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# The Poliovirus 135S Particle Is Infectious

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**The molecular mechanism of cell entry by unenveloped viruses is poorly understood. The picornaviruses poliovirus, human rhinovirus, and coxsackievirus convert to an altered form (the 135S or A particle) upon interaction with receptors on susceptible cells at 37°C. The 135S particle is thought to be a necessary intermediate because it accumulates inside susceptible cells soon after infection and drugs which inhibit conversion of the virus to this form also prevent infection. However, since a variable fraction of the altered 135S particles is reported to elute unproductively from the surface of susceptible cells, their precise role remains unclear. We have found that poliovirus 135S particles can infect Chinese hamster ovary (CHO) and murine L cells, neither of which are susceptible to infection by native poliovirus. The infectivity of the particles in tissue culture appears to be between $10^3$ to $10^5$ times less than that of poliovirus on HeLa cells. The 135S particle infectivity was not sensitive to RNase but was greatly reduced by proteolytic treatment. Proteolysis specifically removed the newly exposed N terminus of VP1, a feature which has previously been shown to mediate interactions of the particle with lipid membranes. These results demonstrate that although the infectivity of the 135S particle appears to be receptor independent, it nonetheless requires some property associated with the protein coat. In particular, the N terminus of VP1 plays an important role in the infection process. Our findings are consistent with the hypothesis that the 135S particle is an intermediate in the normal cell entry pathway of poliovirus infection.**

The first step in viral infection involves the interaction of the virus with a specific receptor on the surface of a susceptible cell. Studies of a number of lipid-enveloped viruses have demonstrated that the fusion of the viral and cellular membranes by the action of viral envelope glycoproteins is a key step in cell entry either at the cell surface or following receptor-mediated endocytosis (31). Less is known about the mechanisms whereby unenveloped viruses, or their genomes, penetrate the membrane of host cells. Picornaviruses, which are among the smallest and simplest unenveloped viruses found in nature, represent a useful system with which to investigate cell entry. These simple viruses incorporate a single-strand positive-sense RNA genome within an icosahedrally symmetric shell formed by 60 copies of four capsid proteins, VP1 to VP4. X-ray crystallographic analysis of several picornaviruses reveals that they have a common core structure for their major capsid proteins (VP1 to VP3) consisting of an eight-stranded β-barrel (1, 21, 30, 40). In the crystal structures, the peptide VP4 and the N termini of VP1 to VP3 are all located at the interior face of the viral capsid. However, it has been shown that for poliovirus, both VP4 and the N terminus of VP1 are reversibly externalized at 37°C (26) and irreversibly externalized after attachment to the receptor (18).

The interaction of a number of different picornaviruses (notably poliovirus, human rhinovirus, and coxsackievirus B3) with susceptible cells (13, 17, 29) and membrane extracts (14, 15, 20, 39) at physiological temperature results in elution of a portion of the input virions in an altered form known as the 135S or A particle (see Fig. 19 in reference 41). In the case of poliovirus, conversion to 135S particles has been demonstrated in vitro by incubation of the virus with the solubilized receptor expressed in insect cells (23). Poliovirus and group A coxsackievirus 135S particles may also be produced simply by incubating the virus at elevated temperatures in hypotonic media in the presence of calcium (12, 28, 43).

The 135S particles are generally reported to lack the ability to bind to susceptible cells and to be noninfectious (13, 17, 22, 27, 29). They are also conformationally altered, having lost VP4 and irreversibly externalized the N terminus of VP1, and sediment more slowly than native virus (160S). The particles display a greater sensitivity to proteases and detergents than native virus and are readily distinguishable from native virus by using immunological probes (18).

Two lines of evidence suggest that the 135S particle is a necessary intermediate in the process of infection. First, particles that appear identical to eluted 135S particles accumulate early in infected cells (18, 19). Second, drugs which inhibit the conversion of virus to the 135S form by binding to and stabilizing the poliovirus capsid are also found to prevent infection (7, 16, 33, 35, 46). However, the elution of a significant fraction of input virus from susceptible cells in the form of reputedly noninfectious 135S particles casts doubt on this hypothesis. If the 135S particle does play an integral role at the stage of cell entry in the infection cycle, then one would expect it to be capable of initiating an infection under appropriate circumstances. Testing this hypothesis required the development of protocols capable of producing large amounts of the 135S particle at high concentrations. We modified an in vitro method for converting purified poliovirus to the altered form (43) in order to generate poliovirus 135S particles. These particles are indistinguishable from 135S particles produced in vivo and were shown to be capable of infecting Chinese hamster ovary (CHO) cells and murine L cells, neither of which are susceptible to infection by native poliovirus. Analysis of the digestion of 135S particles by V8 protease suggests that the

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externalized N terminus of VP1 plays an important role in infection.

## MATERIALS AND METHODS

**Virus growth, labeling, and purification.** Poliovirus serotype 1, Mahoney strain (P1/Mahoney), was grown in HeLa cells in suspension and purified by differential centrifugation and CsCl density gradient fractionation as described previously (44). Poliovirus labeled with [$^3$H]leucine was prepared as described for poliovirus empty capsids (2) except that guanidine hydrochloride was omitted from the infection. To label the virus with [$^{35}$S]methionine, a protocol similar to that published previously (26) was adopted.

**In vitro conversion of poliovirus to 135S particles.** Conversion of poliovirus to 135S particles was performed by using a modification of the method developed by Wetz and Kucinski (43). Purified virus was diluted, normally 20-fold, into prewarmed 20 mM Tris–2 mM $CaCl_2$–0.1% Tween 20 (pH 7.5) (TCT) and incubated at 50°C for 3 min. The degree of conversion was assayed by centrifuging the sample for 45 min at 54,000 rpm on 5 ml of 10 to 30% sucrose gradients.

**Conversion of poliovirus to 135S particles by elution from HeLa cells.** [$^{35}$S] methionine-labeled 135S particles were prepared by elution from HeLa cells at 37°C according to established protocols (18). The eluted particles were concentrated by centrifugation in a Centricon 100 (Amicon) and purified away from residual virions by ultracentrifugation on a 10 to 30% sucrose gradient. Peak fractions containing 135S particles were identified by scintillation counting, pooled, and diluted with growth medium. Aliquots of this preparation were used to inoculate CHO cell monolayers in six-well plates as described below. The inoculum added per well contained 0.09 μg of 135S particles.

**Infectivity assays in CHO and L cells.** Virus and 135S particles were prepared by incubation in hypotonic buffer as described above. Typically, 10 μl at 0.1 mg/ml was diluted in 0.2 ml of phosphate-buffered saline (PBS) containing 0.5 mM $MgCl_2$, 0.7 mM $CaCl_2$, and 0.5% (vol/vol) fetal calf serum (PBSMC-FCS) and allowed to attach to CHO or murine L-cell monolayers in six-well plates for 1 h at room temperature. Unbound particles were removed by washing twice with 1 ml of growth medium consisting of Eagle's minimal essential medium supplemented with antibiotics and containing 2% fetal calf serum and 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES). The cells were overlaid with 1 ml of growth medium and incubated at 37°C. Following incubation, the cells were lysed by three freeze-thaw cycles and the infectivity recovered was measured by plaque assay on HeLa cell monolayers (8).

**Effect of RNase on 135S particle infectivity.** 135S particles (12.5 μg/ml, which contains 3.8 μg of RNA per ml) or RNA extracted from purified poliovirus (3.1 μg/ml) was incubated in PBSMC-FCS with 0 or 2.5 μg of DNase-free RNase per ml for 20 min at room temperature prior to application to CHO cells. After rocking of the cells for 1 h at room temperature, the inoculum was removed and the cells were overlaid with growth medium. The cells were frozen after 8 h at 37°C, and the infectivity in lysates was determined by plaque assay.

The infectivity of the viral RNA preparation and the activity of the RNase used in the experiment were demonstrated in HeLa cells. Serial 10-fold dilutions in Optimem (Bethesda Research Laboratories) of viral RNA in the presence and absence of Lipofectin (Bethesda Research Laboratories) and/or 0.5 μg of RNase per ml were incubated at room temperature for 15 min and added to HeLa cell monolayers in six-well plates. Following incubation at 37°C for 2.5 h, the inoculum was replaced by an agar overlay, and the infectivity was assessed by counting plaques after 2 days at 37°C.

**Effects of antisera on 135S particle infectivity.** Samples containing 10 μl of a 1- to 100-fold dilution of normal serum (prebleed) or an antiserum (αP1) which was raised against a peptide corresponding to amino acids 24 to 40 of the N terminus of VP1 (10) were incubated with 10 μl of 135S (0.1 mg/ml) for 1 h on ice. The complexes were then applied to CHO cells as described above.

To perform sedimentation analysis of $^3$H-labeled 135S particles complexed with twofold-diluted αP1, complexes were prepared as described above and centrifuged on 400 μl of 30 to 45% sucrose gradients at 95,000 rpm for 10 min in a Beckman Airfuge. The gradient was fractionated, and the pellet was recovered by washing with 70 μl of 0.5% sodium dodecyl sulfate (SDS).

**V8 digestion experiments.** Virus or 135S particles were incubated at a concentration of 100 μg/ml for 1 h at 4 and 24°C in the presence of 0, 33, 83, or 167 μg of V8 protease per ml in TCT buffer. Digested samples were analyzed for polypeptide composition, infectivity, and particle integrity. The polypeptides were separated on SDS–12.5% polyacrylamide gels and transferred to nitrocellulose for blotting by three polyclonal antisera raised against peptides corresponding to short sequences from VP1 of P1/Mahoney (αP0, residues 6 to 24; αP1, residues 24 to 40; and αP9, residues 270 to 287) (10) and one raised against isolated VP2 (αVP2) (8). Four identical gels were blotted with a 1:500 dilution of αP0, 1:1,000 dilutions of αP1 and αP9, and a 1:200 dilution of αVP2, using standard protocols. Bound antibodies were detected with a 1:1,000 dilution of anti-rabbit immunoglobulin conjugated with alkaline phosphatase (Vector Laboratories), using the nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate toluidinium substrate (Promega) as instructed by the manufacturer. A blocking buffer consisting of 50 mM Tris, 150 mM NaCl, 0.05% Tween 20, and 1% bovine serum albumin (BSA) (pH 8) was used throughout for washing and antibody dilution. The infectivity of digested poliovirus 135S particles on CHO cells was measured essentially as described above. The infectivity of virus samples was determined directly by plaque assay on HeLa cells. Sedimentation analysis on 10 to 30% sucrose gradients of 135S particles treated with V8 protease was performed as described above.

**Cell binding assays.** A 0.16-μg aliquot of [$^{35}$S]methionine-labeled virus or 135S particles was mixed with 0 to 9.0 μg of [$^3$H]leucine-labeled virus or 135S particles in a total volume of 208 μl of PBS containing 0.1% (wt/vol) BSA (PBSA) and added to 1 ml of HeLa cells in suspension at a density of $10^7$ cells per ml. After 1 h at room temperature with constant rotation, unbound particles were removed by pelleting and washing the cells in PBS. The $^3$H and $^{35}$S activities bound to the cells were determined by resuspending the cells in PBS and mixing them with liquid scintillant. Under the experimental conditions used, virus and 135S particles were found not to compete with one another. The measurements of the amount of $^3$H-labeled virus or 135S competitor bound in the presence of the heterogeneous $^{35}$S-labeled particle were therefore used to determine binding curves for the two particle types.

**Analysis of VP4 content of 135S particles.** [$^{35}$S]methionine-labeled 135S particles were prepared in vitro by the method described above. The particles were concentrated and separated from dissociated VP4 by pelleting in an Airfuge (Beckman) or by centrifugation in a Centricon 100 concentrator. The polypeptides from virus and 135S samples were separated by polyacrylamide gel electrophoresis on 10 to 15% gradient acrylamide gels. Optical densitometry of autoradiograms of the gels was used to quantify the relative VP4 content in each sample.

## RESULTS

**In vitro conversion of poliovirus to 135S particles.** In our hands, the protocol reported by Wetz and Kucinski (43), requiring incubation of poliovirus in 20 mM Tris–2 mM $CaCl_2$ (pH 7.5) at 37°C for 30 min, was incapable of complete conversion to 135S particles and limited to virus concentrations below 2.5 μg/ml. However, we found that incubation of purified poliovirus in the same buffer at 50°C for only 3 min resulted in complete conversion of both P1/Mahoney and P3/Sabin at concentrations of up to 500 μg/ml (Fig. 1a). The degree of conversion was analyzed by centrifuging samples on sucrose gradients. Under our conditions, no virus was detected following incubation in the hypotonic buffer and the vast majority of the sample sedimented at 135S. About 5% of the input material was recovered as 80S empty capsids, particles which have lost VP4 and the viral RNA. An accurate assessment of the yield of 135S particles was difficult because of differential losses of each species on the sucrose gradient.

**Infectivity of poliovirus 135S particles in CHO and L cells.** Infectivity assays were performed with 135S particles prepared in vitro as described above but without any purification on sucrose gradients. On HeLa cell monolayers, 135S particles prepared in this way were approximately 5 orders of magnitude less infectious than intact poliovirus. This low level of infectivity might easily be dismissed as residual unconverted virus in the preparation. However, poliovirus 135S particles were found to infect CHO cells, which are not susceptible to infection by the intact virus (Fig. 1b and c). Following adsorption of 135S particles to the cells, an initial 2-h lag phase was followed by a logarithmic phase of virus replication which ended after a further 5 to 6 h. In striking contrast, incubation of intact virus with the CHO cells resulted in no progressive infection: the number of PFU recovered was simply a constant portion of the inoculum (~0.1%) which remained adsorbed to the cells (see Materials and Methods). For experiments in which 0.5 μg of virus was added to the cells, the small amount of virus remaining adsorbed to the monolayer was sufficient to mask any possible infection. However, even if the amount of input virus was reduced to 0.5 ng, in order to reduce the adsorbed background, there was still no evidence of infection. The observed lack of infectivity of poliovirus on CHO cells is consistent with previous reports (32).

The inocula used in the infectivity assays contained 135S particles and VP4 molecules which had dissociated from them during conversion. To determine whether any VP4 remained

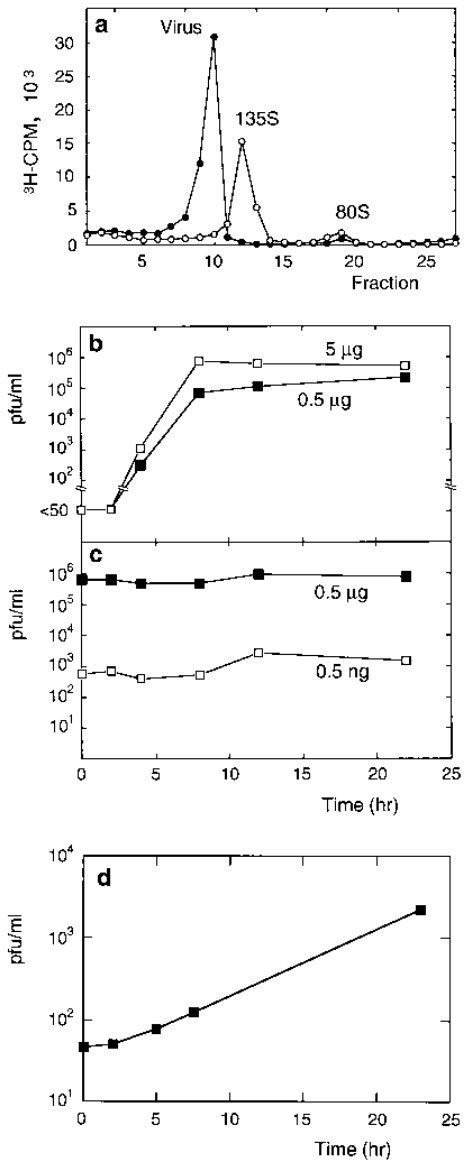


FIG. 1. Poliovirus converted to 135S particles in vitro is infectious in CHO cells. (a) Sedimentation analysis of the in vitro conversion of radiolabeled P1/Mahoney to 135S particles. Virus labeled with [$^3$H]leucine was incubated at a concentration of 200 μg/ml in TCT at 50°C for 3 min (open circles). A control sample was incubated in the same buffer on ice (filled circles). Both samples were centrifuged on 5 ml of 10 to 30% sucrose gradients. No residual virus is visible in the heated sample; about 5% of the input material is converted to 80S empty capsids. Similar results were obtained at a virus concentration of 500 μg/ml. CHO cells are susceptible to infection by 135S particles (b) but not by intact poliovirus (c). Virus and 135S particles were prepared at 100 μg/ml, and dilutions from these stocks were applied to CHO cells as described in Materials and Methods. The total weight of each particle added to each well is indicated. The infection was allowed to proceed at 37°C for the times indicated, after which the cells were frozen at −80°C. The infectivity recovered from lysates after three freeze-thaw cycles was determined by plaque assay on HeLa cells. (d) 135S particles prepared by elution from HeLa cells at 37°C are also capable of infecting CHO cells. 135S particles were prepared, purified, and applied to CHO cells as described in Materials and Methods. In this experiment, 0.09 μg of 135S particles was used to inoculate cell monolayers grown in six-well plates.

physically associated with the particles, we analyzed the polypeptide content of radiolabeled 135S particles which had been isolated from VP4 by ultracentrifugation or filtration (see Materials and Methods). Virus and 135S particle samples handled in this way were subjected to SDS-polyacrylamide gel electrophoresis, and the resulting autoradiograms were measured on an optical densitometer. The 135S particles were found to contain about 27% of the VP4 complement of intact virions. In the infectivity assays, therefore, although the majority of the VP4 dissociated from the particle and remained in the inoculum, a significant proportion of the VP4 stayed attached to the particle. In the normal process of poliovirus infection, VP4 plays an important but undefined role (11, 34); it is conceivable that this polypeptide is also required for the infection of CHO cells by 135S particles.

Murine L cells could also be infected by poliovirus 135S particles but not by intact virus (data not shown), suggesting that the infectivity of the particles may be general and that an interaction with a specific receptor may not be required. The yield of infectious PFU after 24 h from L cells infected by 135S particles was about 1 $\log_{10}$ less than observed in infected CHO cells, a relatively small difference which may, in part, be due to experimental variation.

Poliovirus 135S particles prepared by elution of virus from the surface of HeLa cells at 37°C (18) were also found to be infectious in CHO cells (Fig. 1d). Thus, there is a functional similarity between 135S particles produced in vitro in low-ionic-strength buffers and those observed in vivo by elution from HeLa cells. The infection of CHO cells by 135S particles eluted from HeLa cells was somewhat slower than that observed for particles prepared in vitro; however, the reason for this difference remains unclear.

**Effect of RNase on poliovirus 135S infectivity.** The in vitro conversion to 135S particles invariably generated a small amount of 80S empty capsids, suggesting that samples contained some naked viral RNA. To demonstrate that the infectivity of the 135S particles was not the result of simple transfection by low levels of naked RNA, the infectivities in CHO cells of equimolar amounts of 135S particles and extracted, naked viral RNA were compared in the presence and absence of RNase (Fig. 2). The specific infectivity of 135S particles was found to be at least $10^4$ times greater than that of naked viral RNA. Indeed, the naked RNA did not cause a detectable infection in these assays except in the presence of the transfecting agent Lipofectin. Moreover, the infectivity of the 135S particle was unaffected by RNase. Both findings indicate that properties associated with the particle capsid are required for infectivity.

**Probing the role of the N terminus of VP1 in poliovirus 135S infectivity.** The irreversible externalization of the N terminus of VP1 of the 135S particle distinguishes it from native virus (18). Consequently, a polyclonal antiserum (αP1) raised against a peptide corresponding to residues 24 to 40 in the N terminus of VP1 (10) binds to poliovirus 135S particles (18). Pretreatment of 135S particles with αP1 at 4°C specifically inhibited the infectivity by about 90% (Fig. 3a). αP1 does not recognize the virus at 4°C (26), suggesting that the 135S particle, not the virus, is the agent of infection. Sedimentation analysis showed that αP1 caused aggregation of the 135S particles (Fig. 3b), and this is presumably the mechanism of inhibition of infection.

Poliovirus 135S particles are more susceptible to proteolysis than intact virus (14, 15, 18). Digestion of virus and 135S particles with V8 protease (Endoproteinase Glu-C; Boehringer Mannheim) was analyzed by SDS-polyacrylamide electrophoresis and Western blotting (immunoblotting) using four different polyclonal antisera, three raised against peptides corresponding to fragments of VP1 (αP0, residues 6 to 24; αP1, residues 24 to 40; and αP9, residues 270 to 287) (10, 18) and one raised against VP2 (8) (Fig. 4a). Silver staining showed

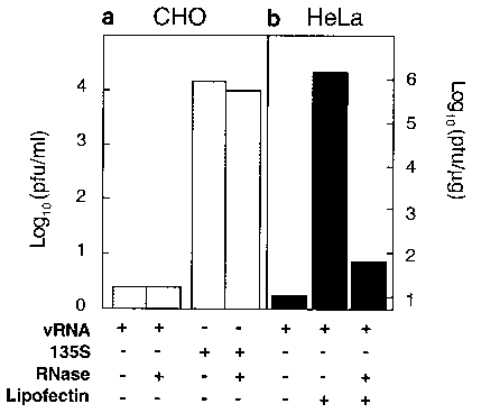


FIG. 2. 135S particles are much more infectious than naked RNA and resistant to RNase treatment. (a) Approximately equimolar amounts of 135S particles or extracted poliovirus RNA (about 3.4 μg of RNA per ml in each case) were incubated in PBSMC-FCS in the absence or presence 2.5 μg of RNase per ml for 20 min at room temperature prior to application to CHO cells. The cells were frozen after 8 h at 37°C, and the infectivity in lysates was determined by plaque assay. No PFU were observed because of the naked viral RNA; the infectivity displayed is an upper estimate. (b) Demonstration of the infectivity of the viral RNA preparation and the activity of the RNase used in the experiment shown in panel a. Serial 10-fold dilutions of viral RNA in the presence and absence of Lipofectin and/or 0.5 μg of RNase per ml were incubated briefly at room temperature and added to HeLa cell monolayers. Following incubation at 37°C for 2.5 h, the inoculum was replaced by an agar overlay and the infection was allowed to proceed at the same temperature for 48 h. The infectivity of each sample was assessed by counting plaques. Use of Lipofectin to transfer the viral RNA into the cells yields a high level of infectious particles. Pretreatment of the RNA with RNase reduces the infectivity under these conditions by over 4 orders of magnitude.

that VP3 remained intact under all conditions tested (data not shown). Although both VP1 and VP2 were cleaved minimally by V8 in virions under our experimental conditions, no concomitant loss of infectivity was observed. For 135S particles, a total of four digestion products arising from VP1 were detected (f1 to f4 [Fig. 4a]), a pattern identical to that observed under conditions (recommended by the manufacturer) which restricted cleavage to the C-terminal side of Glu residues. This result suggests that digestion occurred only in the N-terminal portion of VP1, because the N-terminal 60 residues of VP1 in P1/Mahoney contain five Glu residues (at positions 7, 16, 31, 40, and 48), whereas there are none in the C-terminal 137 residues. Consistent with this, progressive digestion of VP1 led to loss of recognition of the digestion products, first by αP0 and then by αP1, both of which bind within the N terminus. αP0 recognizes only the most slowly migrating fragment f1; αP1 recognizes f1 and the next two digestion products, f2 and f3, but does not bind to f4. However, all four digestion fragments were recognized by αP9, which binds to a sequence only 15 amino acids from the C terminus. Since only four VP1 digestion products were observed, Glu-48 may not be accessible to V8. Digestion of VP2 in 135S particles also occurred but was much less severe; most of VP2 remained intact under all conditions tested.

V8 treatment of 135S particles resulted in a significant loss of infectivity in CHO cells which appeared to be most closely correlated with the degradation of the N terminus of VP1 (Fig. 4a). The digestion of VP2 is unlikely to contribute significantly to the loss of infectivity of 135S particles because it was largely incomplete and similar levels of VP2 digestion in native intact virus had no effect on virus infectivity in HeLa cells. Sedimentation analysis on sucrose gradients of V8-digested 135S particles revealed that they remained intact and reproducibly sedimented slightly faster than untreated 135S (Fig. 4b); moreover, the recovery of V8-digested particles from the gradient was significantly greater than for untreated 135S, suggesting that they are less hydrophobic, consistent with previous findings (18).

That the interaction of the 135S particles with cells is independent of the poliovirus receptor was confirmed by experiments which compared the abilities of virus and 135S particles to compete for binding to HeLa cells. The results show that a greater that 50-fold excess of 135S particles did not compete with virus for attachment to the cell surface (Fig. 5a). The binding of 135S particles was apparently unaffected by a similar excess of virus competitor, although in this case the level of 135S binding (approximately 500 cpm) was too low to determine the significance of this observation (data not shown). Since no competition was observed in either experiment, measurement of the binding of virus or 135S competitor in the presence of low concentrations of the heterogeneous particle allowed us to plot binding curves for these particles (Fig. 5b). Although intact virus binds to a saturable receptor, as expected, the 135S particle appears to attach via a nonsaturable, low-affinity interaction which may simply be binding to the cell membrane. This interpretation is consistent with previous demonstrations that 135S particles bind to lipid membranes (18, 28) and observations that 135S particles bind better to CHO cell monolayers than intact virus (13a).

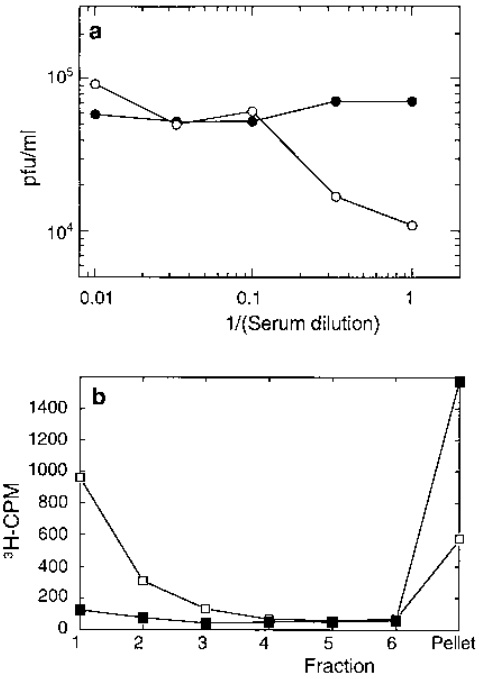


FIG. 3. (a) Antiserum raised against a peptide corresponding to residues 24 to 40 in the N-terminal arm of VP1 (αP1) inhibits the infectivity of poliovirus 135S particles. In each sample, prebleed serum (filled circles) or αP1 (open circles) diluted in PBSA was incubated with 0.1 mg of 135S particles per ml for 1 h on ice. The complexes were then applied to CHO cells (Materials and Methods). (b) Sedimentation analysis of $^3$H-labeled 135S particles complexed with twofold-diluted αP1. Complexes were prepared and centrifuged on 400 μl of 30 to 45% sucrose gradients as described in Materials and Methods. The gradient was fractionated from the top, and the pellet was recovered by washing with 0.5% SDS. αP1 causes the 135S particles to form an aggregate which was recovered in the pellet (filled squares). In the control, prepared by mixing 135S with an equal volume of PBSA, the majority of particles remain at the top of the gradient (open squares).

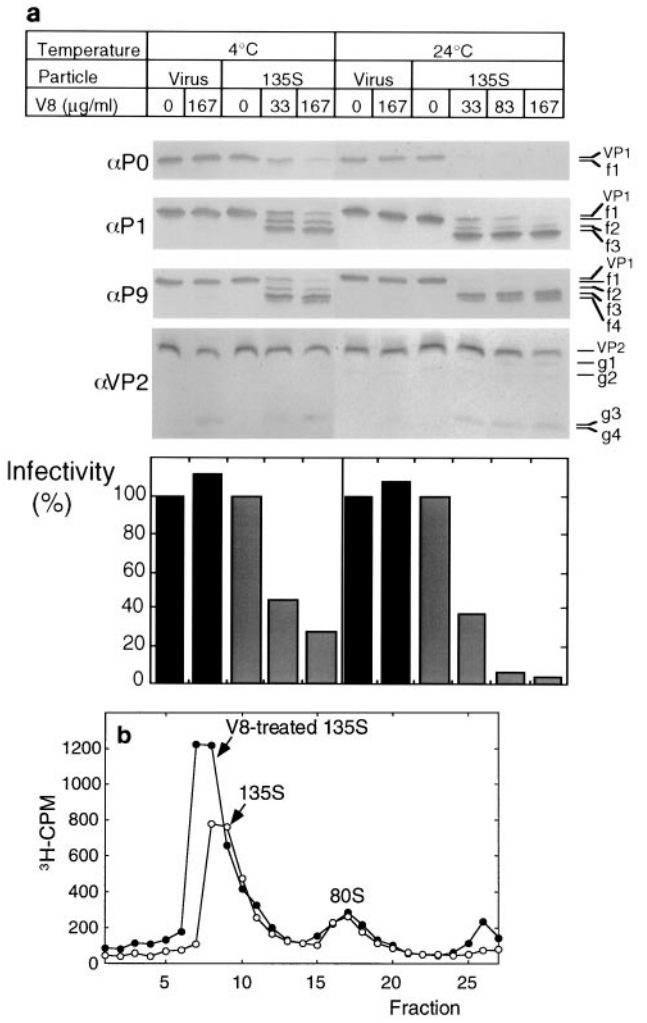


FIG. 4. V8 protease digestion of 135S particles primarily results in cleavage at the N terminus of VP1 and causes loss of infectivity of the particles in CHO cells. Virus and 135S particles (prepared as described in Materials and Methods) were incubated for 1 h at 4 or 24°C in the presence of a range of V8 protease concentrations. (a) The polypeptides of digested samples were analyzed by Western blotting using three polyclonal antisera raised against peptides corresponding to short sequences from VP1 of P1/Mahoney (10) (αP0, residues 6 to 24; αP1, residues 24 to 40; and αP9, residues 270 to 287) and one raised against isolated VP2 (αVP2) (8). αP0 recognizes only one fragment, designated f1. αP1 recognizes this fragment and two smaller ones, f2 and f3. αP9 recognizes a peptide located only 15 amino acids from the C terminus of VP1 and binds, albeit with lower affinity, to all fragments recognized by αP1 as well as to the fastest-migrating fragment of VP1, f4. Four fragments, designated g1 to g4, in addition to intact VP2 are detected with αVP2, although intact VP2 is the main species remaining under all conditions tested. Fragment g2 probably does not arise from V8 digestion, since it is observed in the absence of enzyme at 24°C and not at all at 4°C. The infectivity of V8-treated poliovirus 135S particles on CHO cells was measured essentially as described in Materials and Methods. The infectivity of virus samples was determined directly by plaque assay on HeLa cells. For both particle types, the observed infectivities were normalized relative to their respective controls. (b) Sedimentation analysis on 10 to 30% sucrose gradients of 135S particles incubated in the presence (filled circles) and absence (open circles) of 167 μg of V8 protease per ml. Experimental conditions were identical to those used in Western blot and infectivity experiments.

## DISCUSSION

A primary function of the virus coat, whether it consists lipid or protein or both, is to protect the genome in transit from one susceptible cell to another. During delivery of the genome to the cytosol, both this protective coat and the cell membrane must be breached, a process which invariably requires conformational changes. For a number of lipid-enveloped viruses, such as influenza virus and Semliki Forest virus, endocytosis of the virus follows recognition of a specific receptor. Subsequently, acidification of the endosome triggers a conformational change in a viral membrane glycoprotein which promotes fusion of the viral and endosomal membranes, permitting translocation of the virus nucleocapsid into the cytosol (31). For a number of unenveloped viruses, acid-induced conformational change in the viral capsid following receptor-mediated endocytosis is the most plausible mechanism of cell entry (3, 24, 37, 38).

In the case of unenveloped enteroviruses, such as poliovirus, which must withstand the acidic conditions of the gut of the host animal, the role of acidification in cell entry is less certain (19, 37). Instead, it appears that the binding energy of interaction with the host-cell receptor is engaged to generate a conformational change which primes the virion for cell entry. Thus, the binding of poliovirus and coxsackievirus to susceptible cells is observed to result in conversion of the virion to the conformationally altered 135S or A particle (13, 17). Although some of the 135S particles are shed from the surface of cells, having lost the ability to bind to the cell receptor, circumstantial evidence suggests that they are a genuine intermediate on

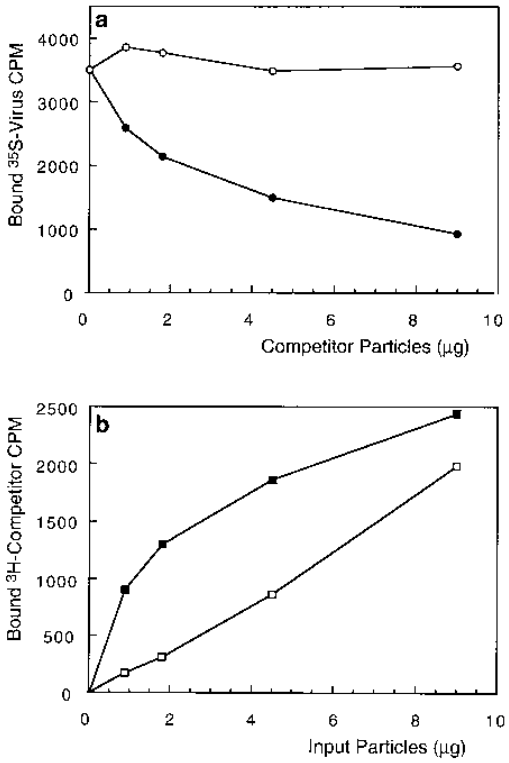


FIG. 5. 135S particles do not compete with themselves or with virus for binding to HeLa cells. A 0.16-μg aliquot of [$^{35}$S]methionine-labeled virus or 135S particles was mixed with excess amounts of competitor [$^{3}$H]leucine-labeled virus or 135S particles in PBSA and added to HeLa cells in suspension (see Materials and Methods). Unbound particles were removed after 1 h at room temperature by pelleting and washing the cells in PBS. The $^{3}$H and $^{35}$S activities bound to the cells were determined by scintillation counting. (a) Excess $^{3}$H-virus competitor (filled circles) but not $^{3}$H-135S competitor (open circles) displaces $^{35}$S-labeled virus. However, neither excess $^{3}$H-virus competitor nor $^{3}$H-135S competitor was observed to affect the binding of $^{35}$S-labeled 135S particles (data not shown). (b) Binding curves for the $^{3}$H-labeled virus (filled squares) and 135S particles (open squares) determined in the presence of a small amount (0.16 μg) of the heterogeneous, noncompeting particle.

the infection pathway. Not only are 135S particles observed to accumulate transiently within cells in the early stages of infection (18, 19), but capsid-stabilizing drugs which prevent conversion to the 135S form are also found to prevent infection (7, 16, 33, 35, 46). The results presented in this report are also consistent with the contention that poliovirus 135S particles are indeed an infection intermediate.

We generated 135S particles in substantial quantities in vitro by brief incubation in a hypotonic buffer in the presence of calcium. By a number of different probes, 135S particles produced in vitro appeared indistinguishable from those observed in vivo. The particles produced in vitro sediment more slowly than virus on sucrose gradients (Fig. 1a), have lost VP4, are proteolytically sensitive (Fig. 4a), and react at 4°C with antibodies directed against peptide sequences within the N terminus of VP1 (Fig. 4a). These properties are all shared by 135S particles observed in vivo either intracellularly shortly after the initiation of infection or following elution from HeLa cells (18).

We found that 135S particles prepared in vitro by incubation in a hypotonic buffer can infect CHO and murine L cells (Fig. 1b). Similarly, 135S particles prepared by elution of virus from HeLa cells at 37°C were also observed to infect CHO cells (Fig. 1d). Neither CHO nor murine L cells express the poliovirus receptor, and consequently both are refractory to infection by the native virus. The observation that CHO and L cells may be infected by 135S particles suggested that they can interact with these cells independently of the poliovirus receptor. The finding that the infectivity of 135S particles in CHO cells is unaffected by RNase but inhibited by antiserum specific to the 135S capsid confirmed that properties associated with the altered capsid are responsible for the mode of infection (Fig. 2 and 3). In particular, by specifically digesting the N terminus of VP1, which is irreversibly externalized upon conversion of poliovirus to the 135S form, we showed that although the particle did not dissociate, its infectivity was severely compromised (Fig. 4).

Thus, infection of CHO cells by poliovirus 135S particles requires the N terminus of VP1 to be externalized and intact; previously it has been suspected that this is also the case for receptor-mediated infection of HeLa cells by native poliovirus. The N terminus of VP1 has been predicted to form an amphipathic helix upon externalization and is known to confer a lipophilicity on the 135S particle that is not possessed by the intact virion (18, 28). We found that 135S particles bind to a low-affinity, nonsaturable receptor on HeLa cells (Fig. 5); the simplest interpretation of this result which is consistent with the findings of Fricks and Hogle (18) is that the particles are binding directly to the lipid membrane. Conceivably several copies of the N terminus of VP1 insert into the cell membrane to form a pore through which the viral RNA may be delivered to the cytosol. This hypothetical mode of action is consistent with the demonstration that a peptide corresponding to the N terminus of human rhinovirus VP1, which is also predicted to form an amphipathic helix (18), has membrane-disrupting activity (45). It also recalls the membrane-fusion activity attributed to the intermediate subviral particle of unenveloped reoviruses (5, 42).

The possibility that VP4 plays a role in the infection of CHO cells by 135S particles was not directly addressed by this study. The inocula used in infectivity assays contained both 135S particles and dissociated VP4 which resulted from conversion of the poliovirion. We found that a minor proportion of the VP4 molecules appeared to remain associated with 135S particles. It is possible that either the associated or the dissociated fractions of VP4 assist the infection attributed to the 135S particle. An early study reported that isolated VP4 may enable the transfection of extracted viral RNA across cell membranes (although the investigation did not rule out the effect of SDS used to purify VP4 from virions) (6). In our experiments with CHO cells, however, VP4 is unlikely to be operating in this manner because the infection was found to be resistant to RNase treatment but sensitive to antibodies and proteases directed against the N terminus of VP1. Thus, if VP4 is playing a role in 135S infection, it is to assist the interaction of the particle with the cell.

Recent genetic evidence demonstrates that VP4 plays a crucial role in the normal course of picornavirus infection (4, 11, 25, 34). The nature of this role is unknown but may well require interaction with the cell membrane, since VP4 is myristoylated at its N terminus (9, 36). In our experiments, VP4 is largely dissociated from the 135S particle before presentation to CHO cells, although it remains in the inoculum. In contrast, when the virus attaches to its receptor on HeLa cells, VP4 and the N terminus of VP1 are externalized together at the cell membrane and may thus be deployed optimally for entry of the RNA genome. This difference may account in part for the relatively poor efficiency of 135S particle infections in CHO cells. Although about 100 poliovirions per HeLa cell are required to guarantee infection of each cell, approximately $5 \times 10^5$ particles per cell were needed to observe saturation of the infection of CHO monolayers by 135S particles. The apparent inefficiency of 135S particle infections may also be attributed to the reduced stability of the particles and their relatively low affinity for cells (Fig. 5).

The ability to infect nonsusceptible cells with in vitro preparations of poliovirus 135S particles will allow us to dissect further the mechanism of cell entry and in particular, with complementation and genetic approaches, to investigate the specific role of VP4.

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