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Poliovirus 2C Region Functions during Encapsidation of Viral RNA

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We have been exploring the mechanism of action of 5-(3,4-dichlorophenyl) methylhydantoin (hydantoin), an antiviral drug that inhibits the replication of poliovirus in culture. By varying the time of drug addition to infected cells, we found that the drug acts at a stage which is late in the replication cycle and subsequent to the step inhibited by guanidine. Furthermore, we detected normal levels of full-length plus-strand virion RNA in hydantoin-treated cultures. A new assembly intermediate in addition to the expected assembly intermediates was detected in drug-treated cultures. This intermediate has properties consistent with that of a packaging intermediate. Drug-resistant mutants were readily isolated. Sequence analysis of three independent drug-resistant mutants identified amino acid substitutions in the 2C coding region. Reconstruction by site-directed mutagenesis confirmed that these single mutations were sufficient to confer drug resistance. Taken together, these data suggest that the poliovirus 2C region is involved in virus encapsidation and that hydantoin inhibits this stage of replication.

Poliovirus (PV) is the prototypical member of the picornavirus family. These RNA viruses contain a single-stranded positive-sense genome. The 7,400-base RNA genome is translated into a single polypeptide which is processed by two virally encoded proteases to yield all known virus-associated structural and nonstructural proteins. The nonstructural proteins are located in the C-terminal two-thirds of the genome and are divided into two roughly equal segments based on the initial proteolytic cleavage. The C-terminal third of the polyprotein, the P3 region, includes the RNA-dependent RNA polymerase ($3D^{pol}$) and the major viral protease ($3C^{pro}$) as well as the genome-linked protein (VPg or 3B) and a hydrophobic protein of poorly defined function (3A). The P2 region contains the other viral protease (2A) and two proteins involved in RNA replication, 2B and 2C.

Studies of the mechanism of action of antiviral drugs have been invaluable in defining various stages of the picornavirus life cycle and the functions of several viral proteins. For example, studies using guanidine-HCl have aided in identifying the role of protein 2C in the replication of viral RNA (5, 29, 30). In addition, capsid-binding inhibitors such as arildone and the WIN compounds were valuable tools for exploring the modes of receptor attachment and uncoating of viral RNA (7, 34, 35). More recently, studies using the inhibitor enviroxime are under way in hopes of providing insights into the function of protein 3AB (17, 18).

In this study, we have explored the mechanism of action of a novel antiviral compound, 5-(3,4-dichlorophenyl) methylhydantoin (referred to here as hydantoin). This compound (Fig. 1) was previously shown to have activity against coxsackievirus A21 (CA21) infection in cell culture and very promising activity in a mouse model (13). Because the drug seemed to have an unusual mode of action, we chose to investigate it more closely. Analyses of hydantoin-induced inhibition of PV serotype 1 (PV1) infection indicates that the target of this antiviral is the encapsidation of viral RNA. We have further determined that the resistance phenotype maps to the 2C coding region of the viral genome. These findings suggest that either the 2C protein or the 2C coding region is directly involved in the encapsidation of viral RNA.

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MATERIALS AND METHODS

Cells and virus stocks. The H-1 HeLa cell line, a gift from R. Rueckert of the University of Wisconsin—Madison, was originally obtained from V. Hamparian, Ohio State University. Cells were grown in suspension and monolayer culture as previously described (17). With the exception of human rhinovirus 14 (RV14) and CA21, all viruses tested were obtained from the American Type Culture Collection. These included PV1 (strain Mahoney), PV2 (strain Lansing), PV3 (strain Leon), coxsackievirus B3 (CB3; strain Nancy), and RV16 (strain 11757). RV14 (strain 1059) was a gift from R. Rueckert. CA21 was a Coe strain variant adapted for efficient growth in mice (40). The production of virus stocks and quantification by plaque assay were carried out as previously described (17). All plaque assays were conducted in the absence of drug unless otherwise noted.

Drug solutions. Hydantoin was dissolved in dimethylsulfoxide (DMSO) at 2,000× concentration and diluted 1/2,000 in culture medium (final DMSO concentration, 0.05%). Guanidine-HCl (Boehringer Mannheim, Indianapolis, Ind.) was dissolved in water at 500× concentration and diluted 1/500 in culture medium. Unless specified, standard final concentrations for all studies were 50 μg/ml for hydantoin and 200 μg/ml for guanidine-HCl. Although DMSO had no discernible effect on virus replication, 0.05% DMSO was added to all drug-free and guanidine-containing solutions.

Spectrum of activity. The effects of 25, 50, and 100 μg of hydantoin per ml were tested against seven representative RV and enterovirus serotypes in single-cycle infections. After inoculation at a multiplicity of infection (MOI) of 1 in the absence of drug, samples were incubated in drug-containing or drug-free medium for 6 h (PV1, PV3, and CB3) or 7 h (PV2 and CA21) at 37°C or for 8 h at 35°C (RV14 and RV16). Virus yields were determined by plaque assay.

Time-of-addition assay. Hydantoin (50 μg/ml) was added to PV1-infected cultures (MOI of 1) at specified times following adsorption. Virus yields were harvested at 6 h postinfection (p.i.) and quantified by plaque assay.

Reversibility of inhibition and comparison of hydantoin activity with guanidine-HCl activity. To determine whether the effects of hydantoin were readily reversible, hydantoin (50 μg/ml) or guanidine-HCl (200 μg/ml) was added to PV1-infected cultures (MOI of 1) immediately after adsorption. At 4 h p.i., cultures were rinsed three times with cold phosphate-buffered saline (PBS; Gibco/BRL, Gaithersburg, Md.), and the medium was replaced with either drug-free or fresh drug-containing medium. To determine whether hydantoin blocks a later step in the life cycle than guanidine, infections were carried out as

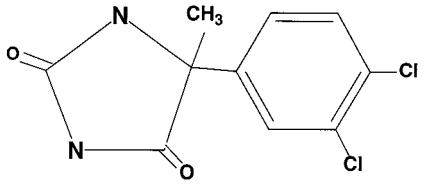

FIG. 1. Structure of 5-(3,4-dichlorophenyl) methylhydantoin.

described for the reversal of inhibition except that instead of receiving drug-free medium, hydantoin-treated cultures received medium containing guanidine and vice versa. Virus was harvested at 8.5 h p.i., and yields were determined by plaque assay.

Positive-strand RNA synthesis. Hydantoin (50 μg/ml) or guanidine-HCl (200 μg/ml) was added to PV1-infected cultures (MOI of 1) immediately after virus adsorption. At 6 h p.i., total cellular RNA was extracted by standard methods (32). Slot blots were prepared by using 10 μg of RNA sample as previously described (17). Another 10 μg of total RNA was subjected to denaturing gel electrophoresis and analyzed on a Northern blot (33), using 1 μg of virion RNA as a size marker. Blots were hybridized with a ^{32}P-labeled strand-specific oligonucleotide probe at 42°C overnight (17). Individual slots were quantified by liquid scintillation counting. The Northern blot was exposed to X-ray film to determine whether the RNA detected was full length.

To examine the inhibitory effect of hydantoin on the linkage of VPg to the RNA, we conducted in vitro uridylylation assays on isolated crude replication complexes of PV1 formed at 5 h p.i. in the presence of 50 μg of compound per ml. The reaction was conducted and analyzed by using procedures previously reported (17).

Analysis of assembly intermediates by sucrose gradients. HeLa cells in suspension (2×10^8 cells) were infected with PV1 at an MOI of 10 in methionine-free or phosphate-free medium (27). At 1 h p.i., hydantoin in DMSO (50 μg/ml, final concentration) or DMSO alone (0.1%, final concentration) was added to one of two duplicate cultures. [^{35}S]methionine (10 μCi/ml, final concentration; specific activity, 1,000 Ci/mmol) or ^{32}P-inorganic phosphate (50 μCi/ml; carrier free) was added to each culture at 3 h p.i. Infections were harvested at 5 or 6 h p.i. Cells (10^8 cells/ml, final concentration) were lysed at 4°C in TNM buffer (10 mM Tris-HCl [pH 7.5], 10 mM NaCl, 1.5 mM $MgCl_2$) supplemented with 0.1% Tween and centrifuged at $900 \times g$ to remove nuclei. Equal cell volumes of supernatants were loaded onto either 6 to 25% (wt/vol) or 15 to 30% (wt/vol) linear sucrose gradients (26). Gradients were centrifuged at 39,000 rpm in a Beckman SW40 rotor for 16 h (6 to 25% gradients) or 2.5 h (15 to 30% gradients). Gradients were fractionated in 300-μl aliquots from the top, and the radioactivity present in each fraction was quantified by liquid scintillation or Cerenkov counting. Samples from peak fractions of [^{35}S]methionine-labeled samples were analyzed on sodium dodecyl sulfate (SDS)–polyacrylamide gels (27).

For the modified label-chase analysis, infected HeLa cell cultures (3×10^8 cells) were treated with hydantoin or DMSO at 1 h p.i. and labeled with [^{35}S]methionine at 3 h p.i. as described above. At 6 h p.i., one-third of the drug-treated and control cultures (10^8 cells) was harvested. The cells in the remainder of the cultures were pelleted, washed with PBS, resuspended in complete minimal essential medium containing standard concentrations of unlabeled methionine, and incubated at 37°C. Cells (10^8) were harvested at 7 and 8 h p.i. and then lysed and analyzed on sucrose gradients as described above.

Selection, mapping, and confirmation of resistant mutants. Mutants of PV1 resistant to 50 μg of hydantoin per ml were isolated as previously described (16). Virion RNA was prepared, and selected regions of the genome encoding structural and nonstructural proteins were sequenced (17) to identify mutations indicating amino acid substitutions. These mutations were constructed into an infectious PV1 cDNA clone by using standard oligonucleotide-directed mutagenesis methods and propagated as described previously (17). Drug resistances of the spontaneous and cDNA-derived mutants were compared by using single-cycle infections. Cultures were infected at an MOI of 1 and incubated for 6 h in medium containing 50 μg of hydantoin per ml or drug-free control medium. Virus yields were quantified by plaque assay.

RESULTS

Spectrum of inhibition of picornaviral infections. Activity against several representative picornaviruses during a single cycle of infection was measured by comparing the ratio of virus titers from drug-treated and mock-treated cells (Table 1). At 50 or 100 μg/ml, the compound was equally active against PV1, PV3, and CA24. PV2 was slightly less sensitive to the compound, whereas the RVs and CB3 showed little or no sensitivity. The degree of inhibition was independent of whether the virus harvest took place in the presence or absence of drug (data not shown). This finding established that the effects of hydantoin occurred during intracellular stages of viral replication and excluded the possibility that the antiviral activity of the drug resulted from an extracellular effect (such as increasing the capsid stability of assembled particles after release). The effects of hydantoin (50 μg/ml) on PV1 infection were analyzed further to characterize its mechanism of action.

TABLE 1. Inhibitory effects of hydantoin on representative picornaviruses

Virus	Relative resistance (% survivors)[a]		
	25 μg/ml	50 μg/ml	100 μg/ml
PV1	3	0.1	0.1
PV2	6	3	0.8
PV3	0.2	0.1	0.1
CA21	0.2	0.2	0.2
CB3	65	80	78
RV14	83	51	14
RV16	101	107	23

[a] Virus yields in PFU were determined following single-cycle infections in monolayer cultures at an MOI of 1. Numbers are relative to no-drug controls.

Stage of antiviral activity. To determine the approximate stage within the life cycle that was inhibited by drug, hydantoin was added to infected cultures at various times p.i., and the percent inhibition relative to no-drug controls was measured (Fig. 2). Inhibition remained near 100% when hydantoin was added as late as 4 h p.i. and had dropped only 15% when hydantoin was added at 5 h p.i. This finding indicates that the site of action is at a late stage of viral replication.

Reversal of inhibition and comparison of hydantoin activity with guanidine-HCl. We also compared hydantoin with a known inhibitor of PV RNA replication, guanidine-HCl (6). We examined whether the effects of hydantoin, like those of guanidine-HCl, were reversible in culture. Midway through the infectious cycle, we rinsed the cells with buffer and concluded the infection in drug-free medium. The activities of both hydantoin and guanidine-HCl were readily reversible under these conditions (Fig. 3). To determine whether one block preceded the other, guanidine-HCl and hydantoin were added in different sequential order. If hydantoin was added first, little antiviral effect remained upon removal of hydantoin and addition

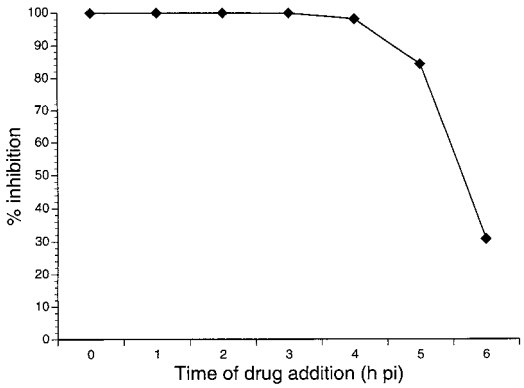

FIG. 2. Inhibitory effect of hydantoin added at hourly intervals to infected cells. Cells infected with PV1 (MOI of 1) were incubated in medium to which 50 μg of hydantoin per ml or 0.05% DMSO alone was added at the indicated times. Virus yields were harvested at 6 h p.i.; titers were determined by plaque assay. Values shown are relative to no-drug controls at each time point.

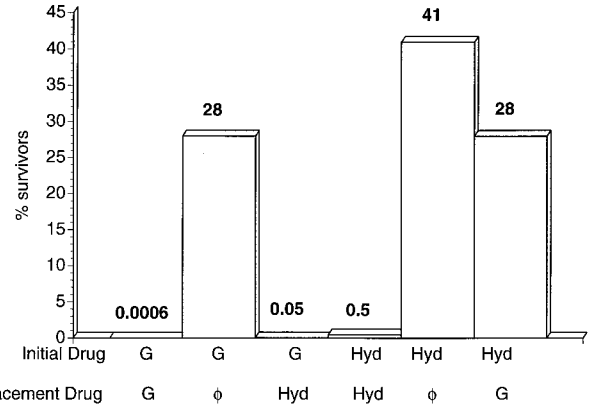

FIG. 3. Reversal of inhibition and comparison of hydantoin activity with guanidine-HCl. PV1-infected cells (MOI of 1) were incubated in medium containing guanidine-HCl (200 μg/ml) or hydantoin (50 μg/ml) for 4 h at 37°C. Cultures were rinsed three times with cold PBS, and the media were replaced as indicated. Virus yields were harvested at 8.5 h p.i. and quantified by plaque assay. Values are relative to no-drug controls. G, guanidine-HCl; ϕ, DMSO control; Hyd, hydantoin.

of guanidine-HCl (Fig. 3). Thus, the function blocked by guanidine-HCl (namely, virion RNA replication) was completed in the presence of the initial hydantoin block. In contrast, when guanidine-HCl is added first, the antiviral effect remained after the guanidine-HCl is replaced with hydantoin. These data indicate that hydantoin acts at a stage later than that inhibited by guanidine-HCl.

Positive-strand RNA synthesis. Within infected cells, RNA synthesis is normally heavily biased toward synthesis of positive-strand RNAs (both mRNA and genomic RNA). To determine whether drug inhibition resulted from decreased synthesis of positive-strand RNA molecules, we analyzed positive-strand RNA production in infections treated with either hydantoin or guanidine-HCl. The hydantoin-treated sample was indistinguishable from the untreated control (Fig. 4). In contrast, guanidine-treated cultures showed nearly complete inhibition of positive-strand RNA production, as expected from previous studies (6). Northern blot analysis of these same samples showed that the RNA produced was full length (Fig. 4, inset). Consistent with this finding, hydantoin treatment had no observable effect on protein synthesis (see below).

To examine the inhibitory effect of hydantoin on the linkage of VPg to the RNA, we conducted in vitro uridylylation assays on isolated crude replication complexes of PV1. In uridylylation assays, the smallest band (approximately 3 kDa) corresponds to VPg covalently linked to one or two uridines; this represents the initiation step of RNA synthesis (36). The heavy smear of higher-molecular-weight RNA represents varying lengths of single and duplex RNAs and the partially double-stranded replicative intermediate forms covalently linked to VPg. The uridylylation reaction was unaffected by the presence of hydantoin (Fig. 5); that is, the pattern of bands observed was identical to that for the untreated control.

Analysis of assembly intermediates by sucrose gradients. Sucrose gradient sedimentation analysis was carried out to determine whether the production of capsid assembly intermediates was altered by the addition of hydantoin (Fig. 6). Consistent with the inhibition of viral titers, few or no mature virions (sedimenting at 150S) were observed in continuously labeled, drug-treated cultures harvested at either 5 or 6 h p.i. This is in contrast to the pronounced peak of mature virions that was observed in the control untreated cultures. There

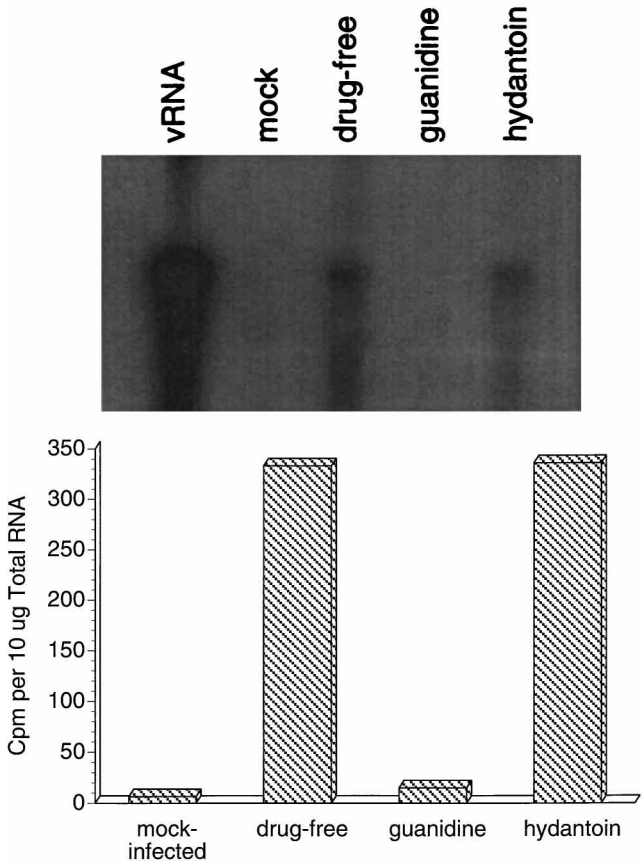

FIG. 4. Positive-strand RNA synthesis in the presence of hydantoin. Cells were infected with PV1 at an MOI of 1, and medium containing hydantoin (50 μg/ml), guanidine-HCl (200 μg/ml), or DMSO alone was added. At 6 h p.i., total cellular RNA was harvested and subjected to slot blot and Northern analysis. Individual slots were quantified by liquid scintillation counting. Background radioactivity (generally about 75 cpm) was subtracted from the values shown. Autoradiography of Northern analysis is shown in the inset. vRNA, virion RNA.

were no apparent differences in the assembly intermediates found in PV-infected drug-treated and control cultures at 5 h p.i. (Fig. 6a and b). However at 6 h p.i., a new peak (105 to 110S) was observed in the drug-treated cultures which sedimented between the 75S empty capsid and the 150S region of the mature virion (Fig. 6d). In addition, accumulation of the 14S pentamer and 75S empty capsid intermediates was consistently observed in the drug-treated cultures at 6 h p.i. (Fig. 6c and d). The accumulation of 14S pentamer and 75S empty capsid intermediates suggested that hydantoin acted by inhibiting viral encapsidation and that the approximately 110S peak might be an encapsidation intermediate.

During assembly of the PV particle, the monomeric assembly-active subunit is a 5S heterotrimeric complex comprised of one copy each of capsid proteins VP1, VP3, and VP0. VP0 is the precursor protein of VP2 and VP4. Five monomers are assembled to form the 14S pentamer intermediate, an obligate intermediate during the assembly process. The empty capsid is formed subsequently by assembling 12 pentamers. Although it remains unknown whether packaging occurs via nucleation of 14S pentamers around the RNA genome or via threading the RNA genome into the empty capsid, cleavage of VP0 to VP2 and VP4 occurs only after RNA encapsidation (11, 12, 20) and is required for viral infectivity (1). This cleavage allows the occurrence of conformational rearrangements that are associ-

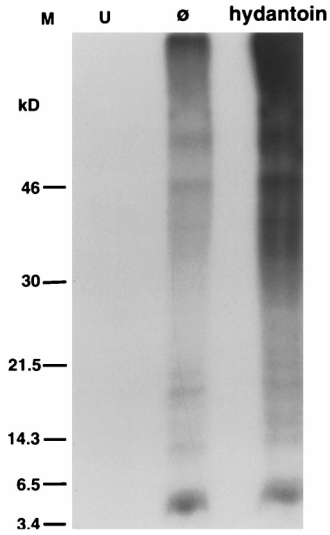

FIG. 5. Uridylylation assay showing the effect of hydantoin on the initiation and elongation of plus-strand RNA. HeLa cell suspensions were infected at an MOI of 1 and incubated in medium containing DMSO alone (ϕ) or 50 μg of hydantoin per ml for 5 h. Uridylylation reactions were performed on harvested crude replication complexes by incubation for 3 h at 30°C with creatine phosphate and creatine phosphate kinase supplemented with [^{32}P]UTP, and the products were immunoprecipitated with anti-3B antiserum and electrophoresed on SDS–12% polyacrylamide gels.

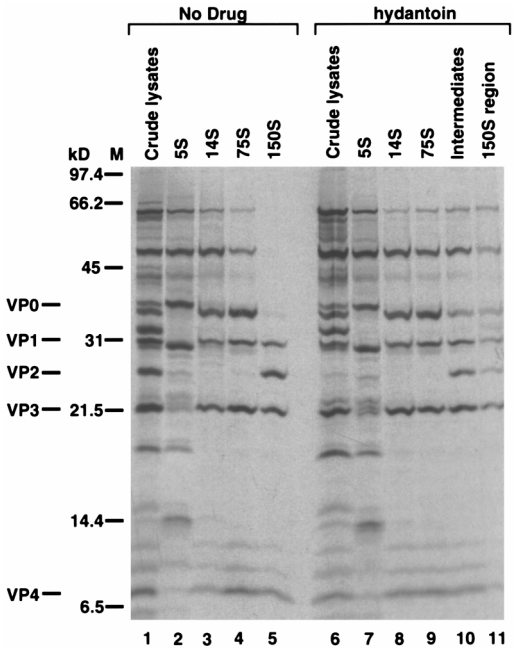

FIG. 7. Protein composition of assembly intermediates. Aliquots from the crude cell lysates (lanes 1 and 6) and peak fractions corresponding to 5S (lanes 2 and 7), 14S (lanes 3 and 8), 75S (lanes 4 and 9), and 110S (lane 10) intermediates and 150S mature virion (lane 5) of control (lanes 1 to 5) and hydantoin-treated (lanes 6 to 11) samples were analyzed by SDS-polyacrylamide gel electrophoresis. Lane 11 corresponds to the 150S region of the gradient of the drug-treated samples. M, molecular size standards.

ated with maturation of the virion and generation of the mature infectious 150S virion (3).

Samples from each of the peak fractions were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 7). As expected, VP0, VP1, and VP3 are the major protein species in the 14S and 75S fractions of both drug-treated and control samples and are present in roughly equimolar concentrations (Fig. 7, lanes 3, 4, 7, and 8). In addition, VP0 was largely cleaved to VP2 and VP4 in the mature infectious 150S virion (Fig. 7, lane 5). In contrast, the new 110S peak from the hydantoin-treated samples contained visible quantities of both VP0 and VP2, indicating that partial cleavage of VP0 had occurred (Fig. 7, lane 10). Because cleavage of VP0 occurs only after RNA encapsidation (11, 12, 15), this result suggests that the 110S peak reflects the accumulation of an RNA-containing intermediate and that hydantoin is interfering with viral encapsidation and maturation. This inference was additionally supported by the observation that label was associated with the 110S region

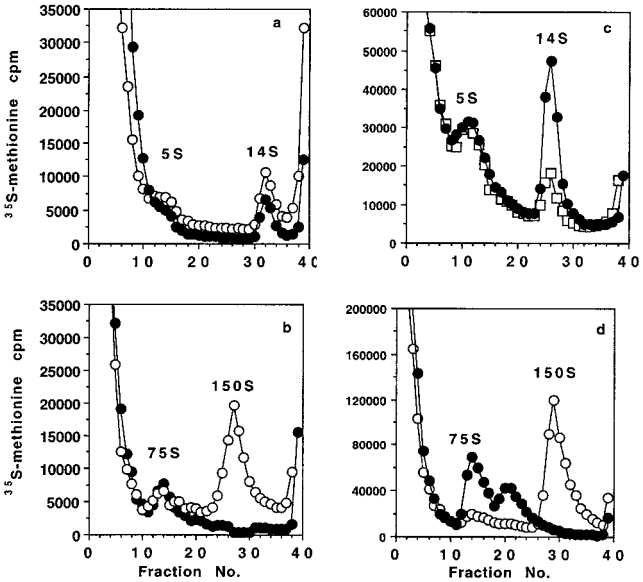

FIG. 6. Sucrose gradient analysis of hydantoin-treated PV-infected cells. Virus-infected cells were harvested at 5 h (a and b) and 6 h (c and d) p.i. from hydantoin-treated (●) and control (○) cultures. Lysates were loaded onto 6 to 25% (a and c), or 15 to 30% (b and d) sucrose gradients. Gradients were fractionated and counted.

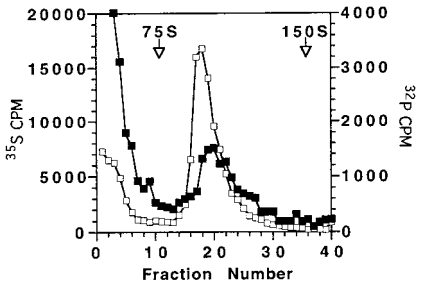

FIG. 8. ^{32}P label is associated with the hydantoin-induced 110S peak. Duplicate cultures of infected cells were incubated with hydantoin at 1 h p.i. and then labeled with either [^{35}S]methionine (open squares) or ^{32}P-inorganic phosphate (closed squares). Lysates from cells harvested at 6 h p.i. were sedimented through a 15 to 30% sucrose gradient. Because high background levels of unincorporated ^{32}P were present throughout this gradient, fractions corresponding to the 110S peak were pooled and rerun on another 15 to 30% sucrose gradient. Samples were fractionated, and the radioactivity in each fraction was determined by Cerenkov radiation (^{32}P) or by liquid scintillation (^{35}S). The arrows indicate the positions of 75S empty capsids and 150S mature virions that were run as markers in the second gradients. Sedimentation occurs from left to right.

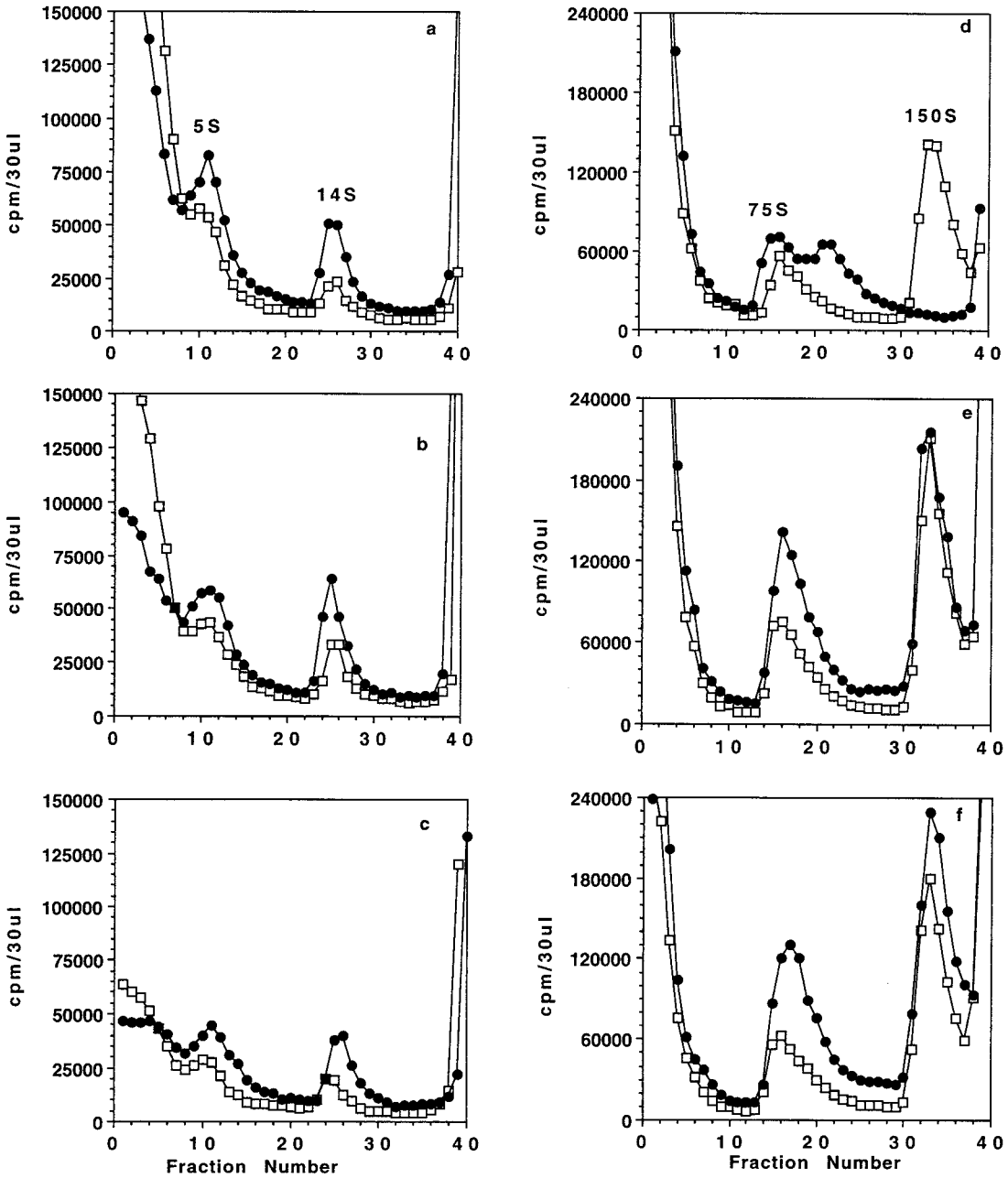

FIG. 9. Fate of 110S intermediate after release from hydantoin inhibition. Virus-infected cells were treated with hydantoin (●) or DMSO (○) at 1 h p.i. and labeled with [^{35}S]methionine at 3 h p.i. Samples were harvested at 6 h p.i. (panels a and d). The remaining cultures were released from hydantoin inhibition and cultured for an additional 2 h in the presence of unlabeled methionine. Additional samples were harvested at 1 h (b and e) and 2 h (c and f) after release. The cell lysates were analyzed for the 5S monomer and 14S pentamer assembly intermediates on 6 to 25% sucrose gradients (a to c) and for the 75S empty particle, 110S intermediate, and 150S mature virion on 15 to 30% sucrose gradients (d to f).

when hydantoin-treated, infected cells were labeled with ^{32}P-inorganic phosphate (Fig. 8).

The antiviral effect was reversible when hydantoin was removed from the media. Thus, the resultant infectious virus could be due to de novo assembly of new virus which occurs after drug removal or to subsequent maturation of virus from the 110S intermediate which accumulated during drug treatment. To address this question, we determined the fate of the 110S intermediate after drug removal. Samples were labeled from 3 to 6 h p.i. in the presence of hydantoin to accumulate detectable amounts of 110S intermediate. The drug was removed, and cells were further incubated in nonradioactive media. Consistent with the reversibility of hydantoin's antiviral effects, assembly of mature virus sedimenting at 150S was observed in infected cells after release from drug inhibition (Fig. 9). The 110S intermediate decreased over time as the mature 150S virus peak increased (Fig. 9d to f). Appearance of the 150S peak clearly included material derived from the 5S and 14S peaks. However, VP2 protein was observed only in the 110S peak of blocked samples and 150S of released samples and no other part of the gradient, suggesting that formation of 150S particles included material from the 110S peak also.

TABLE 2. Resistance to hydantoin at 50 μg/ml

Virus	2C mutation	Resistance (% survivors)[a]	
		Spontaneous	cDNA
Parent	None	0.16	0.06
Mutant 1	Q65/R	100	ND
Mutant 3	L125/V	64	25
Mutant 6	V218/I	20	44

[a] Virus yields in PFU were determined following single-cycle infections in monolayer cultures at an MOI of 1. Numbers are relative to no-drug controls. ND, not determined.

These data suggested that the 110S intermediate was a normal intermediate along the encapsidation pathway that accumulated during drug exposure rather than the result of a dead-end assembly pathway induced by drug.

Selection, mapping, and confirmation of mutants resistant to hydantoin. Mutants resistant to 50 μg of hydantoin per ml were isolated at a frequency of about 10^{-3}, a frequency which predicted that resistance could result from single amino acid changes (19). Selected regions of the genomes of three mutants were sequenced. Each mutant was found to contain a different single amino acid change in the 2C coding region: Q65/R, L125/V, or V218/I. Two of these mutations were constructed into the wild-type PV1 cDNA, and the resistance of the cDNA-derived viruses was compared to that of the spontaneous isolates. In both cases, the resistance levels of the spontaneous and cDNA-derived mutants were the same (Table 2), indicating that a single nucleotide substitution was sufficient to confer resistance to hydantoin.

DISCUSSION

In this report, we have presented evidence that a new assembly intermediate is produced in the presence of hydantoin. This intermediate has characteristics consistent with those predicted for an encapsidation intermediate: (i) the intermediate appears at late times of infection; (ii) it has a sedimentation coefficient between those of the empty capsid and the fully encapsidated mature virion; (iii) VP0 is partially cleaved, indicating the presence of RNA, and (iv) during label-chase experiments, the 110S peak is initially labeled; upon drug release, label decreases in the 110S peak and increases in the 150S peak (the only other VP2-containing peak), suggesting a precursor-product relationship. Thus, hydantoin appears to inhibit viral encapsidation and maturation. Analysis of RNA synthesis in the presence of the drug revealed no effect on the amount of plus-strand viral RNA made or on the size of the RNA product. These data also argue for a direct effect on the packaging of virion RNA. We have also determined that mutants conferring resistance to hydantoin map to the 2C coding region of the PV1 genome, implying a role for 2C in this late stage of viral replication.

Numerous studies of PV replication have produced evidence suggesting that the PV 2C protein is directly involved in RNA replication. Viruses containing site-specific mutations in the 2C protein show defects in viral RNA synthesis (22). In addition, mutants resistant to or dependent on guanidine-HCl, a compound that inhibits RNA synthesis and causes the accumulation of empty capsids (6, 8, 20), contain point mutations in the 2C coding region (2, 21, 29, 30). In a recent publication, it was shown that mutations in the 2C coding region of PV could compensate for a block in RNA production induced by growth at 25°C (10). Consistent with its involvement in RNA synthesis, sequence analysis of picornaviral 2C proteins has identified a nucleotide triphosphate-binding motif in these proteins (9, 14). Genetic analysis has revealed that mutations in these conserved regions cause defects in viral RNA synthesis (24, 38). Recent biochemical data have confirmed that the 2C protein possesses ATPase and GTPase activities (25, 31, 39). Finally, biochemical and ultrastructural data show that proteins of the P2 region are associated with intracellular membranes and with the viral RNA in the membrane-associated replication complex (4, 5, 28, 37).

In contrast to the body of data linking 2C to replication, there has been only one previous report suggesting a role for 2C in assembly or encapsidation. While studying a temperature-sensitive virus that contained an insertion in 2C, Li and Baltimore (23) isolated a revertant that contained two additional amino acid substitutions in 2C. This revertant virus proved to be cold sensitive for uncoating, implying that 2C is involved in determining virion structure.

Mutants highly resistant to hydantoin were easily isolated.

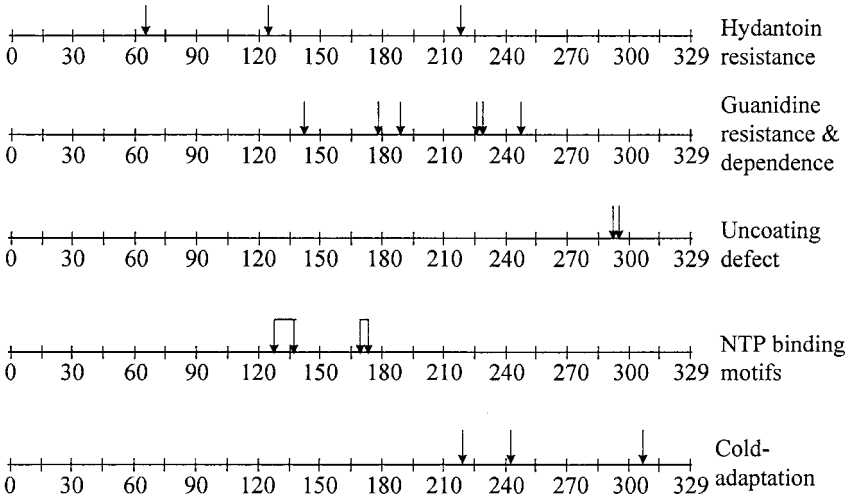

FIG. 10. Locations of mutations conferring resistance to hydantoin relative to known motifs in the 2C region (numbering refers to amino acid positions). Line 1, sites to which hydantoin resistance maps; line 2, sites to which guanidine resistance and dependence map (21, 29); line 3, sites to which an uncoating defect was mapped (23); line 4, nucleoside triphosphate (NTP)-binding motifs (24); line 5, sites to which cold adaptation maps (10).

Sequence analysis and genetic reconstruction of these mutants demonstrated that single nucleotide substitutions in the 2C coding region are sufficient to confer the resistance phenotype (mutants were at least 400 times more drug resistant than wild-type virus). The mutations were distributed throughout the 2C coding region, suggesting no obvious relationship between them and other known replication motifs (2, 9, 14, 21, 29, 30) or uncoating mutations (23) (Fig. 10).

Taken together, our data support a role for 2C in the assembly of mature virions. Perhaps 2C affects the association of capsid precursors with each other or with the RNA to facilitate the encapsidation of the RNA. These observations are consistent with a model in which protein 2C is involved in the release of the viral RNA from the replication complex and/or association of the RNA with the waiting capsid precursors (5). Further studies will be required to determine the exact mechanism of action and to exclude the possibility that hydantoin inhibits encapsidation by altering the RNA structure or sequence within the 2C coding region. Though no specific RNA secondary structure has been suggested to exist in this region, it is possible that encapsidation signals or required binding sites for capsid precursors are located in the 2C coding region. It is interesting that one of the residues identified here as having a role in encapsidation (V218/I) is identical to one of the mutations found to overcome the block to RNA replication at 25°C (Fig. 10) (10). This finding suggests that some residues of 2C may contribute to more than one functionally distinct domain. The specific inhibition demonstrated by the hydantoin described here may prove to be a useful tool for further study of the role of 2C during virus assembly and for establishing whether viral encapsidation is coupled to genomic RNA synthesis.

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