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Distribution of Drug Resistance Mutations in Type 3 Poliovirus Identifies Three Regions Involved in Uncoating Functions

ANNE G. MOSSER,* JEAN-YVES SGRO, AND ROLAND R. RUECKERT
Institute for Molecular Virology, University of Wisconsin, Madison, Wisconsin 53706

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We have previously described the use of an uncoating inhibitor, WIN 51711, to select drug-resistant mutants of the Sabin strain of poliovirus type 3. Two-thirds of the mutants proved to be dependent on the drug for plaque formation because of extreme thermolability (A. G. Mosser and R. R. Rueckert, J. Virol. 67:1246–1254, 1993). Here we report the responsible mutations; all were traced to single amino acid substitutions. Mutations conferring dependence and thermolability occurred in all four capsid proteins (VP1 to VP4), but all were clustered near residue 53 of VP4 at the inner capsid surface. Amino acid substitutions of the remaining non-drug-dependent mutants were mapped to three distinct loci: (i) on or near the inner capsid surface, at VP4 residue 46 or VP1 residue 129, in the vicinity of the drug dependence substitutions; (ii) at residues 192, 194, and 260 in the lining of the VP1 beta barrel, which is the drug-binding site; and (iii) at VP1 residue 105 on the edge of the canyon surrounding the fivefold axis of symmetry, the putative receptor-binding site. All of the mutations increased the eclipse rate of cell-attached virus. Such mutants help identify parts of the capsid that play a role in viral uncoating functions.

Picornaviruses have a single-stranded, positive-sense RNA surrounded by an icosahedral protein shell constructed from 60 copies of each of four capsid proteins, VP1, VP2, VP3, and VP4. The three larger proteins, VP1, VP2, and VP3, make up the bulk of the capsid and share a common core structural motif, the eight-stranded, antiparallel beta barrel (18, 38). VP4 is smaller and is confined to the inner surface of the capsid, in contact with the viral RNA and with the amino termini of VP1 and VP3. The VP1 proteins are contiguous around the fivefold axes of symmetry, and loops connecting the beta strands, especially the B-C loops, build star-shaped plateaus at the fivefold axes. Surrounding these plateaus are 25-Å (2.5-nm)-deep canyons that are acceptors for cellular receptors for many human rhinoviruses (5, 35) and probably also for polioviruses.

For many picornaviruses whose structures have been solved crystallographically (10, 18, 34), the space within the VP1 beta barrel is occupied by a ligand interpreted as hydrophobic molecules whose sizes are strain specific (Fig. 1, pocket factor). Electron density has not been observed within the beta barrel of crystallized human rhinovirus 14 (HRV-14) (34), possibly because such pocket molecules are lost during purification of the virus. Nothing is known about the function of the pocket factor.

The viral protein shell has a dual role; to protect the viral RNA against degradation while outside of the cell and to deliver the genome intact into the cytoplasm of its host cell. When picornaviruses come in contact with their host cells, they bind to specific cellular receptors, recruiting additional receptors to initiate envelopment by the plasma membrane (Fig. 1 A and B). Receptor molecules have been identified for several picornaviruses, among them ICAM-1 for the majority of human rhinoviruses (14, 41). The poliovirus receptor has been cloned and identified as a member of the immunoglobulin supergroup (29), but its function in the human host has not yet been identified. For these viruses, interaction with the cellular receptor, whether on the surfaces of cells or free in solution, initiates changes in the viral capsid that have been associated with the first step in viral uncoating (8, 12, 20, 24). This is the formation of the A particle, a noninfectious particle with a reduced sedimentation coefficient that has lost VP4, extruded the amino terminus of VP1, and is considerably more hydrophobic than the native virion (7, 12, 13) (Fig. 1C). Although conversion to the A particle takes place on the surfaces of cells and many of the altered particles elute from the cells (19), such altered particles are also found within the endosomic vesicles of newly infected cells (8). After alteration to the A particle, virions must still deliver their RNAs across the plasma or endosomic membrane; it has been speculated that the extruded portions of the capsid (VP4 and the amino termini of VP1 molecules) are instrumental in this process, perhaps by forming a channel for RNA transfer (12, 31) (Fig. 1D). It is this step which appears to be blocked in rhinovirus mutants defective in maturation cleavage (22) and in certain poliovirus VP4 mutants (21, 31).

Little is known about what portions of the viral capsid are instrumental in these early stages of the viral infectious cycle. Our laboratory is exploring this problem by mapping mutations in mutants resistant to capsid-binding drugs that block attachment or uncoating (11, 27, 36). These drugs insert into a hydrophobic space within the VP1 beta barrel normally occupied by pocket factor (2, 40). The drugs act as analogs of pocket factor but have higher affinities, i.e., longer residence times within the pocket. Thus, it can be anticipated that studies on mutants resistant to these drugs will help illuminate the role of natural pocket factor. When capsid-binding drugs replace the pocket factor within the VP1 beta barrel, they make it more rigid, stabilizing the virus against denaturation as a result of heat or extremes of pH (3, 11, 15, 37) and blocking conversion to the A particle (8).

A study of HRV-14 mutants resistant to several of these drugs showed that drug resistance could be acquired via single amino acid substitutions (16). Insertion of the drug elevates the floor of the canyon of HRV-14 and blocks its attachment to cells (36, 39). Drug-resistant mutants displayed two phenotypes which could be distinguished by the ability of bound drug

* Corresponding author. Mailing address: Institute for Molecular Virology, University of Wisconsin, 1525 Linden Dr., Madison, WI 53706. Phone: (608) 262-7891. Fax: (608) 262-7414. Electronic mail address: annemosser@psl.wisc.edu.

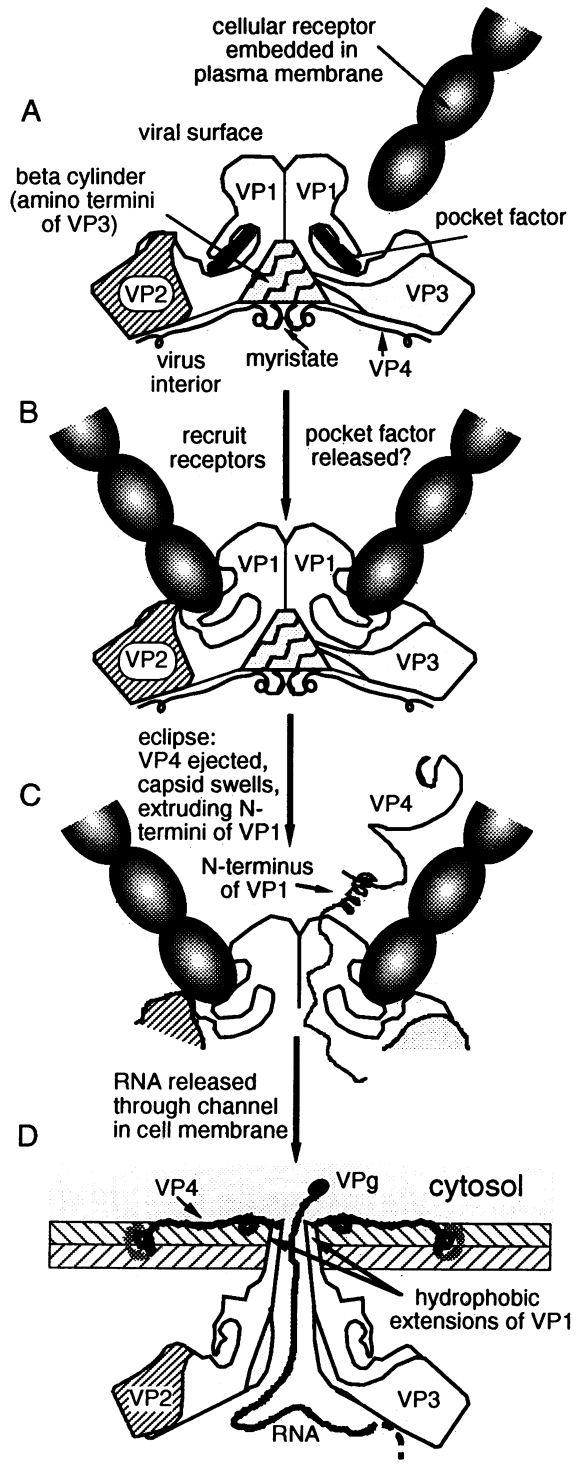


FIG. 1. Model showing how attachment of receptors might mediate early events in uncoating. Panels depict a side view of a single viral pentamer with sections removed to reveal the relationship between receptor, canyon, drug-binding pocket, and interior surface in contact with the RNA genome. (A) The cellular receptor binds into the canyon. (B) The fate of pocket factor is not known, but it has been speculated that its release during the attachment process might sensitize the shell for uncoating by promoting flexibility of the protein (34). (C) Conformational changes, propagated either through the VP1 beta barrel or at protomer-protomer interfaces, result in the expulsion of VP4 and exposure of the amino terminus of VP1 (12). (D) VP4 and the amino termini of VP1 form a channel through the cell membrane, allowing the viral RNA to penetrate into the cytoplasm. Many parts of this model are speculative, and successful penetration of intact vRNA into the cytoplasm may be a rare event (22). Typically, 100 or more picornavirus virions are needed to establish a single productive infection.

to protect mutants against thermal inactivation: drug-exclusion mutants substituted bulky amino acids for smaller amino acids in the lining of the drug-binding pocket, thus greatly reducing drug-binding affinity, and drug-compensation mutants had amino acid substitutions in the floor of the canyon which altered the interaction of the virus with its cellular receptor (17, 39).

We initiated a study with the Sabin strain of poliovirus type 3 (P3/Sabin) and the drug disoxaril (WIN 51711) (27) which prevents uncoating but not attachment. Drug-escape mutants should therefore provide information on those parts of the viral capsid important for formation of the A particle and crucial for viral uncoating. Of 22 independently derived drug-resistant mutants, 14 proved drug dependent (33). All 14 drug-dependent mutants produced the same yield of infectious progeny with or without drug, but they were dependent on the presence of drug for plaque formation. Once released from cells the virions were extremely thermolabile without drug and decayed at 37°C to particles with all the characteristics of the A particle. Thus, they had a marked propensity to undergo the first stage in uncoating, even without interaction with the cellular receptor. This article describes the amino acid substitutions in the capsid proteins of these mutants and of the remaining eight mutants which were capable of forming plaques in either the presence or absence of drug.

MATERIALS AND METHODS

Viruses and cells. HeLa cells, obtained from V. Hamparian of Ohio State University, were cloned in our laboratory, cured of a mycoplasma infection (22), and maintained in shake cultures in medium B (28). These cells are available from the American Type Culture Collection as cell line ATCC CRL 1958 (H1 HeLa). P3/Sabin was obtained from Olen Kew, Centers for Disease Control and Prevention, Atlanta, Ga.

Solutions. WIN 51711 was a gift from Guy Diana, Sterling Winthrop Pharmaceuticals Research Laboratories, Malvern, Pa. It was dissolved at 4 mg/ml in dimethyl sulfoxide and diluted 2,000-fold into medium or phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 1.47 mM KH_2HPO_4, 4.86 mM Na_2HPO_4, 0.68 mM $CaCl_2$, 0.49 mM $MgCl_2$) containing 0.4% bovine serum albumin (BSA).

Preparation of virus stocks and extraction of the viral RNA. Wild-type and mutant virus stocks were prepared for sequencing by growth in suspension culture. To minimize the introduction of spurious mutations, sequencing was carried out on viral stocks not more than two amplifications beyond plaque selection. Virus at a multiplicity of 5 to 10 PFU per cell was added to washed HeLa cells at a final concentration of 4×10^7 cells per ml in supplemented medium P5 (28, 33) containing 2 μg of WIN 51711 per ml for mutant viruses. Virus was allowed to attach with gentle mixing for 30 min at room temperature, and then the cells were diluted 10-fold with the same medium, prewarmed to 37°C. Cells were incubated at 37°C for 6.5 h and sedimented by low-speed centrifugation. They were resuspended at 4×10^7 cells per ml and lysed by treatment with 0.5% Nonidet P-40 for 5 min at room temperature. Cell debris was removed by centrifugation for 10 min at 10,000 rpm in a

Sorvall SS34 rotor, and the supernatant was layered over a sucrose step gradient consisting of three 0.75-ml layers of 10, 20, and 30% sucrose in 1 M NaCl–25 mM Tris-acetate, pH 7.5. Tubes were centrifuged at 15°C for 120 min at 40,000 rpm in a Beckman SW41 rotor, and the supernatant was decanted. RNA was extracted from the pellet by the guanidinium thiocyanate procedure of Chomczynski and Sacchi (4) after resuspension of the viral pellet in solution D.

Sequence determination. Viral RNA was sequenced directly with a set of 15- to 16-base DNA oligonucleotides by the procedure of Air (1) for sequencing within 30 to 150 bases of the primer and by the procedure of Fichot and Girard (9) for sequencing 100 to 220 bases from the primer. Approximately 60% of the capsid region of the genome of the wild-type RNA (primarily VP1, VP3, and VP4 coding sequences) has been sequenced, and no changes from the published sequence (42) were found.

Selection of revertants of drug-dependent mutants. Dilutions of drug-dependent mutants (200 μl) in PBS containing 0.4% BSA were applied to washed HeLa cell monolayers, and the virus was allowed to attach for 30 min at room temperature. The cells were then overlaid with 2.5 ml of supplemented medium P5 containing 0.8% Noble agar and 2.5 ml of the same medium without agar. No drug was present. After approximately 48 h at 37°C, the liquid overlay was replaced with a solution of 0.01% neutral red–0.1% glucose in Earle's saline. The plates were incubated at 37°C for an additional 1 to 2 h before removal of the liquid layer and picking of the agar plugs overlying the plaques with Pasteur pipettes. Agar plugs were expelled into 0.5 ml of PBS containing 0.4% BSA, and viruses were released from cells by freezing and thawing. Virus stocks grown from such plaques always demonstrated a drug-related phenotype different from that of the parental drug-dependent mutant.

Thermostabilization assay. Virus which had been grown without drug was diluted to 10^7 or 10^8 PFU/ml with PBS containing 0.4% BSA with or without 2 μg of WIN 51711 per ml. Samples (150 μl) were pipetted into warmed, capped glass tubes immersed in a water bath. After 2.5 min, the tubes were withdrawn and quick cooled in an ice bath. Virus was diluted into PBS containing 0.4% BSA and 2 μg of WIN 51711 per ml, and infectivity was determined by plaque assay. Heated samples were compared with control samples pipetted directly into tubes in an ice bath.

Assay of viral eclipse. All virus stocks were prepared in the absence of drug. HeLa cells were sedimented and washed once in room temperature medium A (28). They were resuspended in supplemented medium P5 plus virus inoculum to a concentration of 4×10^7 cells per ml and 10 PFU per cell. Virus was allowed to attach for 30 min at room temperature, and then unattached virus was removed by sedimenting the cells and washing once in room temperature medium A. Infected cells were resuspended to 4×10^7 cells per ml in supplemented medium P5 at room temperature and then diluted into a ninefold volume of medium P5 at the desired temperature. Samples were taken immediately, and at intervals thereafter, virus was released by three cycles of freezing and thawing and then infectivity was quantitated by plaque assay.

RESULTS

Amino acid substitutions in drug-dependent mutants. We have previously described the isolation of 22 drug-resistant mutants which could be further sorted into two phenotypes, dependent and nondependent, by replating each resistant isolate in the presence and absence of drug (Table 1). All 14 of the drug-dependent mutants, but none of the 8 nondependent ones, were hyperlabile. Hyperlabile mutants displayed a half-life of less than 1 min at their normal growth temperature, 37°C, in the absence of drug. They were able to grow in the absence of drug because they became thermosensitive only after release from the cell (33).

TABLE 1. Locus of drug resistance mutations[a]

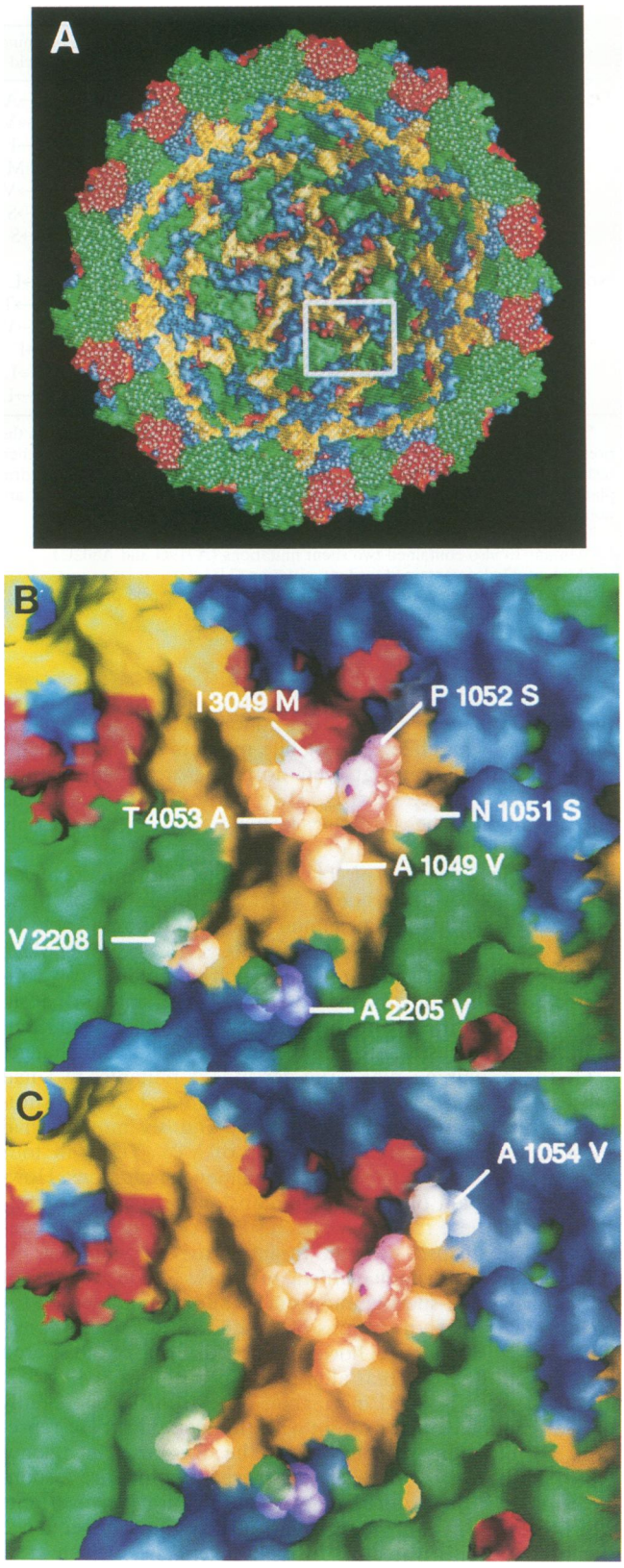
Phenotype	Mutation	Mutant	Substitution	
			Position	Amino acid
Dependent	A908G[b]	8, 13,[c] 15, 16, 22[d]	4053[e]	T→A
	C1569U	20	2205	A→V
	G1577A	7[f]	2208	V→I
	A1915G	18	3049	I→M
	C2628U	3,4	1049	A→V
	A2634G	17	1051	N→S
	C2636G	2, 10, 19	1052	P→S
Nondependent	C888U	1[g]	4046	S→L
	U2796C	11, 21	1105	M→T
	A2868U	6, 12	1129	D→V
	A3056U	14	1192	I→F
	G3062C	9	1194	V→L
	A3260C	5	1260	M→L

[a] Mutants were first selected for the ability of P3/Sabin to form plaques in the presence of WIN 51711 at 2 μg/ml. Each of these drug-resistant isolates was then tested for ability to form plaques in the absence of drug; those unable to form plaques in the absence of drug are dependent; those able to do so are nondependent (33).
[b] Mutation of base A to base G at position 908 in the RNA genome.
[c] Mutant 13 also contained two silent mutations (A719G and A802U).
[d] Mutant 22 also contained a silent mutation (U814C).
[e] Threonine was replaced by alanine at position 53 in capsid protein VP4.
[f] Mutant 7 also contained a silent mutation (C1837U).
[g] Mutant 1 also contained a silent mutation (G2731).

Sequencing this set of 14 hyperlabile mutants revealed mutations at seven positions in the genome; all of these mutations resulted in single amino acid substitutions (Table 1). Five of the 14 dependent mutants (no. 8, 13, 15, 16, and 22) displayed identical mutations in which threonine was converted to alanine at residue 53 in VP4, the smallest capsid protein. Two of these, mutants 13 and 22, also contained silent mutations, i.e., base changes involving no amino acid change.

Of the remaining nine drug-dependent mutants, two had substitutions in the beta G strand of VP2, one had a substitution in the amino terminus of VP3, and six mutants had substitutions in the amino terminus of VP1. Thus, all four capsid proteins were involved in dependence mutations. However, examination of the three-dimensional structure (Fig. 2) showed that these residues were clustered on or near the inner surface of the capsid near the threefold axis of symmetry.

Revertants of drug-dependent mutants. Reconstruction of mutations by site-specific mutagenesis affords one method for confirming that the mutations observed are actually responsible for drug dependence; however, the clustering of the altered residues and the fact that alterations at positions 1049, 1052, and 4053 were isolated more than once from independently derived mutants are consistent with the idea that these represent the mutations responsible for the drug-resistant phenotype. Another way of showing that these mutations are responsible for drug dependence is to isolate phenotypic revertants which are wild type in their drug sensitivity and to sequence their RNAs. This was done by isolating plaques which grew when drug-dependent mutants were plated in the absence of drug (33). Some resembled the wild-type virus in drug sensi-



tivity, and others were partially resistant; none of these revertants was drug dependent. Revertants were isolated from dependent mutants representing each of the seven amino acid substitutions shown in Table 1. We selected 17 revertants for sequence analysis, choosing only those revertants having the same drug sensitivity as the wild-type virus. Several of these mutants were also tested for thermal stability at 37°C, and they resembled the wild-type virus in this characteristic as well (data not shown). Sequencing revealed that 8 of the 17 were true genetic revertants that had the wild-type sequence at the site of the original mutation, confirming that the mutations at 1049, 1051, 1052, and 4053 were capable of conferring drug dependence (Table 2). The remaining nine were pseudorevertants; they had retained their original amino acid substitutions. Eight of these pseudorevertants had identical second-site C to U mutations at nucleotide 2643, conferring an alanine to valine change in VP1 amino acid 54 (Fig. 2C). The second-site mutation for the remaining pseudorevertant has not yet been located.

Nondependent drug-resistant mutants. As noted previously, eight drug-resistant mutants were nondependent. They produced approximately equal numbers of plaques in either the presence or absence of drug (33). Sequencing revealed that nondependent resistance was conferred by single amino acid substitutions, primarily in capsid protein VP1 (Table 1). The positions of these residues in the structure of the virion fell into three loci (Fig. 3). The first location was in the lining of the drug-binding pocket, at residues 192, 194, and 260 of VP1. The side chains of these residues project toward the pocket lumen (Fig. 3A). In the drug-resistant mutants, isoleucine 192 and valine 194 were replaced by amino acids with bulkier side chains.

The second location was in the canyon lining. Two mutants had identical substitutions at residue 105 (Table 1), on the "north" wall of the canyon surface (Fig. 3B and C). The remaining two mutations were in the third location, on or near the inner capsid surface, but removed from the cluster of mutations conferring drug dependence. One of these mutants had a change in amino acid 4046. In two others, aspartic acid 1129 was replaced by valine. This amino acid faces away from the drug-binding pocket, and the tip of its side chain lies just under a loop of VP4 from a neighboring protomer (Fig. 3D). In this case, the amino acid substitution involved a charge change which might have relatively large effects on local structure.

Evidence that nondependent mutants still bind drug. Drug resistance studies with HRV-14 showed that some mutants lost the ability to bind drug; others did not (17). This was demonstrated by the inability of drug to thermostabilize viral mutants which had lost drug-binding ability. Application of thermostabilization analysis to our mutants showed that none had

FIG. 2. Location in type 3 poliovirus of mutations conferring drug dependence (thermal uncoating in the absence of drug). (A) Half shell with RNA removed from the central cavity. VP1 is blue, VP2 is green, VP3 is red, and VP4 is yellow. Atoms appear as white dots where the protein shell was cut in cross-section. (B) Detail of boxed area in panel A shows the location of amino acid substitutions in drug-dependent mutants (spheres) in one of the 60 protomers. Residues 1052, 2208, and 3049 are partially exposed on the surface. Residues are colored pink, but buried portions are discolored by the overlying translucent surface. (C) Location of pseudorevertant substitution at VP1 residue 54 and its proximity to the residues shown in panel B. This figure was prepared using the program GRASP, using structural coordinates provided by James Hogle.

TABLE 2. Amino acid substitutions in drug-sensitive revertants of drug-dependent mutants

Virus	Amino acid at position[a]							
	1049	1051	1052	1054	2205	2208	3049	4053
Wild type	Ala	Asn	Pro	Ala	Ala	Val	Ile	Thr
A1049V[b]	Val							
A1049VR4.1	Ala							
A1049VR4.3	Val			Val				
N1051S		Ser						
N1051SR17.2		Ser		Val				
N1051SR17.3		Asn						
P1052S			Ser					
P1052SR10.3			Pro					
P1052SR10.4			Pro					
A2205V					Val			
A2205VR20.1				Val	Val			
A2205VR22.2				Val	Val			
V2208I						Ile		
V2208IR7.1				Ala		Ile[c]		
V2208IR7.2				Val		Ile		
I3049M							Met	
I3049MR18.2				Val			Met	
I3049MR18.3				Val			Met	
T4053A								Ala
T4053AR8.2, 13.2, 16.1, 22.2				Ala				Thr
T4053AR22.1				Val				Ala

[a] The first digit is the number of the viral capsid protein; the next three digits give the number of the amino acid in that protein.

[b] Mutants are designated with the letter code for the wild-type amino acid followed by the capsid protein and amino acid numbers and the letter code for the mutant amino acid. Revertants are numbered according to the drug-dependent mutant they were selected from, appended to this designation.

[c] Mutation conferring reversion to drug sensitivity not yet found.

completely lost the ability to bind drug (Table 3). Wild-type virus was rapidly inactivated when heated to 42°C in the absence of drug. Most of the mutants were more thermolabile than wild-type virus; however, all were significantly thermostabilized by drug. This test was performed at only one fairly high drug concentration, 2 μg/ml, and therefore cannot be used as a source of information on the relative drug-binding affinity of wild-type virus or mutants. However, mutants 9 and 14, which have substituted bulkier amino acids into the lining of the drug-binding pocket, were poorly thermostabilized by drug, suggesting that they may have a lower affinity for drug. On the other hand, mutant 5, which has substituted leucine for methionine at VP1 amino acid 260 in the drug pocket lining, was thermostabilized by drug as well as the wild-type virus.

Evidence that all drug-resistant mutants have a lowered eclipse temperature. All of the nondependent drug-resistant mutants bind drug. If so, how do they escape the inhibitory effect of bound drug on uncoating? One possibility is that the mutations lower the temperature at which the viruses eclipse, that is, undergo the transition to the A particle in the presence of cells. Figure 4A shows that the wild-type virus eclipsed in the presence of cells fairly rapidly at 35°C, but as the temperature was lowered to 30°C, eclipse slowed dramatically. Eclipse was not measurable at 25°C. Eclipse of the wild-type virus was then compared with eclipse of non-drug-dependent mutants that had been grown in the absence of drug. These mutants showed some variability, but all eclipsed faster at 28°C than wild-type virus did (Fig. 4B). When two of the drug-dependent mutants were grown without drug and tested in this assay, they eclipsed markedly faster than either the wild-type virus or the drug-resistant mutants that were not drug dependent (Fig. 4C). For these experiments, all virus stocks were prepared in the absence of drug; this was possible because the drug-dependent mutants produce infectious virus equally well in either the presence or absence of drug, as long as stocks are not heated to 37°C after release from cells.

DISCUSSION

We have isolated P3/Sabin mutants resistant to 2 μg of WIN 51711 per ml, a drug which blocks viral uncoating by preventing conversion to the A particle. Of 22 drug-resistant mutants, 8 made approximately equal numbers of plaques in the presence or absence of drug, and the other 14 were dependent on the presence of drug for plaque formation because of extreme thermolability when outside of the cell (33). Sequencing of all 22 mutants revealed a total of 13 different amino acid substitutions: 7 that produced drug dependence, and 6 that produced virus that could form plaques in either the presence or absence of drug. The variety of amino acid substitutions suggests that there are many routes to drug resistance, and it therefore seems likely that new mutations will be located if more drug-resistant mutants are selected.

The locations of these amino acid substitutions (Fig. 5) point to three regions of the viral capsid as important in uncoating functions: (i) the wall of the canyon surrounding the fivefold axis of symmetry, (ii) the lining of the drug-binding pocket in the interior of the VP1 beta barrel, and (iii) the inner surface of the protein capsid.

The canyon wall. Two nondependent mutants had a methionine to threonine substitution at position 1105. This amino acid lies on the surface of the virion, on the canyon wall (Fig. 3B and C and Fig. 5, arrow 1). Although there is no direct evidence in the case of P3/Sabin, the canyon is thought to be the binding site for the cellular receptor, and amino acids in the B-C loop of VP1 (amino acids 94 to 102) have been shown to permit infection of mice (26), which are not normal hosts for polioviruses. This region of the capsid may play a role in triggering conversion to the A particle after receptor binding.

The same mutation has been reported (25) in a non-temperature-sensitive type 3 poliovirus (EM47) isolated from a healthy vaccinee, but this isolate also had several other amino acid substitutions, including the Ala to Val substitution at 1054 found in our pseudorevertants. Since the substitution at 1054 appears to be capable of suppressing the temperature-sensitive phenotype, it is not clear that the substitution at 1105 had any effect.

The lining of the drug-binding pocket. Three of the eight nondependent mutants had substitutions of amino acids which project into the lumen of the drug-binding pocket (Fig. 3A and Fig. 5, arrow 2). The substitution of Phe for Ile at 1192 or of Leu for Val at 1194 involves the placement of bulkier amino acids into the pocket walls. These two mutants were thermostabilized by drug but less strongly than the other mutants (Table 3), suggesting they have reduced affinity for drug. Alternatively, these mutations might confer increased conformational flexibility in the VP1 beta barrel, thus allowing uncoating to occur even with drug present.

The inner surface of the protein capsid. Three of the nondependent mutants and all of the drug-dependent mutants had amino acid substitutions in the inside lining of the capsid, either exposed on the inner surface or in its vicinity. It is not yet

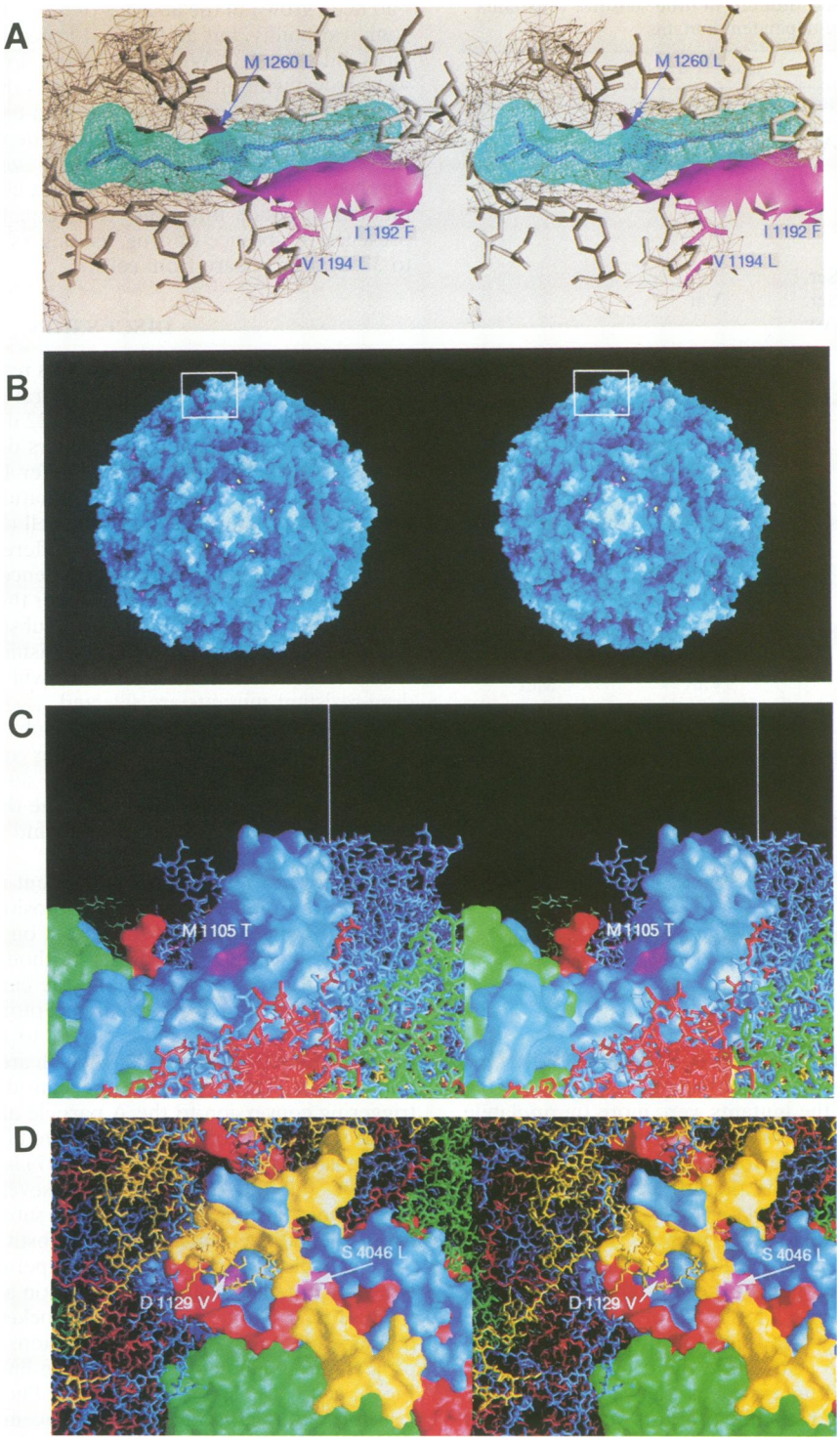


FIG. 3. Stereo pairs depicting locations of three classes of nondependent resistance mutations. (A) Grey mesh depicts the back wall of the drug-binding pocket. The outside of the virion is toward the top; the interior is toward the bottom. Pocket factor modeled as sphingosine is colored light blue. Stick models identify amino acid residues forming the pocket. Pink regions identify surface contributed by mutated residues (labeled residues). (B) Exterior surface of type 3 poliovirus showing rugged topography and location of M-1105 (pink) near the base of the north wall of the canyon. Pocket factor (yellow) is visible in the central pentamer through pores in the bottom of the canyon. (C) Color-coded detail of residue 1105 (pink) shown in the boxed area in panel B. VP1 is blue, VP2 is green, and VP3 is red. The surface is modeled for only one of the five protomers; amino acids in adjacent protomers are rendered as stick figures. (D) Detail of the internal cavity surface, with mutated residues shown in pink. Color code and approximate location are as described for the boxed area of Fig. 2A. The surface of a single protomer is shown; adjacent protomers are rendered as stick figures. The residues shown in Fig. 2B (dependence mutations) are located nearby in the vicinity of the VP4 (yellow) segment in the bottom right corner.

TABLE 3. Effect of heating non-drug-dependent mutants to 42°C in the presence and absence of 2 μg of WIN 51711 per ml

Mutant no.	% Survival[a]	
	+Drug	−Drug
Wild type	90	0.6
1	70	2
5	100	0.1
6	4	0.05
9	0.08	0.005
11	80	0.5
12	20	0.1
14	1	0.04
21	90	0.08

[a] Virus grown in the absence of drug was preincubated in buffer with or without 2 μg of WIN 51711 per ml, and then one-half of each virus sample was heated to 42°C for 2.5 min. Surviving infectivity in both heated and unheated samples was determined by plaque assay.

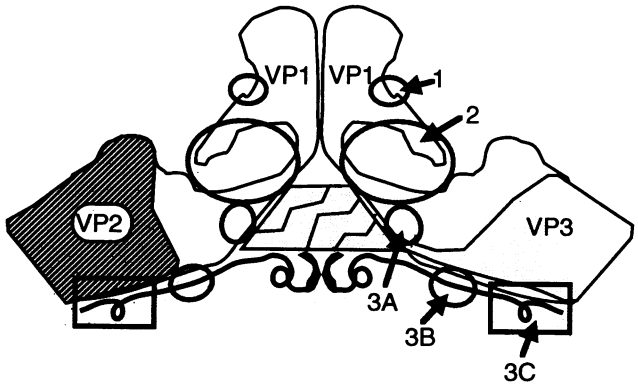


FIG. 5. Schematic diagram showing locations of mutations conferring drug resistance, both dependent (rectangles) and nondependent (ellipses). A side view of a single viral pentamer is shown as described in the legend to Fig. 1. Mutations were found on the canyon surface, where they might affect the interaction of virus and cellular receptor (arrow 1), on the lining of the drug-binding pocket (arrow 2), and on or near to the interior surface of the capsid (arrows 3A to C). These locations suggest a pathway from the receptor-binding site through the drug-binding pocket to those structures which are extruded during the formation of the A particle.

clear to us whether the mutations conferring drug dependence form a unique functional site and those associated with nondependent drug resistance form a separate site; we discuss these together because all point to a region that must be involved in the conformational changes that take place during uncoating.

In two of the nondependent mutants, Asp-1129 was changed to valine. When viewed from the inside of the capsid, this residue lies just below a loop of VP4 contributed by an adjacent protomer (Fig. 3D and Fig. 5, arrow 3A). The side chain oxygens of the wild-type aspartic acid are within hydrogen bonding distances of residues in two adjacent beta strands. Therefore, replacement of aspartic acid with valine would cause the loss of two potential hydrogen bonds and increased flexibility of the VP1 beta barrel.

Another nondependent mutant had an amino acid substitution on the interior surface of the capsid at position 4046 (Fig. 5, arrow 3B), and all of the mutations in the drug-dependent mutants were tightly clustered either on the interior surface or close to it (Fig. 5, arrow 3C). Many of the amino acid substitutions in these mutants involve conservative exchanges of hydrophobic amino acids such as the substitution of valine for alanine, and yet these changes can have enormous effects on the stability of the virion. This part of the capsid may control the delicate balance between the need for structural

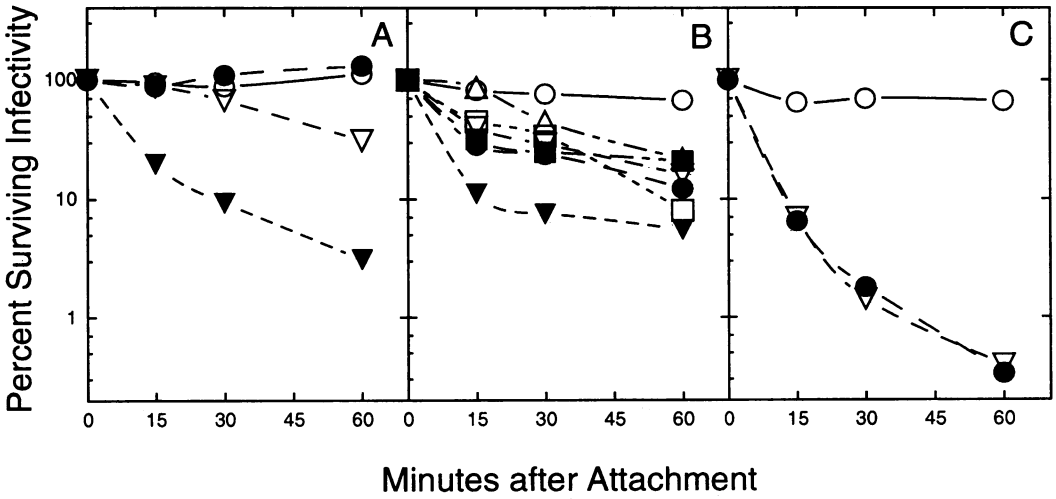


FIG. 4. Evidence for enhanced eclipse rate by drug-resistant mutants. (A) Wild-type virus was allowed to attach to 4×10^7 HeLa cells at 22°C for 30 min. Cells were pelleted and washed once to remove unattached virus. Infected cells were resuspended in 1 ml of medium at 22°C and then diluted into medium at the following temperatures: 22°C (○), 25°C (●), 30°C (▽), 35°C (▼). At the times shown, cells were sampled and broken by freezing and thawing, and virus titer was determined by plaque assay. Little or no eclipse occurred below 25°C. (B) Eclipse of non-dependent, drug-resistant mutants at 28°C compared with that of wild-type virus. Cells were infected with virus grown in the absence of drug, and virus was harvested as described for panel A. ○, wild-type virus; ●, mutant 1; ▽, mutant 5; ▼, mutant 9; □, mutant 12; ■, mutant 14; △, mutant 21. (C) Eclipse of drug-dependent mutants at 28°C compared with that of wild-type virus. Cells were infected with virus grown in the absence of drug, and virus was harvested as described for panel A. ○, wild-type virus; ●, mutant 7; ▽, mutant 15.

stability and the requirement for the ability to undergo the conformational changes involved in uncoating.

Several other laboratories have demonstrated the importance of residues on the amino terminus of VP1 to early events in the virus infection cycle. Moss and Racaniello (32) found that the mouse-virulent P2/Lansing strain of poliovirus could be rendered noninfectious in mice by replacing the VP1 B-C loop with the corresponding sequence from the P1/Mahoney strain. Two passages of this chimeric virus in mice led to the selection of neurovirulent progeny with changes not in the VP1 B-C loop, which is on the surface of the virion, but at residues 1040 and 1054. Since the VP1 protein of P3/Sabin has a two-amino-acid deletion between residues 6 and 10 relative to types 1 and 2, position 1054 in poliovirus type 2 corresponds to 1052 in P3/Sabin, for which three of our drug-dependent mutants have the same proline to serine substitution. Thus, one of the residues identified here as a destabilizing mutation predisposing the virus to formation of the A particle has also been identified as a key residue in allowing the infection of mice even when the surface residues do not have ideal sequences for contact with the mouse cellular receptor. Couderc et al. (6) identified substitutions at two additional residues, 1022 and 2031, also on the virion interior, that were capable of conferring mouse neurovirulence on the normally mouse-avirulent P1/Mahoney strain.

The residues identified in this article as responsible for the drug-dependent phenotype all make the virion unstable at 37°C (33). When plaques were isolated in the absence of drug, many of the stocks from these plaques were phenotypically like wild-type virus in both their thermolability and drug sensitivity. About half of these were true genetic revertants, but the remainder were second-site revertants and almost all of these had substituted valine for alanine at 1054. This mutation has also commonly been found in non-temperature-sensitive isolates of poliovirus type 3 from either healthy vaccinees or from type 3 vaccine-associated cases of poliomyelitis (30). Thus, this amino acid substitution appears to be generally stabilizing. Minor et al. (30) found this mutation was always accompanied by one or more additional mutations. We have not found evidence for such additional mutations in this study, and the frequency of isolation of these revertants, about 1 in 1,000, argues against multiple nucleotide changes.

Role of pocket factor in picornavirus stability and uncoating. Polioviruses and some rhinoviruses have a ligand, termed pocket factor, in the hydrophobic interior of the VP1 beta barrel (34). The properties of our drug-dependent mutants support the idea that the presence of a hydrophobic molecule such as pocket factor within the drug-binding pocket stabilizes the virus during transit between cells. In order to undergo the large conformational changes involved in uncoating, pocket factor probably must be released from the virion. Perhaps the conformational changes induced by interaction with the cellular receptor trigger this release (Fig. 1). Capsid-binding drugs such as WIN 51711 bind to virus with higher affinities than pocket factor, displacing it and further stabilizing the virus. They block the first step in uncoating because they are less likely to be released when the virion interacts with the cellular receptor.

Formation of the A particle by extrusion of VP4 and the amino terminus of VP1 after interaction with cellular receptor is irreversible. Yet limited conformational changes of these same structures may be part of the normal repertory of the viral capsid. Li et al. (23) showed that antisera specific for VP4 and amino-terminal sequences of VP1 were capable of neutralizing virus when mixed at 37°C. Virus that had been heated to 37°C but mixed with one of these antisera at 25°C was not neutralized. Thus, the virus capsid is capable of reversibly exposing sequences that are internal in the static X-ray crystallographic structure. This "breathing" of the capsid was not prevented by the presence of capsid-binding drugs (23) that block formation of the A particle, such as the WIN compounds. This suggests that the irreversible formation of the A particle involves more extensive capsid rearrangements than those involved in capsid breathing.

The uncoating thermostat. Another way of considering the early stages of virion uncoating is to measure the thermal input required to initiate conversion to the A particle. Each picornavirus strain has a characteristic temperature at which it spontaneously converts to the A particle. The temperature is lowered when the virion binds to its cellular receptor and is raised when the VP1 pocket contains drug rather than pocket factor. The wild-type P3/Sabin is rapidly converted to the A particle at 42°C in the absence of drug or cellular receptor. In the presence of cells, the conversion occurs slowly at 30°C and more rapidly at 35°C (Fig. 4A). The drug-dependent mutants convert to A particles very rapidly at 37°C even without cellular receptor (33); with receptor binding, they convert rapidly at 28°C (Fig. 4C). In other words, the amino acid substitutions that confer drug dependency also lower the temperature for uncoating. The non-drug-dependent mutants also uncoat more rapidly than does wild-type virus at 28°C (Fig. 4B); their uncoating temperature must lie between that of the wild type and the drug-dependent mutants.

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