of Picornaviruses. *In* "Comprehensive virology" (H. Fraenkel-Conrat, and R. Wagner, Eds.), pp. 131-213. Plenum Press, N.Y.

Sabin, A. B., and Boulger, L. R. (1973). History of Sabin attenuated poliovirus oral live vaccine strains. *J. Biol. Stand.* **1,** 115-118.

Tsang, S. K., McDermott, B. M., Racaniello, V. R., and Hogle, J. M. (2001). Kinetic analysis of the effect of poliovirus receptor on viral uncoating: the receptor as a catalyst. *Journal of Virology* **75,** 4984-4989.

TABLE 1

Presence of theVP4 protein in the particles separated in sucrose gradient after incubation of S1 or S1(2Y-1I) with PVR-IgG2a at various concentrations

Virus [a]	PVR-IgG2a (nM)	Incubation at 37°C [b]	Peak analyzed [c]	Presence of VP4 [d]
S1	5	-	160S	+
	5	+	135S	-
	20	-	147S	+
	40	-	135S	+
	40	-	80S	-
S1(2Y-1I)	1.25	-	147S	+
	1.25	+	135S	-
	20	-	135S	+
	20	-	80S	-

[a] Radiolabeled virus (2.10^{10} particles) was incubated for 2.5 h at 0°C with PVR-IgG2a.

[b] + and – indicate incubation for 7 min at 37°C or no incubation after adsorption.

[c] The position of peaks was determined in sucrose gradient (15-30%). S1 160S, 135S A and 80S particles were used as markers in parallel gradients.

[d] Fractions corresponding to the top of the peak were analyzed by SDS-PAGE. Radioactivity associated with each of the four capsid proteins was quantified using a STORM 820 (see Materials and Methods) and is expressed as a percentage of the total radioactivity. The presence of VP4 is noted + when the percentage of radioactivity corresponding to VP4 was greater than 2.5% and – when it was 0%.

Legend to figure 1: PVR-induced conformational transition on HEp-2c cells of the parental Sabin 1 virus and of the S1(2Y-1I) persistent mutant. Cells were incubated with [^{35}S]methionine-labeled viruses for 2.5 h at 0°C, and for 0, 3 or 10 min at 37°C, washed and lysed as described (Pelletier, Duncan, and Colbère-Garapin, 1998). Cell lysates were subjected to centrifugation in 15-30% sucrose gradients. Empty capsids were used as markers in parallel gradients.

Legend to figure 2: Protein analysis of 147S particles. [^{35}S]methionine-labeled S1(2Y-1I) mutant was incubated with HEp-2c cells for 2.5 h at 0°C and lysed. Cell lysates were fractionated in sucrose gradients. **A**- The [^{35}S] radioactivity of an aliquot of each fraction was counted. **B**- The fractions (8-15) corresponding to the 147S peak were analyzed by 14% SDS-PAGE. The 160S and 135S A particles from S1 and S1(2Y-1I) were used as markers. VP1 protein from strain S1 and from the S1(2Y-1I) mutant migrated between VP2 and VP3 as previously reported (Pelletier et al., 1991). **C-** Radioactivity of each of the four capsid proteins was quantified using a STORM 820 apparatus (Molecular Dynamics®) and is expressed as a percentage of the total radioactivity with the Image Quant software.

Legend to figure 3: Determination of the density of S1(2Y-1I) 147S particles in CsCl gradients: Cells were incubated with radiolabeled S1 or S1(2Y-1I) virions for 2.5 h at 0°C, and half of each sample was analyzed in a sucrose gradient (left-hand panel) and half in a CsCl gradient (right-hand panel).

Legend to figure 4: Analysis in sucrose gradients (15-30%) of transitions of S1 and S1(2Y-1I) viruses with various concentrations of PVR-IgG2a in a cell-free system. Radiolabeled S1 and S1(2Y-1I) viruses were incubated for 2.5 h at 0°C: **A**- without PVR-IgG2a or **B**- with PVR-IgG2a at various concentrations (1.25 to 20 nM). **C**- An additional high concentration of PVR-IgG2a, (40 nM) was used with the S1 virus. **D**- Viruses were incubated with PVR-IgG2a at various concentrations (1.25 to 20 nM) for 2.5 h at 0°C, then for 7 min at 37°C.

Legend to figure 5: Susceptibility of 147S particles to V8 protease. Radiolabeled S1 and S1(2Y-1I) viruses were incubated for 2.5 h at 0°C with or without PVR-IgG2a, then incubated or not incubated for 7 min at 37°C. An aliquot of each sample was layered onto a sucrose gradient to verify the position of the peak. The samples were exposed to digestion by various concentrations (0, 5 and 10 μg/ml) of V8 for 1h at 4°C, and then analyzed by 12.5% SDS-PAGE as described in Materials and Methods.

Legend to figure 6: Detection of PVR-IgG2a cosedimenting with viral particles in sucrose gradients. Radiolabeled S1, CVB1 and and S1(2Y-1I) viruses were incubated with the indicated PVR-IgG2a concentration for 2.5 h at 0°C and samples were analyzed in sucrose gradients (15-30%). The 147S particles of S1 and S1(2Y-1I) were obtained with 20 nM and 1.25 nM PVR-IgG2a, respectively. The radioactivity in an aliquot of each fraction was counted, and another aliquot was used in a slot blot assay to detect PVR-IgG2a with an anti-mouse IgG Fc fragment conjugated with peroxidase (see Materials and Methods). PVR-IgG2a was quantified with a MultiImager, using a range of known concentrations of PVR-IgG2a for reference.