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WIN51711-Resistant Mutants of Poliovirus Type 3: Capsid Residues Important in Uncoating Functions

BY ANNE G. MOSSER, JEAN-YVES SGRO AND ROLAND R. RUECKERT

University of Wisconsin, Institute for Molecular Virology, 1525 Linden Drive, Madison, WI 53706, USA

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Abstract

Capsid-binding drugs that inhibit the first stage of picornaviral uncoating were used to select drug-resistant mutants of the Sabin strain of poliovirus type 3. Such mutants provide information about parts of the capsid that are important for functions blocked by the drugs, and also about pathways to drug resistance. Amino-acid substitutions allowing virus to produce progeny in the presence of drug were mapped to 13 different residues occupying three distinct locations: (I) the canyon base; (II) the lining of the drug-binding pocket; and (III) the base of the protomer. These loci might be thought of as action points for transmitting the uncoating signal from receptor, through the pocket, and to the base of the protomer. All of the mutations in a special class of drug-dependent mutants were clustered at site (III) and all were hyperlabile, *i.e.*, uncoated spontaneously (without receptor) at growth temperature unless prevented from doing so by the presence of drug in the pocket. Thus, site (III) seems to represent a kind of thermostat which regulates the temperature at which the uncoating transition (release of VP4 to form *A* particles) is triggered.

Introduction

Picornaviruses have 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 β-barrel (Rossmann *et al.*, 1985; Hogle, Chow & Filman, 1985). The amino-terminal half of the VP4 proteins cluster at the base of each fivefold axis, on the inner surface of the capsid, in contact with the viral RNA and with the amino termini of VP1 and VP3; the carboxyl-terminal portions approach the threefold axes. The VP1 proteins are contiguous around the fivefold axes of symmetry and some of the loops connecting the β-strands, especially the *B–C* loops, build star-shaped plateaux on the outside of the virion at the fivefold axes. Surrounding these plateaux are canyons 25 Å deep that are the sites for cellular receptor binding for most, if not all, human rhinoviruses (Colonno *et al.*, 1988; Olson *et al.*, 1993), and probably also for polioviruses. For many picornaviruses whose structures have been solved crystallographically (Hogle *et al.*, 1985; Filman *et al.*, 1989; Oliveira *et al.*, 1993), the space within the VP1 β-barrel is occupied by a hydrophobic ligand whose size is strain specific ('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 the next cell to be infected. 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 most human rhinoviruses (Greve *et al.*, 1989; Staunton *et al.*, 1989). The poliovirus receptor has been cloned and identified as a member of the immunoglobulin supergroup (Mendelsohn, Wimmer & Racaniello, 1989), but its function in the human host has not yet been identified. For these viruses, interaction with the cellular receptor initiates changes in the viral capsid that have been associated with the first step in viral uncoating (Everaert, Vrijsen & Boeyé, 1989; Fricks & Hogle, 1990; Lonberg-Holm, Gosser & Kauer, 1975; Kaplan, Freistadt & Racaniello, 1990). This involves release of VP4 with formation of the *A* particle, a non-infectious particle with a reduced sedimentation coefficient that is considerably more hydrophobic than the native virion, probably due to extrusion of the amino terminus of VP1 (DeSena & Mandell, 1977; Fricks & Hogle, 1990; Gomez Yafal, Kaplan, Racaniello & Hogle, 1993) (Fig. 1, panel *C*). Conversion of virions to *A* particles can take place on the surface of cells, and many of the altered particles elute from the cells (Joklik & Darnell, 1961). Altered particles are also found within endosomic vesicles of newly infected cells (Everaert *et al.*, 1989). After alteration to the *A* particle, virions still must deliver their RNAs across the plasma or endosomic membrane, and 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 (Fricks & Hogle, 1990; Moscufo, Gomez Yafal, Rogove, Hogle & Chow, 1993) (Fig. 1, *D*). It is this step which appears to be blocked in rhinovirus mutants defective in maturation cleavage (Lee, Monroe & Rueckert, 1993) and in certain poliovirus mutants with changes in VP4

(Moscufo *et al.*, 1993) or the extreme amino terminus of VP1 (Kirkegaard, 1990).

Little is known about what portions of the viral capsid are instrumental in these early stages of the viral infectious cycle. Our approach has been to map mutations in mutants resistant to drugs that block attachment or uncoating. We have studied several of the neutralizing antiviral drugs manufactured by ViroPharma Inc., Collegeville, PA, USA (Diana, McKinlay, Otto, Akullian & Oglesby, 1985). These drugs insert into the space within the VP1 β-barrel normally occupied by pocket factor (Badger *et al.*, 1988; Smith *et al.*, 1986). The capsid-binding drugs appear to act as analogs of pocket factor but have higher affinities for the viral pocket. When capsid-binding drugs replace the pocket factor within the VP1 β-barrel, they make it more rigid, stabilizing the virus against denaturation as a result of heat or extremes of pH (Caliguiri, McSharry & Lawrence, 1980; Fox, Otto & McKinlay, 1986; Gruenberger, Pevear, Diana, Keuchler & Blaas, 1991; Rombaut, Andries & Boeyé, 1991). This stabilization apparently also blocks the conversion to the *A* particle (Everaert *et al.*, 1989; Mosser & Rueckert, 1993).

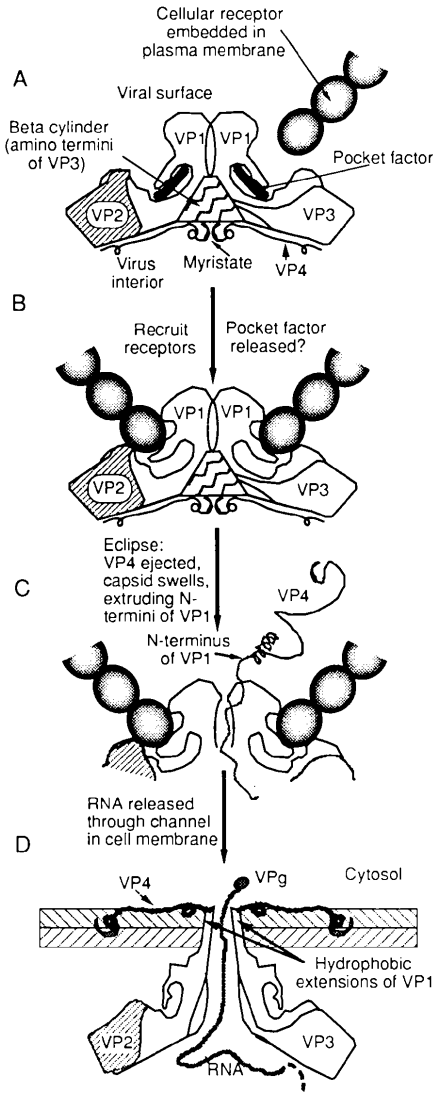


Fig. 1. Model showing how binding of a picornavirus to its receptor might mediate uncoating. This diagram shows single viral pentamers, with sections removed to reveal the relationship between receptor, canyon, drug-binding pocket and interior surface in contact with the genome. The outside of the virus is at the top of each figure. Panel *A*, we hypothesize that the cellular receptor makes contact with some part of the canyon. *B*, it is proposed that this contact changes when the pocket factor diffuses out, an event which may be promoted by receptor binding or by recruitment of additional receptors. *C*, conformational changes are propagated through the VP1 β-barrel and result in the expulsion of VP4 and the amino termini of VP1 from the particle. *D*, an artist's version depicting how VP4 and the amino termini of VP1 may form a channel through the cell membrane that allows the viral RNA to penetrate into the cytoplasm. Successful penetration of intact vRNA into the cytoplasm may be a rare event. Typically 100 or more virions are needed to establish a single infection.

The selection of naturally occurring drug-resistant mutants is relatively easy because of the high mutation rates in these viruses. Viruses with single-stranded RNA genomes typically incorporate an incorrect base per 10^4–10^5 bases (Holland *et al.*, 1982). Since there is no repair function for these genomes, such missense mutations go uncorrected. Many lead to non-viable mutations, but vigorous mutants resistant to neutralizing monoclonal antibodies or drug may be isolated with frequencies of 10^{-3} to 10^{-5}.

A study of human rhinovirus type 14 (HRV14) mutants resistant to several capsid-binding drugs (Heinz *et al.*, 1989) showed that single amino-acid substitutions were sufficient to confer drug resistance. Moreover, the location of these substitutions pinpointed important functional elements of the protein. For HRV14, insertion of the drug elevates the floor of the canyon and blocks cellular attachment (Pevear *et al.*, 1989; Shepard, Heinz & Rueckert, 1993). Drug-resistant mutants showed that the virus had two methods for circumventing the drug action. Some drug-resistant mutants ('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. Other mutants ('drug-compensation mutants') had amino-acid substitutions in the floor of the canyon which altered the interaction of the virus with its cellular receptor (Heinz, Shepard & Rueckert 1990; Shepard *et al.*, 1993) in such a way as to allow attachment and uncoating of mutants even in the presence of drug.

We initiated a study with the Sabin strain of poliovirus type 3 (P3/Sabin) using disoxaril (WIN51711) (McKinlay, 1985) because this drug selectively prevents uncoating, but not attachment for this virus (Fox *et al.*, 1986). We hoped, therefore, that drug-escape mutants would 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 to be drug dependent (Mosser & Rueckert, 1993). All 14 of these drug-dependent mutants produced equal yields 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 became extremely thermolabile without drug and decayed at 310 K to particles with all of the characteristics of the *A* particle. In other words, by warming to 310 K the mutants could be induced to undergo the first stage in uncoating spontaneously, even in the absence of the cellular receptor. Here we describe the amino-acid substitutions in the capsid proteins of these dependent mutants. We also report the positions of mutations in the remaining eight 'non-dependent' mutants, which were capable of forming plaques in either the presence or absence of drug.

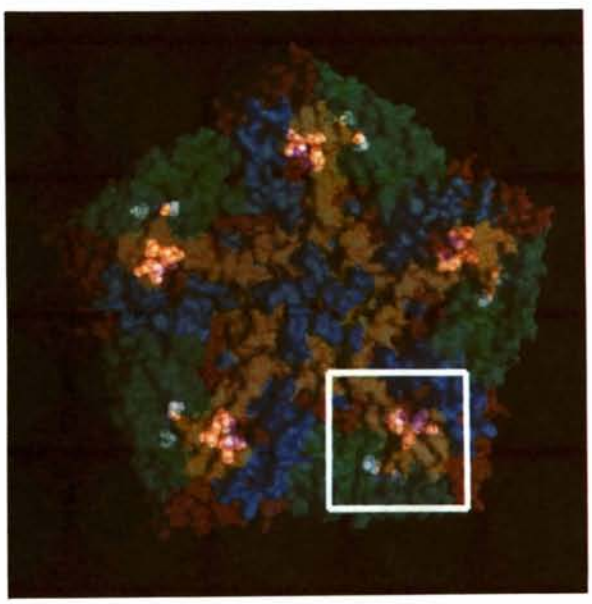
Results and discussion

Amino-acid substitutions in drug-dependent mutants

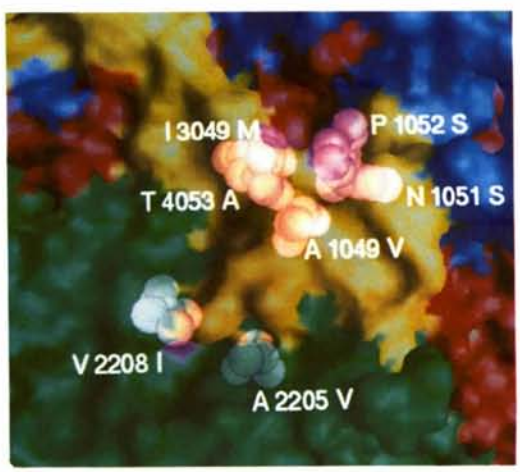
All 14 of our drug-dependent mutants shared a curious property: their infectivity was hyperlabile at their normal growth temperature, 310 K in the absence of drug. Moreover, this extreme thermolability was only manifested after the mutants were released from the cell, suggesting dissociation of a protective substance, perhaps pocket factor (Mosser & Rueckert, 1993). Stocks of these mutants were grown, virus was partially purified and the RNA was extracted. Viral RNA was sequenced directly using a set of 15–16 base DNA oligonucleotides as primers (Mosser, Sgro & Rueckert, 1994). Sequencing revealed seven relevant mutations (Table 1). Six of the mutants had substitutions in the amino terminus of VP1; two had substitutions in the βG strand of VP2, one had a substitution in the amino terminus of VP3 and five had identical mutations leading to the substitution of alanine for threonine 53 in VP4. 'Silent' base changes in the coat gene and the 5′ non-coding region were also identified for three of these mutants.

Although all four viral capsid proteins were sites of mutations, an examination of the three-dimensional structure showed that the mutations are clustered on or near the interior lining of the capsid, near the threefold axis of symmetry (Fig. 2). The amino-acid changes in VP1, VP3 and VP4 are tightly clustered within 4–8 Å of each other. The amino-acid substitutions in VP2 are located about 12–15 Å away from this cluster, but residue 2205 is within 6–7 Å of the VP4 chain (residue 4058).

Several other laboratories have demonstrated the importance of residues in the capsid interior to early events in the virus infection cycle. Some strains of poliovirus are capable of infecting mice, a property which has been localized to the amino-acid sequence in the *B–C* loop of VP1 (Martin *et al.*, 1988). However, mouse infectivity can be conferred on normally mouse-avirulent strains of poliovirus by amino-acid substitutions in the amino termini of VP1 and VP2 (Moss & Racaniello, 1991; Couderc, Hogle, LeBlay, Horaud & Blondel, 1993). These changes presumably allow virions to uncoat productively even when they interact less efficiently with the mouse cellular receptor. This work demonstrates a link between receptor binding and the conformational changes associated with uncoating. One of the amino-acid substitutions capable of conferring virulence for



(*a*)



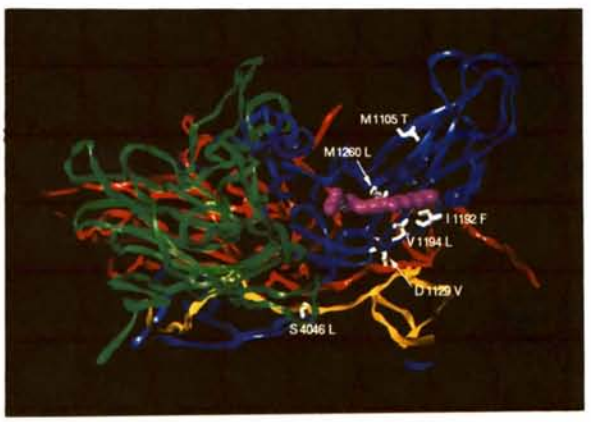
(*b*)

Fig. 2. (*a*) View of the interior surface of a pentamer of P3/Sabin from the inside. Area within the box is enlarged in (*b*). VP1 is shown in blue, VP2 in green, VP3 in red and VP4 in yellow. The residues showing amino-acid substitutions in the drug-dependent mutants are shown in pale yellow. Where these residues are exposed on the interior surface of the capsid, they are colored pink. This figure was produced using the program *GRASP*, using atomic coordinates supplied by J. Hogle.

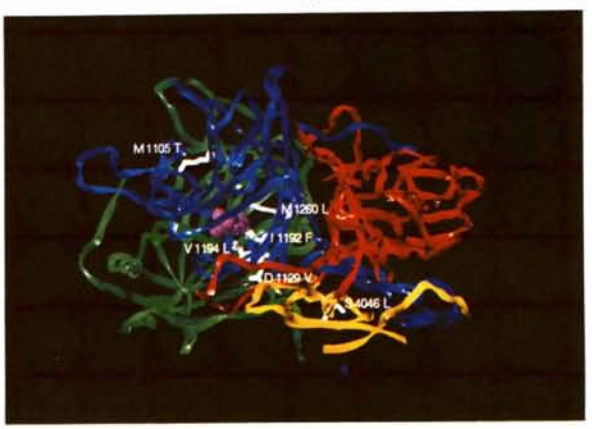
mice corresponds to the proline to serine substitution at 1052 in drug-dependent mutants 2, 10 and 19 (Table 1). The amino-acid substitutions we have identified in our drug-dependent mutants allow them to uncoat in the presence of drug by conferring instability that favors spontaneous transition to the *A* particle when the temperature is raised to 310 K (Mosser & Rueckert, 1993).

Non-dependent drug-resistant mutants

The remaining eight mutants were capable of making plaques in either the presence or absence of drug. Sequencing revealed (Table 2) that all but one of the mutations are in VP1. When these mutations were located in the viral structure, they fell into three loci (Figs. 3 & 4).



(a)



(b)

Fig. 3. Location of amino-acid substitutions in non-dependent drug-resistant mutants. (*a*) Ribbon diagram of a single protomer of P3/Sabin from the side. The top corresponds to the outside of the virion; the bottom is the interior. Color code as in Fig. 2. Pocket factor modeled as sphingosine is colored pink. Side chains of mutated residues are shown in white and labeled. (*b*) The view shown in Panel *A* has been rotated 90° around the *Z* axis. The observer is looking down the axis of the pocket factor from the viewpoint of the fivefold axis of symmetry. Note that the side chain of D1129 points away from the drug. This figure was prepared using the program *SYBYL* (Tripos Associates, 1992).

Table 1. *Positions of amino-acid substitutions in drug-dependent mutants*

Protein	Position*	Substitution	Mutants
VP1	1049	Ala→Val	3,4
VP1	1051	Asn→Ser	17
VP1	1052	Pro→Ser	2,10,19
VP2	2205	Ala→Val	20
VP2	2208	Val→Ile	7†
VP3	3049	Ile→Met	18
VP4	4053	Thr→Ala	8,13†,15,16,22†

* The first digit specifies the capsid protein; the last three digits give the amino-acid position. For example, 1049 indicates amino-acid residue 49 in VP1.

† These mutants also had silent mutations within the capsid-coding region of the genome. Mutant 13 had an A to G change at nucleotide 719 in the 5′ non-coding region as well.

Table 2. *Positions of amino-acid substitutions in non-drug-dependent mutants*

Protein	Position*	Substitution	Mutants	Comments
VP1	1105	Met→Thr	11,21	Outer surface, canyon wall
VP1	1129	Asp→Val	6,12	Near cavity surface
VP1	1192	Ile→Phe	14	Pocket lining
VP1	1194	Val→Leu	9	Pocket lining
VP1	1260	Met→Leu	5	Pocket lining
VP4	4046	Ser→Leu	1†	Surface of central cavity

* Positions are indicated as in Table 1.

† This mutant also had a silent mutation within the capsid-coding region of the genome.

Three mutants had substituted amino-acids whose side chains projected into the lining of the drug-binding pocket. The substitutions of Phe for Ile at 1192 or Leu for Val at 1194 involve the placement of bulkier amino acids into the pocket walls. The Met to Leu substitution at 1260 also involves an amino acid that points in toward the drug. The Mahoney strain of poliovirus, which is much less sensitive to this drug, has a leucine at the equivalent position. The mutants bearing these mutations were thermostabilized by drug (Mosser *et al.*, 1994), indicating that they were still capable of binding drug.

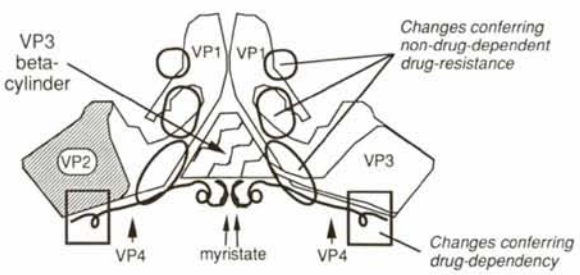


Fig. 4. Summary of locations of drug-resistance mutations. Schematic diagram of a side view of a single viral pentamer as in Fig. 1. The ovals mark regions where mutations were observed in drug-resistant but non-dependent mutants. The rectangular box marks the cluster of mutations observed in drug-dependent mutants.

Two of the non-dependent drug-resistant mutants substituted threonine for methionine at residue 1105. This amino acid lies in the lining of the canyon surrounding the fivefold axis of symmetry. This amino-acid substitution may affect the interaction between virion and cellular receptor in such a way as to promote uncoating.

The remaining two mutations conferring the non-dependent phenotype were in amino acids located on or near the inner surface of the protein capsid, but nearer to the fivefold axis of symmetry than the cluster of mutations conferring drug-dependence. One mutant substituted alanine for threonine 4046, which lies on the inner capsid surface. Two others have valine substituted for aspartic acid 1129. On the capsid lining, this residue is covered by a loop of VP4 contributed by an adjacent protomer. The side-chain O atoms of the wild-type aspartic acid 1129 are within hydrogen-bonding distances of amino acids in the two adjacent β-strands in the VP1 β-barrel. Therefore, the replacement of this charged amino acid with valine leads to the loss of hydrogen bonds and may cause alterations in the shape or flexibility of the VP1 β-barrel.

Concluding remarks

Of 22 drug-resistant mutants selected, a total of 13 different amino-acid substitutions were found, seven that produced drug dependence and six 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 is likely, therefore, that new mutations would be located if more drug-resistant mutants were selected.

The amino-acid substitutions in our mutants illustrate the flexibility of the viral structure in evading drug action. Three different functional regions of the capsid were involved in drug resistance (Fig. 4): the drug-binding pocket, the canyon surrounding the fivefold axes of symmetry and the inner surface of the protein capsid. Changes in the lining of the drug-binding pocket probably reduce the binding affinities for both drug and pocket factor, making it more likely that they would be released from virions bound to cells. These substitutions, therefore, allow the mutants to uncoat in the presence of drug, but also tend to destabilize the virus in the absence of drug because of reduced binding of pocket factor. We have demonstrated that these three mutants are less stable than the wild-type virus at 315 K (Mosser *et al.*, 1994).

The non-dependent mutants with an amino-acid substitution at M1105, at the base of the canyon wall, may have an altered interaction with the cellular receptor. This region of the capsid may play a role in triggering the conversion to the *A* particle after receptor binding.

Three of the non-dependent 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 clear to us whether the mutations conferring drug-dependence form a unique functional site distinct from those associated with the non-dependent mutants; both groups of mutations point to a region that must be involved in the conformational changes that take place during uncoating.

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