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A Mutation in VP4 Defines a New Step in the Late Stages of Cell Entry by Poliovirus

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During the entry of poliovirus into cells, a conformational transition occurs within the virion that is dependent upon its binding to the cell surface receptor. This conformational rearrangement generates an altered particle of 135S, results in the extrusion of capsid protein VP4 and the amino terminus of VP1 from the virion interior, and leads to the acquisition of membrane-binding properties by the 135S particle. Although the subsequent fate of VP4 is unknown, its apparent absence from purified 135S particles has long suggested that VP4 is not directly involved during virus entry. We report here the construction by site-specific mutagenesis of a nonviable VP4 mutant that upon transfection of the cDNA appears to form mature virus particles. These particles, upon interaction with the cellular receptor, undergo the 135S conformational transition but are defective at a subsequent stage in virus entry. The results demonstrate that the participation of VP4 is required during cell entry of poliovirus. In addition, these data indicate the existence of additional stages in the cell entry process beyond receptor binding and the transition to 135S particles. These post-135S stages must include the poorly understood processes by which nonenveloped viruses cross the cell membrane, uncoat, and deliver their genomes into the cytoplasm.

Poliovirus is a nonenveloped virus composed of 60 copies of each of four proteins (VP1 to VP4) which form an icosahedrally symmetrical spherical shell (approximately 30 nm in diameter) and a single copy of a plus-strand RNA genome of approximately 7,400 nucleotides, polyadenylated at the 3′ end and covalently linked at the 5′ end to a small protein, VPg (14). VP4 is a myristate-modified (C14:0) 7-kDa capsid protein which is found on the inner surface of the poliovirus virion (1). Within the high-resolution atomic structure of poliovirus, a hydrogen bond is observed between Thr-28 of VP4 (4028T) and the myristate carbonyl moiety. This interaction is between two fivefold symmetry-related copies of VP4, and it is one of several myristoyl-mediated interactions that appear to stabilize the virus particle (1, 12).

So that the role of this intersubunit interaction during viral replication could be studied, residue 4028T within an infectious poliovirus cDNA clone (pPVM) was site-specifically replaced with several different amino acids, including Gly. Upon transfection of the mutant cDNAs or RNA transcripts into HeLa cells, it was observed that the glycine substitution was a lethal mutation for the virus (12). To identify the lethal stage in virus replication, a high-efficiency transfection system which involves cDNA transfection of vTF7-3-infected cells was used (6). Poliovirus infection is initiated upon in vivo synthesis of RNA transcripts of the mutant poliovirus genome by the T7 RNA polymerase, which is also expressed in these cells (15). Lysates were isolated from vTF7-3-infected cells which were transfected with either wild-type or glycine mutant (4028T.G) cDNAs and continuously labeled with [^{35}S]methionine after the addition of dactinomycin (13, 15). All viral proteins were synthesized with appropriate stoichiometries (Fig. 1, lanes 1 and 6). In particular, the presence of VP2 was observed in 4028T.G-transfected lysates. Capsid proteins VP2 and VP4 are generated by cleavage of VP0 during virus assembly. This cleavage occurs after RNA encapsidation to form the provirion during the final stages of virion maturation. Thus, the presence of VP2 in the 4028T.G-transfected cell lysate indicates that mature viral particles are formed.

A previous characterization of viable 4028T mutants had indicated that this residue participated in interactions necessary for maintaining assembly intermediates in their assembly-competent conformations (12). Thus, it was possible that the lethal defect was due to gross structural anomalies resulting from defective assembly of the 4028T.G particle or to incomplete maturation of the provirion to the infectious 150S particle. The fully assembled 150S mutant virus particle was purified on 15 to 30% sucrose gradients, and its protein composition was indistinguishable from that of wild-type virus (Fig. 1, lanes 5 and 10). Moreover, like wild-type poliovirus, the 4028T.G particle is stable to high-salt or sodium dodecyl sulfate (SDS)-EDTA treatment, is resistant to proteolysis by trypsin or *Staphylococcus aureus* V8 protease, and is precipitated by monoclonal antibodies recognizing each of the three major neutralizing antigenic sites on the virion surface (data not shown). Thus, by several different biochemical criteria, the structure of the 4028T.G virus appears identical to that of the wild-type virus and suggests that the capsid assembly pathway generates, overall, a particle of normal structure. Indeed, sucrose gradient

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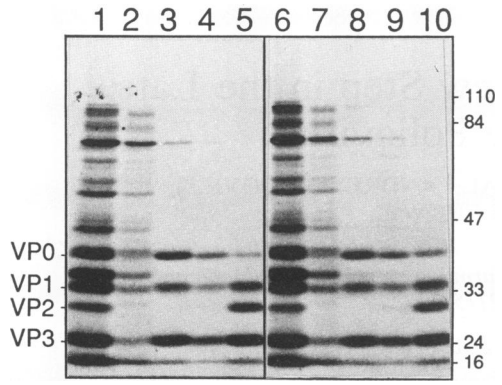

FIG. 1. Poliovirus capsid protein expression in wild-type- or 4028T.G-transfected cells. vTF7-3-infected cells were transfected with wild-type or 4028T.G mutant cDNA (15), labeled with [^{35}S]methionine at 3 h posttransfection in the presence of dactinomycin (5 μg/ml), harvested at 7 h posttransfection, and lysed in TNM buffer (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 10 mM $MgCl_2$) plus 1% sodium deoxycholate–1% Brij 58. Virus assembly intermediates were fractionated on sucrose gradients and analyzed on SDS-polyacrylamide gels (13). The autoradiograph displays wild-type (lanes 1 to 5) and 4028T.G mutant (lanes 6 to 10) fractions. Cell lysates from transfected cells (lanes 1 and 6), 5S protomer (i.e., monomer) fractions (lanes 2 and 7), 14S pentamer assembly intermediates (lanes 3 and 8), 75S empty capsid intermediates (lanes 4 and 9), and 150S mature virions (lanes 5 and 10) are shown. The migration of prestained protein molecular weight markers (in thousands) and the positions of the poliovirus capsid proteins are as indicated.

profiles of assembly intermediates (5S monomer, 14S pentamer, and 75S empty capsid structures) present in lysates from 4028T.G-transfected cells (Fig. 2) and the protein composition of the mutant assembly intermediates appeared like those of the wild type (Fig. 1, lanes 7 to 9).

Although there appear to be no qualitative differences in the assembly pathways of the 4028T.G mutant and wild-type virus, there are quantitative differences. Despite similar concentrations of 14S pentamer and 75S empty capsid intermediates in lysates from wild-type and mutant transfected cells, yields of fully assembled mutant viral particles are

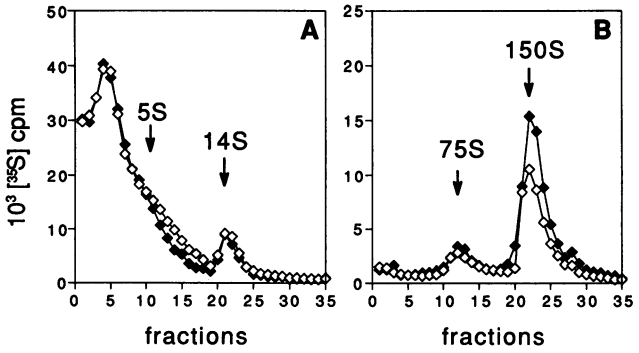

FIG. 2. Analysis of 4028T.G capsid assembly intermediates. The assembly intermediates in [^{35}S]methionine-labeled cell lysates from wild-type (◆) and 4028T.G (◇) cDNA-transfected cells were separated on 6 to 20% (monomer [5S] and pentamer [14S] intermediates) and 15 to 30% (empty capsids [73S] and mature virions [150S]) sucrose gradients (13). Gradients were fractionated from the top, and the radioactivity in each fraction was determined.

consistently approximately 70 to 80% of that observed for the wild type (Fig. 2B). The difference in yields can be accounted for in part by the presence of mutant capsid protein aggregates that are found as an insoluble pellet at the bottom of the sucrose gradient or are typically removed during the low-speed spin that removes nuclei and cellular debris. In addition, a greater fraction of the 4028T.G 75S intermediates than the wild-type assembly intermediates fail to maintain an assembly-competent structural conformation. This is detected in vitro by the incomplete dissociation of mutant 75S empty capsids into 14S pentamers at pH 8.3 (data not shown). Although not identical, the quantitative differences observed for the nonviable 4028T.G mutant are similar to the assembly phenotypes observed for the viable 4028T.V and 4028T.S mutants (12). (These viable mutants also appear to assemble virus particles that appear structurally indistinguishable from the wild-type virus but are less efficient at assembling the viral particles because a larger fraction of the assembly intermediates are also not in an assembly-competent conformation.) Thus, the threonine residue (4028T) provides interactions (not present in the valine-, serine-, or glycine-substituted mutants) that enable efficient assembly of the virion. However, because the assembly phenotype of 4028T.G is similar to those of the viable 4028T.S and 4028T.V mutants, it suggests that the nonviability of the 4028T.G mutant is not likely to be due to faulty capsid assembly.

The apparently normal structure of the particle suggested that the lethal defect in the 4028T.G mutant occurred at early stages of the infection process, during virus entry into cells. Cell entry is initiated upon virus binding to the poliovirus receptor on the cell surface. This receptor is a member of the immunoglobulin G superfamily and is the principal host-range determinant for poliovirus infections (8, 11). For the wild-type virus, receptor interaction initiates a conformational transition that generates an RNA-containing, altered particle sedimenting at 135S (2, 4, 7). This 135S particle is thought to be a necessary intermediate in the cell entry process (3, 9). To establish whether the 4028T.G mutant particle was capable of receptor binding and undergoing the 150-to-135S transition, [^{35}S]methionine-labeled virus particles were incubated in vitro with a detergent-solubilized form of the poliovirus receptor and analyzed on sucrose gradients. In the presence of the receptor, both mutant and wild-type virions were converted to the 135S particle form upon a 1-h incubation at 37°C (Fig. 3). Continued incubation for up to 2 h at 37°C of either wild-type or mutant 135S particles with the receptor did not alter the observed sedimentation profiles (data not shown), indicating that these 135S particles are stable under these conditions. The protein profile of these mutant 135S particles is indistinguishable from that of the wild type (Fig. 4). Most notable is the disappearance of VP4 from these purified 135S particles and the slightly smaller size of the VP1 capsid protein in the 135S particles. Conversion to the 135S particle results in externalization from the particle interior of not only VP4 but also the amino-terminal sequences of VP1 (5). These exposed VP1 sequences can be cleaved with proteases, and in these in vitro experiments, VP1 is slightly smaller, likely because of cleavage of these amino-terminal sequences by endogenous protease activities present in the extract (5). (The apparent lower intensity of VP1 present in the 135S particles is due to the loss of two of the five methionines present in the protein upon removal of these VP1 amino-terminal sequences.) The similar protein compositions and the slightly smaller VP1 protein of both wild-type and mutant 135S particles

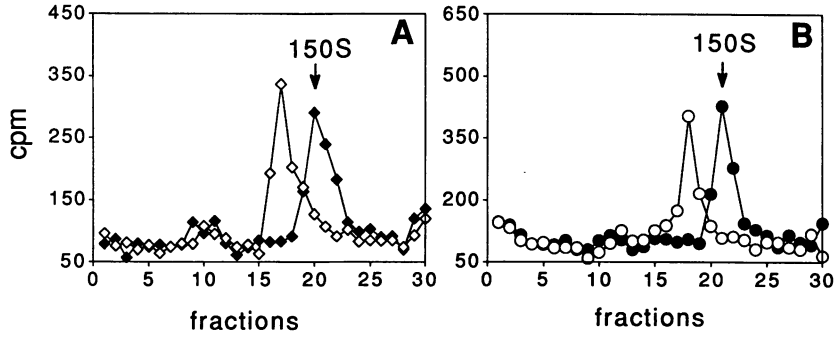

FIG. 3. Receptor-mediated in vitro conversion of 150S virions to 135S particles. Sucrose-purified wild-type (A) or 4028T.G (B) 150S virus particles were incubated with a detergent-solubilized cell extract containing the poliovirus receptor for 1 h at 4°C (closed symbols) or 37°C (open symbols). Samples were analyzed on 15 to 30% sucrose density gradients in TNM (see the legend to Fig. 1)–0.05% Nonidet P-40. The sedimentation direction is from left to right.

indicate that the mutant particle functionally binds to the poliovirus receptor and that the conformational transition, triggered by interaction with the poliovirus receptor, is similar for both wild-type and mutant particles. The 150-to-135S particle transition was also examined in vivo. Upon the binding of labeled mutant or wild-type virus to HeLa cells and its incubation at 37°C for 20 or 40 min, the appearance of 135S particles is induced (Fig. 5). No production of infective particles was detected in 4028T.G-infected cells upon continued incubation of these cells at 37°C (as determined by plaque assays), confirming the nonviability of this mutant. In contrast, incubation of these wild-type-infected cells resulted in total cell lysis and the production of infective virus (data not shown). Thus, both in vitro and in vivo data indicate that this mutant can initiate the cell entry process upon binding to the cell receptor but that it is defective at later stages in the virus life cycle. Because virus particles are synthesized upon transfection of either the mutant cDNA or RNA transcripts (Fig. 1), the lethal defect of the 4028T.G mutant occurs at a previously undefined stage(s) of cell entry which leads to uncoating and delivery of the viral genome into the cell cytoplasm. In addition, this mutant demonstrates the involvement of VP4 sequences during these later stages (following receptor binding and the 135S capsid transition).

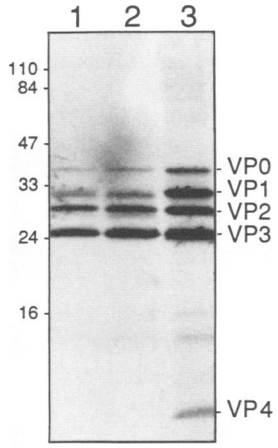

FIG. 4. Protein composition of mutant 135S particles. Mutant and wild-type 135S particles, generated upon incubation of [^{35}S]methionine-labeled 150S particles with poliovirus receptor-containing cell extracts, were isolated on 15 to 30% sucrose gradients and analyzed on 13% polyacrylamide gels. Lanes: 1, wild-type 135S particles; 2, 4028T.G mutant 135S particles; 3, 4028T.G 150S particles. The migration of unlabeled capsid proteins from 150S wild-type virions and that of prestained molecular weight markers (in thousands) are indicated.

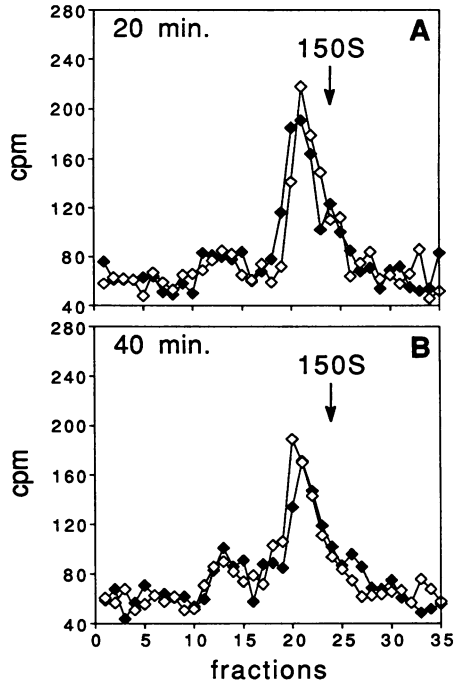

FIG. 5. In vivo receptor-mediated conversion of 150S virions to 135S particles. [^{35}S]methionine-labeled 150S sucrose-purified wild-type (closed symbols) or mutant 4028T.G (open symbols) virions were bound to HeLa cells (multiplicity of infection of approximately 100 virions per cell) at 4°C for 30 min. Cells were washed in phosphate-buffered saline (PBS) to remove unbound virions, resuspended in medium (minimal essential medium–5% fetal calf serum), and incubated at 37°C to initiate cell entry. Aliquots of the cell suspension were taken at the indicated times, washed twice with PBS to remove any eluted virions, and lysed in TNM buffer (see the legend to Fig. 1) plus 0.5% Nonidet P-40. Lysates were analyzed on sucrose density gradients. Samples were taken after 20 (A) or 40 (B) min at 37°C. The sedimentation direction is from left to right.

Cell entry remains a poorly characterized stage of viral infection. For enveloped viruses, such as influenza virus, receptor binding followed by fusion of the viral membrane with the plasma or endosomal membrane delivers the viral genome and proteins into the cytoplasm (10). For the nonenveloped viruses, such as poliovirus, the topological problems of traversing the cellular membrane and of delivering the genome to the cytoplasm remain even after binding to the cell surface receptor. The phenotype of the 4028T.G mutant enables the stages of receptor binding and virus uptake to be differentiated from the subsequent events leading to cytoplasmic entry of the viral genome. Thus, receptor interaction leads to exposure of VP4 sequences from the interior of the poliovirus particle and the participation of these externalized sequences at later stages of the cell entry process. Because purified 135S particles appear not to contain VP4, it has been assumed that the role of protein did not extend beyond the 135S transition. The 4028T.G mutant demonstrates that this myristoyl-modified protein is involved in the membrane-mediated uncoating of poliovirus particles, perhaps in targeting and/or transport of the viral genome across the membrane.

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REFERENCES

1. **Chow, M., J. F. E. Newman, D. Filman, J. M. Hogle, D. J. Rowlands, and F. Brown.** 1987. Myristylation of picornavirus capsid protein VP4 and its structural significance. Nature (London) **327**:482–486.
2. **De Sena, J., and B. Mandel.** 1977. Studies on the *in vitro* uncoating of poliovirus. II. Characteristics of the membrane-modified particle. Virology **78**:554–566.
3. **Everaert, L., R. Vrijsen, and A. Boeyé.** 1989. Eclipse products of poliovirus after cold-synchronized infection of HeLa cells. Virology **171**:76–82.
4. **Fenwick, M. L., and P. D. Cooper.** 1962. Early interactions between poliovirus and ERK cells. Some observations on the nature and significance of the rejected particles. Virology **18**: 212–223.
5. **Fricks, C. E., and J. M. Hogle.** 1990. Cell-induced conformational change in poliovirus: externalization of the amino terminus of VP1 is responsible for liposome binding. J. Virol. **64**:1934–1945.
6. **Fuerst, T. R., E. G. Niles, F. W. Studier, and B. Moss.** 1986. Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. Proc. Natl. Acad. Sci. USA **83**:8122–8126.
7. **Joklik, W. K., and J. E. Darnell.** 1961. The adsorption and early fate of purified poliovirus in HeLa cells. Virology **13**:439–447.
8. **Koike, S., H. Horie, I. Ise, A. Okitsu, M. Yoshida, N. Lizuka, K. Takeuchi, T. Takegami, and A. Nomoto.** 1990. The poliovirus receptor protein is produced both as membrane-bound and secreted forms. EMBO J. **9**:3217–3224.
9. **Lonberg-Holm, K., L. B. Gosser, and J. C. Kauer.** 1975. Early alteration of poliovirus in infected cells and its specific inhibition. J. Gen. Virol. **27**:329–345.
10. **Matlin, K. S., H. Reggio, A. Helenius, and K. Simons.** 1981. Infectious entry pathway of influenza virus in a canine kidney cell line. J. Cell Biol. **91**:601–613.
11. **Mendelsohn, C. L., E. Wimmer, and V. R. Racaniello.** 1989. Cellular receptor for poliovirus: molecular cloning nucleotide sequence, and expression of a new member of the immunoglobulin superfamily. Cell **56**:855–865.
12. **Moscufo, N., and M. Chow.** 1992. Myristate-protein interactions in poliovirus: interactions of VP4 threonine-28 contribute to the structural conformation of assembly intermediates and the stability of assembled virions. J. Virol. **66**:6849–6857.
13. **Moscufo, N., J. Simons, and M. Chow.** 1991. Myristoylation is important at multiple stages in poliovirus assembly. J. Virol. **65**:2372–2380.
14. **Rueckert, R. R.** 1990. Picornaviridae and their replication, p. 507–548. *In* B. N. Fields and D. M. Knipe (ed.), Virology. Raven Press, New York.
15. **Simons, J., A. Rogove, N. Moscufo, C. Reynolds, and M. Chow.** 1993. Efficient analysis of nonviable poliovirus capsid mutants. J. Virol. **67**:1734–1738.