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WIN 52035-2 Inhibits both Attachment and Eclipse of Human Rhinovirus 14

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WIN compounds inhibit attachment of human rhinovirus 14 by binding to a hydrophobic pocket within the capsid and inducing conformational changes in the canyon floor, the region that binds the cellular receptor. To study the basis of drug resistance, we isolated and characterized a family of human rhinovirus 14 mutants resistant to WIN 52035-2. Thermostabilization data and single-cycle growth curves provided evidence for two classes of resistant mutants. One class, here called exclusion mutants, showed a marked decrease in drug-binding affinity and was characterized by substitution to bulkier amino acid side chains at two sites lining the hydrophobic pocket. The other class, called compensation mutants, displayed single-amino-acid substitutions in the drug-deformable regions of the canyon; these mutants were able to attach to cells despite the presence of bound drug. A delay in the rise period of the growth curves of compensation mutants indicated a second locus of drug action. WIN 52035-2 was found to inhibit the first step of uncoating, release of VP4. Attempts to identify this site of drug action by using single-step growth curves were obscured by abortive elution of a major fraction of cell-attached virus. The drug had no effect on the rate of this process but did affect the spectrum of particles produced.

WIN compounds, which neutralize picornaviruses by binding reversibly to the protein coat, are a second generation of antiviral agents derived from compounds such as arildone (23) and rhodanine (8). They block the uncoating of poliovirus type 1 (37), type 2 (11, 37), and type 3 (25) and of human rhinovirus 1A (HRV1A [16]) and HRV2 (11).

The location of the drug-binding pocket was first identified by x-ray crystallographic studies (35) of HRV14 (Fig. 1A). For this virus, however, studies with membrane receptor preparations (29) or intact HeLa cells (14) showed that the primary effect of drug was to block attachment, apparently by deforming regions of the canyon floor (3, 29) lying just above the drug-binding pocket (Fig. 1B).

Drug-resistant variants may be selected with a frequency on the order of 10^{-4} to 10^{-5} from picornaviruses grown in the presence of WIN compounds (13). In HRV14, resistance could be traced to single-amino-acid substitutions that were assigned to two groups, suggesting two mechanisms of resistance (13). One group of resistance mutations mapped to residues Cys-1199 and Val-1188, amino acids whose side chains project into the drug-binding pocket (squares in Fig. 1B). These two residues were always replaced by amino acids with bulkier side chains, suggesting that they might exclude the WIN compound from the drug-binding pocket (13). This interpretation is supported by crystallographic analysis of the Cys-1199→Tyr mutant, which confirms that the bulkier tyrosine residue decreases the space available within the pocket and may actually block drug entry into the pocket (2). Studies reported here support this mechanism of resistance by drug exclusion.

The second group of resistance mutations mapped to the drug-deformable region of the canyon floor (circles in Fig. 1B). We show here that these mutations allowed the virus to alleviate the attachment-inhibitory effect of the drug by compensating for the deforming effect of bound drug. Studies on the growth curve of one such compensation mutant revealed a second effect of the drug, inhibition of uncoating. We demonstrate that WIN 52035-2 blocks the release of VP4 (eclipse), the first step of a two-step process leading to release of the RNA genome.

MATERIALS AND METHODS

Cells, media, and plaque assay conditions. A cloned H-1 HeLa cell line was cultured in suspension in medium B or as monolayers in medium A, as previously described (24). Infections to generate virus stocks were carried out in medium A. Radiolabeled virus stocks were produced from infections in medium AL. Medium AL is medium A that is lacking amino acids and is supplemented with 25 mM *N*-2-hydroxyethylpiperazine-*N*-2′-ethanesulfonic acid (HEPES). Plaque assays were done by using medium P6 (34) with a 2.5-ml 0.8% agar layer (1:1 mixture of P62X and 1.6% Noble agar) and a 2.5-ml liquid overlay (1:1 mixture of P62X and distilled water) supplemented with 4 mM glutamine, 2 mM pyruvate, 4 mM oxaloacetate, and 0.2% glucose. All plaque assay mixtures in which drug was present were supplemented with 0.4% bovine serum albumin (BSA). Plaque assay plates were incubated at 35°C under 5% CO_2 for 45 to 51 h for quantitation of PFU and 72 h for mutant selection. Plates used for PFU quantitation were fixed with 4% formalin and stained with 0.1% crystal violet. Plates used for virus selection were stained for 30 to 60 min in the presence of 0.12 mg of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) per ml.

A stock solution of 5-[5-[4-(4,5-dihydro-2-oxazolyl)phenoxy]pentyl]-3-methylisoxazole (WIN 52035-2) was dissolved in dimethyl sulfoxide to a concentration of 10 mg/ml. From this stock, dilutions to 1,000 times the desired final concentration were made in dimethyl sulfoxide. This was

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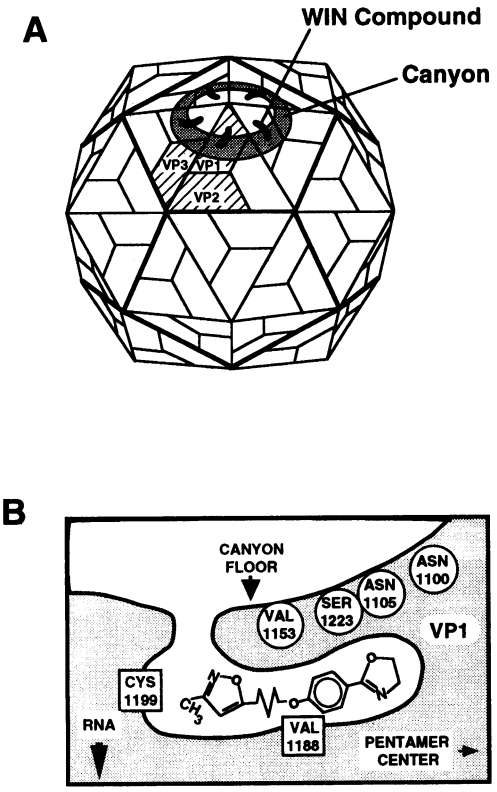

FIG. 1. (A) The capsid of HRV14 consists of 60 protomers (one is shown hatched), each composed of three external polypeptides, VP1, VP2, and VP3, and one internal polypeptide, VP4. Protomers are organized as 12 pentamers (bold outline). Encircling the fivefold axis of each pentamer is a deep depression known as the canyon (stippled), only one of which is shown. The canyon binds the cellular receptor ICAM-1 (12, 27, 36). Each protomer is capable of binding one molecule of drug in a hydrophobic pocket just below the canyon floor (35). (B) Schematic representation of the HRV14 drug-binding pocket and canyon floor region. The amino acids indicated (all in VP1) are the sites of spontaneous mutations known to confer resistance to WIN 52035-2 (13, 14). For the designation of amino acids, the first number indicates the viral protein and the remaining numbers indicate the residue. WIN 52035-2, the drug shown in the pocket, binds in the orientation shown (3). Binding of the drug deforms the floor of the canyon and inhibits attachment of wild-type HRV14.

then further diluted 1:1,000 into phosphate-buffered saline (PBS) (34) or medium A, each containing 0.4% BSA. The BSA was present to buffer the cells against the cytotoxic effects of the WIN compound. The final concentration of dimethyl sulfoxide was 0.1% in both drug-free controls and drug-treated samples.

Virus stocks. HRV14 strain 1059, obtained from V. V. Hamparian, Ohio State University, was passaged in tissue culture, and its identity was confirmed by antiserum neutralization assays. Mutant viruses resistant to WIN 52035-2 were selected and isolated as described previously (13). Virus stocks were generated by infecting HeLa cells in suspension or HeLa cell monolayers. Infections in suspension were done at a multiplicity of infection (MOI) of 0.5 PFU per cell. After an 8-h incubation at 35°C, the progeny virus was harvested by sedimenting the cells, freezing and thawing the resuspended cells three times in medium A supplemented with 37.5 mM HEPES, and sedimenting the suspension at low speed to pellet the cell debris.

For monolayer infections, 0.3 ml of virus stock was inoculated onto a 100-mm HeLa cell monolayer and incubated with medium A containing 0.4% BSA at 35°C in a 5% CO_2 atmosphere. The plate was incubated until a cytopathic effect was seen. The cells were scraped from the plate, supplemented with 25 mM HEPES, and lysed by three cycles of freezing and thawing.

Radiolabeled-virus preparation. Radiolabeled virus for attachment assays was grown and purified by using the following procedure (18). Briefly, HeLa cells were infected with HRV14 at an MOI of 10 PFU per cell in medium AL. At 3 to 4 h postinfection, [^{35}S]methionine (0.5 mCi/10^8 cells) was added. Virus was harvested at 8 h postinfection, and the cells were lysed with Nonidet P-40 (NP-40). Cell debris were removed by low-speed centrifugation (11,950 × *g* for 10 min), and the supernatant was treated with RNase A, EDTA, 2-mercaptoethanol, and *N*-lauroylsarcosine. The virus was pelleted through a 30% sucrose cushion, resuspended, and sedimented in a preformed 7.5 to 45% sucrose gradient.

For radiolabeled virus prepared for all other types of assays, the procedure was modified. Following lysis of the cells with NP-40, the supernatant was treated with 2-mercaptoethanol and *N*-lauroylsarcosine but not RNase A or EDTA. After being pelleted through a 30% sucrose cushion to remove unincorporated [^{35}S]methionine, the virus was resuspended in medium A containing 0.4% BSA and lacking phenol red. No further purification steps were performed.

Thermostabilization assays. For time course studies, virus was diluted into PBS containing 0.4% BSA with or without drug and incubated for 1 h at room temperature to ensure sufficient time for virus and WIN compound to form complexes; samples were then held overnight at 4°C before the start of the experiment. A 0.15-ml aliquot was removed from each preincubation mixture and kept on ice as the unheated control. The remaining 1.35 ml was transferred to a prewarmed glass vial in a 52°C circulating water bath. At desired intervals, 0.15-ml aliquots were removed and chilled in an ice-water bath. Heated and unheated samples were diluted 1:100 into PBS containing 0.4% BSA and allowed to stand 1 h at room temperature to allow drug release from the virus before the infectivity titer was determined by plaque assay.

For single-time-point measurements of thermal inactivation, the assay described above was used with one modification. The virus and drug were preincubated in a volume of 0.3 ml. The preincubation mixture was split into two 0.15-ml aliquots, with one aliquot serving as the unheated control and the other being heated at 52°C for 4 min.

Attachment assays with [^{35}S]methionine-labeled virus. [^{35}S]methionine-labeled virus was preincubated for 1 h at room temperature and overnight at 4°C with or without WIN 52035-2 in medium A containing 0.4% BSA. The medium A used in these assays was prepared without the phenol red indicator, to prevent color quenching during scintillation counting. HeLa cells, at a density of 4 × 10^7 cells per ml, were infected with the virus at an MOI of 10 PFU per cell. The attachment step was done in 15-ml polypropylene conical tubes coated with a 1% BSA solution. The tubes containing 0.75 ml of infected-cell suspension were gently agitated for 30 min at room temperature to allow virus to attach to the cells. The cells were sedimented and washed once with PBS with or without drug. Triplicate 100-μl aliquots of each of the following samples—the input virus, the supernatant containing the unattached virus, and the PBS wash—were counted for ^{35}S. The cells were resus-

pended in medium A containing 0.4% BSA with or without drug. Triplicate 100-μl aliquots of the cell suspension were placed in scintillation vials, and the cells were lysed with NaOH at a final concentration of 0.01 N for 5 min at room temperature. To each vial, 5 ml of EcoScint scintillation cocktail (National Diagnostics, Manville, N.J.) was added, and the samples were counted for ^{35}S.

Single-cycle growth curves. Virus was preincubated for 1 h at room temperature and overnight at 4°C with or without WIN 52035-2 (7 μg/ml) in medium A containing 0.4% BSA. HeLa cells (4×10^7 cells per ml) were inoculated with 1.5 ml of virus (10 PFU per cell) in 15-ml polypropylene conical tubes. The tubes were gently agitated for 30 min at room temperature to allow virus to attach to the cells. The virus-cell complexes were then sedimented and washed with PBS with or without drug to remove unattached virus. The cells were resuspended to a density of 4×10^6 cells per ml in medium A containing 0.4% BSA with or without drug and transferred to a silanized Erlenmeyer flask. A 0.5-ml sample of the cell suspension was removed and designated the zero-time sample; this provided a measure of the percentage of the input virus that was associated with cells. HEPES was added to the zero-time sample to a final concentration of 37.5 mM. The sample was frozen in an ethanol-dry ice bath to stop the infection and stored at −70°C.

The cell suspension was then placed in a 35°C shaking water bath. Over an 8-h period, 0.5-ml aliquots of the cell suspension were removed and frozen as above. All of the samples from the growth curve were frozen and thawed three times to release virus, and the cell debris were pelleted. Each sample was diluted 1:100 in PBS containing 0.4% BSA and allowed to stand 1 h at room temperature to promote drug release from the virus before measuring infectivity by the plaque assay.

Time course of viral RNA synthesis and incorporation into virions. To measure viral RNA synthesis, we used a modified version (17) of a procedure reported previously (30). Virus, preincubated overnight with or without 7 μg of WIN 52035-2 per ml in medium A containing 0.4% BSA, was attached to a suspension of 6×10^7 HeLa cells at an MOI of 20 PFU per cell. The infected-cell suspension (4×10^7 cells per ml) was agitated gently in a 15-ml conical tube for a 30-min attachment period at room temperature. The cells were pelleted, washed with PBS, and resuspended in 15 ml of medium A containing 0.4% BSA and 5 μg of actinomycin D per ml, with or without 7 μg of WIN 52035-2 per ml, into a silanized Erlenmeyer flask. The flasks were incubated in a shaking water bath at 35°C. At 1 h postinfection, 0.6 mCi (1 mCi/10^8 cells) of [^3H]uridine was added to the cell suspension. A 0.4-ml aliquot was removed immediately to provide a measurement of background counts per minute (cpm). Similar aliquots were removed at hourly intervals through 11 h postinfection. For each time point aliquot, the cells were pelleted and washed twice with cold PBS containing 0.1% BSA. The cell pellet was resuspended in 50 μl of PBS containing 0.1% BSA and lysed for 10 min at room temperature in 100 mM NaCl–1 mM EDTA–0.5% sodium dodecyl sulfate (SDS)–10 mM Tris-HCl (pH 8.0). Cold trichloroacetic acid was added to the lysate at a final concentration of 10%. After a 10-min incubation on ice, the resulting precipitate was pelleted at 16,000 × *g* for 10 min, washed once with 10% trichloroacetic acid and once with 75% ethanol, and dried. The pellet was resuspended in 0.1 N NaOH and boiled 5 to 10 min until dissolved. The sample was counted for ^3H.

From the same infected-cell suspension used to measure viral RNA synthesis, aliquots were also removed to determine the incorporation of the RNA into virions (17). Each aliquot was supplemented with HEPES to a concentration of 37.5 mM. After three cycles of freezing and thawing to release virus, the lysate was clarified by centrifugation at 10,000 × *g* for 5 min. In duplicate, 100 μl of supernatant was added to 200 μl of PBS (lacking Ca^{2+} and Mg^{2+}) containing 0.1% BSA, 10 mM EDTA, and 20 μg of RNase A per ml. Following a 10-min incubation at 35°C, 100 μl of PBS containing 0.1% BSA and 1.25 μg of anti-HRV14 monoclonal antibody 17 was added. Virus-antibody complexes were allowed to form for 3 to 4 h at room temperature. Then 20 μl of IgGSorb (The Enzyme Center, Inc., Malden, Mass.; IGSL10: binding capacity, 1.8 μg of immunoglobulin G per μl) was added, and the mixture was incubated for 1 h at room temperature with frequent, gentle vortexing. The immune complexes were pelleted at 10,000 × *g* at 8°C for 2 min and washed three times with 300 μl of RIPA buffer (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 1% SDS). Because 5 to 10% of the supernatant was left with the pellet after each wash to avoid disturbing the immune complexes, the supernatant of the last wash was counted to determine the cpm contributed by the supernatant. The pellet was transferred to a scintillation vial with three 100-μl washes of PBS containing 0.1% BSA and 0.1% SDS. Following the addition of 50 μl of 0.3 N NaOH to solubilize the pellet and a 15-min incubation at room temperature, the samples were counted for ^3H.

Isolation and characterization of cell-associated and eluted viral particles. Virus radiolabeled with [^{35}S]methionine was used to infect HeLa cells by the procedure for single-cycle growth curves described above. The medium A used in these assays was prepared without phenol red indicator to prevent color quenching during subsequent scintillation counting. Attachment was carried out in BSA-coated, 15-ml polypropylene conical tubes containing either 1.5 or 3.0 ml of infected-cell suspension.

For isolation of cell-associated viral particles, the cell suspension was diluted to 4×10^6 cells per ml following attachment and split into equivalent volumes, each containing 3×10^7 cells, in silanized Erlenmeyer flasks. Each flask was incubated for a different length of time at 35°C. The cells were then sedimented and washed once with PBS with or without drug. After resuspension in 1.0 ml of medium A containing 0.4% BSA with or without drug, the cells were lysed with a mixture of 1% NP-40 and 0.5% sodium deoxycholate for 10 min at 0°C (9). Cell debris were removed by low-speed centrifugation, and a 0.5-ml aliquot of the clarified suspension was layered on a 5 to 20% (wt/wt) sucrose gradient. For the gradient solutions, sucrose was dissolved in PBS containing 0.01% BSA with or without 7 μg of WIN 52035-2 per ml. The virus was sedimented in a SW41 rotor for 55 min at 40,000 rpm at 20°C. The gradient was fractionated from the bottom, by drop, into 20-drop fractions in either scintillation vials or sample cups coated with 0.01% BSA. All or a portion of each fraction was counted for ^{35}S.

To isolate eluted viral particles, we maintained the cell suspension at a density of 4×10^7 cells per ml during the 35°C incubation. The cells were then sedimented, and a 0.5-ml aliquot of the supernatant was layered on a 5 to 20% (wt/wt) sucrose gradient and sedimented and fractionated as described above.

To determine the protein composition, we concentrated viral particles from gradient fractions by pelleting in a TLA-100.3 fixed-angle rotor for 90 min at 40,000 rpm at 25°C and electrophoresed them in a tricine-SDS-polyacrylamide gel system (32). The gel size was 15 by 15 cm with 1.5-mm

spacers. The separating gel consisted of 16.5% total acrylamide (acrylamide and bisacrylamide) and 3% cross-linker relative to the total acrylamide concentration. Electrophoresis was done at 110 to 120 V (constant voltage) for 11 h. The gels were soaked in Amplify (Amersham International, Arlington Heights, Ill.), and the bands were visualized by fluorography.

Kinetics of elution. HeLa cells were infected with [^{35}S]methionine-labeled virus by the procedure for single-cycle growth curves. To quantitate eluted virus, aliquots of the cell suspension were removed at the desired intervals, the cells were pelleted in a BSA-coated 1.5-ml microcentrifuge tube, and replicate aliquots of the supernatant were removed to count for ^{35}S. Cell-associated virus was quantitated by resuspending the cell pellets from the above samples in clear medium A containing 0.4% BSA and lysing aliquots of the cell suspension in scintillation vials with 0.01 N NaOH. After a 5-min incubation at room temperature, the samples were counted for ^{35}S.

RESULTS

Use of thermostabilization by bound drug to distinguish two types of drug resistance: exclusion and compensation. Earlier studies showed that arildone (6) and WIN compounds (11) protect poliovirus against thermal inactivation. The finding that HRV14 is similarly stabilized by WIN compounds (14) provided a tool for distinguishing drug-resistant mutants which lose their ability to bind drug from those which do not. To this end, we used WIN 52035-2 at 7 μg/ml, the same concentration at which drug-resistant mutants were selected by their ability to form plaques. This method will be illustrated by using one exclusion mutant, Cys-1199→Tyr, and one compensation mutant, Val-1153→Ile.

In the absence of drug, wild-type HRV14 was inactivated rapidly when heated at 52°C, with less than 0.5% of the infectivity surviving after 6 min (Fig. 2A). An initial lag in the inactivation curve reflected the time required to reach 52°C after the sample (1.35 ml) was placed into the water bath. In the presence of drug, greater than 70% infectivity survived.

X-ray crystallographic studies suggested that in the Cys-1199→Tyr mutant, the bulkier side chain of the tyrosine residue would interfere with the binding of drug (2). Comparison of the thermostability of this mutant with that of wild-type virus in the absence of drug demonstrated that replacing the cysteine residue with tyrosine has a significant thermostabilizing effect (Fig. 2B). The presence of WIN 52035-2 provided little, if any, additional thermoprotective effect, as predicted if the drug was excluded from this mutant. This result supported the idea that thermostabilization requires drug bound within the binding pocket and is not merely a surface adsorption effect of the drug. A similar low level of thermoprotection by drug indicated that the Val-1188→Met mutant is also a drug exclusion mutant (Table 1). However, the slight thermoprotective effect of drug seen with both of these mutants suggested that drug is not entirely excluded.

Figure 2C illustrates the behavior of mutant Val-1153→Ile, a member of a second class of resistant mutants that was strongly thermostabilized by the drug. We refer to such mutants as compensation mutants because they are able to grow even though the thermostabilization assay indicates that they have not lost the ability to bind drug. By this criterion, all four of the mutants with mutations that mapped to the drug-deformable region at the bottom of the canyon (Table 1) were compensation mutants.

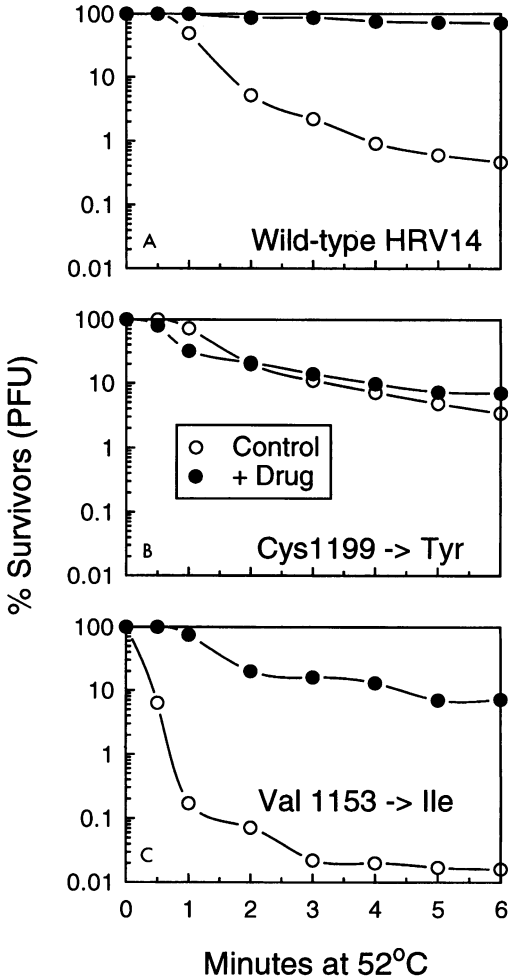

FIG. 2. Thermostabilization curves of wild-type HRV14 (A) and two drug-resistant mutants, Cys-1199→Tyr (B) and Val-1153→Ile (C), in the presence and absence of 7 μg of WIN 52035-2 per ml. Virus was pretreated in PBS containing 0.4% BSA, with or without drug. The virus-drug complexes (1.35 ml) were heated at 52°C, and aliquots were removed at intervals. Chilled samples were diluted 1:100 and incubated for 1 h at room temperature to allow drug release before the infectivity titer was determined by plaque assay.

Exclusion and compensation mutants attach to HeLa cells in the presence of WIN 52035-2. Studies with radiolabeled virus under standard attachment conditions showed that WIN 52035-2 inhibited the attachment of wild-type HRV14 to HeLa cells to 5% of control levels (Table 2). As expected, the drug-resistant mutants overcame the strong attachment-inhibitory effect of drug, although not completely. Attachment of exclusion mutant Cys-1199→Tyr with drug was 68% of control levels whereas that of the Val-1153→Ile mutant was only 22% of control levels. The mutations also exerted an effect on attachment. In the absence of drug, attachment of the Cys-1199→Tyr mutant was slightly poorer than that of the wild type (Table 2, column 2) whereas attachment of the Val-1153→Ile mutant was slightly better. Attachment of the wild-type and mutant viruses was blocked by a monoclonal antibody, RR1/1, specific for the cell adhesion molecule ICAM-1 (36) (data not shown).

Single-cycle growth curves of the compensation mutant, Val-1153→Ile, reveal a second locus of drug action. The

TABLE 1. Thermostabilization of virus as a measurement of drug binding

Virus	% of survivors (after 4 min at 52°C)[a]		Resistance mechanism
	Control	WIN 52035-2[b]	
Wild type	0.3	47	
Cys-1199→Tyr	1	4	Exclusion
Val-1188→Met	0.4	3	Exclusion
Ser-1223→Gly	0.9	49	Compensation
Val-1153→Ile	0.002	36	Compensation
Asn-1100→Ser	0.01	53	Compensation
Asn-1105→Ser	0.8	60	Compensation

[a] Thermostabilization was measured as described in the legend to Fig. 2, except that the volume of the heated sample was 0.15 ml.
[b] Present at 7 μg/ml.

growth curve of wild-type virus in the absence of drug showed a marked decline (eclipse) in cell-associated infectivity from 0.6 PFU per cell at zero time to 0.01 PFU per cell after 3 h (Fig. 3A). The rise period began at 3.5 h postattachment, and synthesis of viral progeny was essentially complete by 8 h. The strong inhibitory effect of the drug on attachment to cells was manifest by the low recovery (0.01 PFU per cell) of cell-associated infectivity at the 0-h time point. A small rise in infectivity beginning at about 6 h produced only about 1/1,000 of a normal yield by 8 h. The progeny was enriched 240-fold for resistant mutants (data not shown) and probably resulted from infection of a small fraction of cells by resistant mutants present in the wild-type population.

The growth of compensation mutant Val-1153→Ile in the absence of drug was similar to that of wild-type virus (Fig. 3B). Attachment of this mutant in the presence of drug was inhibited considerably less strongly in this case (55% of control) than was observed using radioactive particles, when attachment was inhibited to 22% of control (Table 2). At least two processes contribute to this discrepancy. First, as will be shown below, approximately 20% of the Val-1153→Ile particles normally eclipse (lose VP4) during the 30-min attachment period at room temperature; this eclipse is blocked by the presence of drug. Second, only about 40% of attached, radioactive Val-1153→Ile particles are normally released by freeze-thaw lysis of cells in the absence of WIN 52035-2, whereas 65% are released in the presence of drug (data not shown). The nonreleased fraction may represent multivalent attachment associated with receptor recruitment, a process that would be slowed by an inhibitory effect

TABLE 2. Effect of WIN 52035-2 on virus particle attachment

Virus	% of input particles attached[a]		Attachment with drug (% of control)
	Control	WIN 52035-2[b]	
Wild type	31 ± 8	1.6 ± 0.9	5
Cys-1199→Tyr	22 ± 6	15 ± 6	68
Val-1153→Ile	42 ± 8	9.4 ± 2.4	22

[a] As measured after 30 min at room temperature with purified, [^{35}S]methionine-labeled virus at an MOI of 10 PFU per cell incubated with 4 × 10^7 HeLa cells per ml in medium A–0.4% BSA with or without drug. Virus particles were released from cells by lysis with 0.01 N NaOH in scintillation vials. Average values (± standard deviation) from three experiments are shown.
[b] Present at 7 μg/ml.

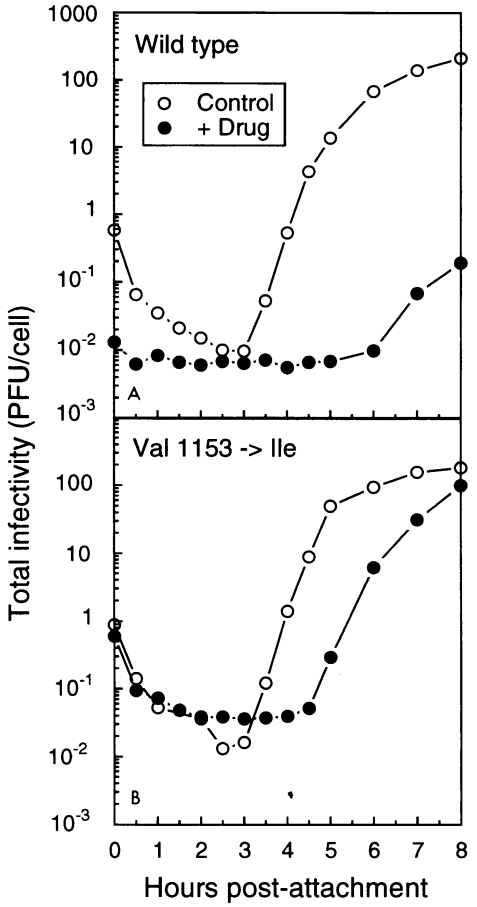

FIG. 3. Single-cycle growth curves of wild-type HRV14 (A) and the drug-resistant mutant Val-1153→Ile (B) in HeLa cell suspension in the presence and absence of 7 μg of WIN 52035-2 per ml. Virus (10 PFU per cell), pretreated in the presence and absence of drug, was allowed to attach to cells for 30 min at room temperature. Unattached virus was removed, the cell suspension was diluted 1:10, and the zero-time sample was taken. The infected-cell suspension was incubated at 35°C for 8 h, and aliquots were removed at intervals. The virus was released from the cells and diluted 1:100 to remove the drug, and infectivity titers were determined.

of drug on binding affinity. Both processes favor recovery of infectivity in the presence of drug.

The major effect of drug on this mutant was a 1.5-h delay in the rise period. As seen in Fig. 3B, the drug had essentially no effect on the kinetics of eclipse, suggesting that it interfered with some later step such as RNA release, RNA synthesis, or virion assembly. Similar results were obtained with the compensation mutants Ser-1223→Gly, Asn-1100→Ser, and Asn-1105→Ser (data not shown).

Preliminary evidence for an effect of drug on uncoating of mutant Val-1153→Ile. To examine the effect of WIN 52035-2 on viral RNA synthesis, drug was added to infected-cell suspensions at 0, 1, or 2 h postattachment and cumulative RNA synthesis was measured by incorporation of [^{3}H]uridine into trichloroacetic acid-insoluble material. The drug caused a slight delay in viral RNA synthesis when added at 0 h postattachment but had no effect at 1 and 2 h postattachment (data not shown). The delay observed when the drug was added at 0 h postattachment suggested an inhibitory effect on an early step in infection, such as uncoating.

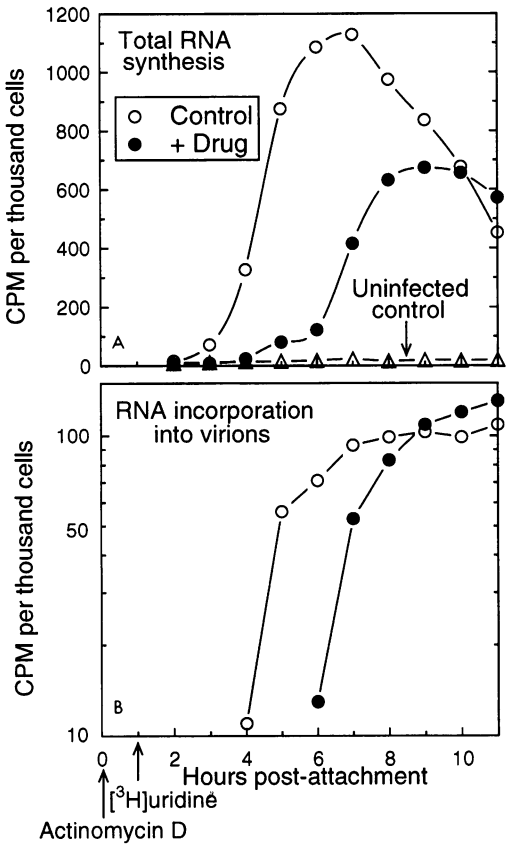

FIG. 4. Time course of total viral RNA synthesis and RNA incorporation into virions for the Val-1153→Ile mutant, in the presence and absence of 7 μg of WIN 52035-2 per ml. Virus was pretreated overnight in growth medium containing 0.4% BSA, with or without drug. HeLa cells were infected at an input MOI of 20 PFU per cell for 30 min at room temperature. Unattached virus was removed, and the cells were resuspended in growth medium containing 0.4% BSA and 5 μg of actinomycin D per ml, with or without drug. Cell suspensions were incubated at 35°C; at 1 h postinfection, 0.6 mCi of [^{3}H]uridine was added to the medium. Aliquots were removed at hourly intervals. Total RNA was isolated by trichloroacetic acid precipitation and counted for ^{3}H (A). Immune complexes of virions were precipitated from cell lysates by using staphylococcal protein A (IgGSorb) and counted for ^{3}H (B).

To investigate whether the drug did interfere with an early step in infection instead of a later step such as assembly, we measured the cumulative synthesis of viral RNA and its incorporation into virions over an 11-h period in the presence and absence of drug (Fig. 4). Virus (20 PFU per cell), which had been preincubated in the presence or absence of drug, was attached to cells under standard conditions for 30 min at room temperature. The virus-cell complexes were sedimented, washed, resuspended into growth medium to 4 × 10^6 cells per ml, and incubated at 35°C. Synthesis of viral RNA was measured by cumulative incorporation of [^{3}H] uridine in the presence of actinomycin D.

Viral RNA synthesis rose to maximum levels at 6 to 7 h in the absence of drug and declined thereafter (Fig. 4A). In the presence of the drug, RNA synthesis was delayed by several hours and the maximum yield was reduced by 40%. The long delay in RNA synthesis reinforced the notion of an inhibitory effect of the drug on uncoating. We attribute the reduced yield to the inhibitory effect of the drug on attachment (Table 2) and probably uncoating even with this resistant mutant.

The delay in RNA synthesis was also reflected by a delay in packaging of RNA into virions (Fig. 4B). However, the interval between half-maximal RNA synthesis and half-maximal packaging was the same in the absence of drug (5.2 h − 4.4 h = 0.8 h) as in its presence (7.4 − 6.6 h = 0.8 h). We conclude that the drug had no measurable effect on virion assembly.

Evidence that WIN 52035-2 inhibits the release of VP4, the first step in the uncoating process. Studies with HRV2 and HRV14 (20) suggest that the pathway of infection following attachment of virus to HeLa cell receptors involves the loss of VP4 to yield noninfectious A particles (eclipse) followed by release of RNA to yield empty capsids. The lack of any effect of drug on the eclipse kinetics of mutant Val-1153→Ile (Fig. 3B) suggested that the drug was delaying release of RNA; however, this interpretation proved erroneous. In what follows, we will first demonstrate that the drug does not, in fact, delay the release of RNA from A particles. We will then show that the true effect of the drug is to delay the release of VP4; we will also show that this action of the drug is not reflected in the eclipse kinetics, because more than 70% of the cell-attached virions flow into a drug-insensitive pathway of abortive elution.

The first clue that the drug was not delaying RNA release was provided by examining the fate of radioactive virions after warming virus-cell complexes to 35°C (Fig. 5). Virus-cell complexes were prepared by the standard 30-min attachment procedure, this time with radiolabeled virions. The cell suspension was washed, resuspended in growth medium, and divided into three aliquots that were incubated at 35°C for 0, 30, or 60 min. For each time point, the cells were concentrated by centrifugation and lysed with detergent. The fate of the radiolabeled virus particles was then examined by sedimentation on sucrose density gradients.

As seen in Fig. 5A, corresponding to the 0-min time point in the absence of drug, the bulk of the virus was present as intact 149S particles. Two smaller components, one sedimenting at 125S (A particles) and the other sedimenting at 90S (empty capsids, not labeled in Fig. 5A) were also observed. This suggested that, in the absence of drug, a small fraction of virions began to uncoat even before the temperature was raised to 35°C. The profiles at 30 and 60 min showed a flow of radioactivity from the 149S virions into 125S A particles into 90S empty capsids (Fig. 5B and C).

If, as suggested by the eclipse kinetics in Fig. 3B, the drug delayed the release of RNA from the Val-1153→Ile mutant, accumulation of A particles would be predicted. That this was not the case is shown in Fig. 5D through F. In the presence of drug, conversion of native virus to A particles was delayed, indicating that the drug blocks the eclipse step (release of VP4), not the RNA release step. The conclusion that the drug blocks release of VP4 contradicts the interpretation from the growth curve that it blocks the release of RNA. How, then, can the virtually identical eclipse kinetics in the presence and absence of drug (Fig. 3B) be reconciled with the conclusion that drug actually blocks the release of VP4? As will be shown below, the explanation to this apparent paradox came from attempts to determine the fate of virus not accounted for in recovery of radioactivity from the sucrose gradients (see "Recovery" insets in Fig. 5B, C, E, and F).

Evidence for elution of cell-associated Val-1153→Ile particles. When the infection process was initiated by elevating

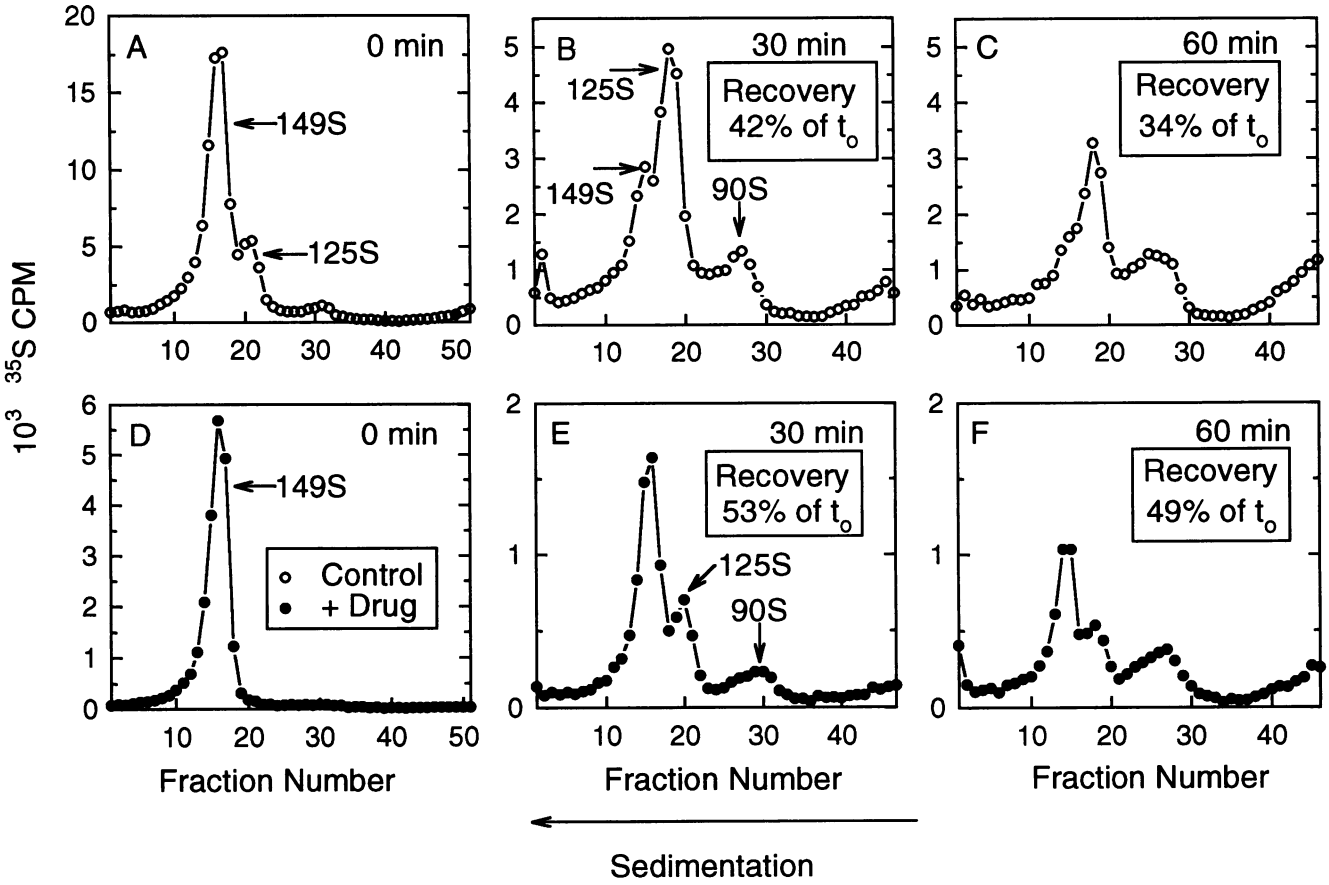

FIG. 5. Sedimentation profiles of cell-associated particles of the Val-1153→Ile mutant in the presence and absence of 7 μg of WIN 52035-2 per ml. [^{35}S]methionine-labeled virus, pretreated with or without drug, was used to infect HeLa cells for 30 min at room temperature. After removal of unattached virus, the infected-cell suspension was diluted 1:10 and split into equivalent volumes, each containing 3×10^7 cells. These suspensions were incubated at 35°C for 0 min (A and D), 30 min (B and E) or 60 min (C and F) before being lysed with 1% NP-40 and 0.5% sodium deoxycholate (9). The lysates were sedimented through 5 to 20% (wt/wt) sucrose gradients. The gradients were fractionated from the bottom, and fractions were counted for ^{35}S. The recoveries in panels B, C, E, and F were determined by the number of ^{35}S cpm present in each gradient relative to the ^{35}S cpm present in the gradients containing samples not incubated at 35°C (panels A and D). On the basis of a sedimentation coefficient of 149S for native virus (31), sedimentation coefficients of other particles were determined relative to 149S, assuming isokinetic sedimentation conditions.

the temperature of virus-cell complexes from room temperature to 35°C in the absence of the drug, a substantial fraction of the ^{35}S-labeled protein was released into the growth medium (Fig. 6A). Thus, 50% was eluted by 1 h, and 70% was eluted by 2 h. This accounted for the decreasing recoveries of cell-associated radioactivity observed in Fig. 5. Moreover, as seen in Fig. 6B, the drug had no significant effect on the kinetics of this process.

The nature of eluted particles was determined by sedimentation analysis. As seen in Fig. 7A, three components were eluted in the absence of the drug: one at 125S, one at 90S, and a peak of soluble material at the top of the gradient. In the presence of the drug (Fig. 7B), a fourth component, corresponding to intact virions sedimenting at 149S, was observed. This reinforced the conclusion that the drug interferes with conversion of virions to 125S particles and showed that, although the drug had no effect on the kinetics of elution, it did alter the spectrum of particles which are produced.

Characterization of eluted and cell-associated Val-1153→Ile subviral particles. The specific infectivity of eluted and cell-associated particles was determined. The 149S particles were fully infectious, whereas the 125S and 90S particles were not infectious (data not shown). Only the 149S particles contained all four viral proteins (Fig. 8, lanes 3 and 4), although VP2 and VP3 were not resolved. All of the eluted and cell-associated subparticles (125S and 90S) lacked VP4 (lanes 1, 2, 5, 6, and 7). A minor band migrating slightly faster than VP1, possibly a fragment of VP1, was always observed in cell-associated 125S particles (lanes 5 and 6) but not in eluted 125S particles (lane 1). The band migrating at approximately 18.4 kDa was always present and probably represents a species produced by degradation of a viral protein. Dual-label experiments with [^{35}S]methionine and [^{3}H]uridine confirmed that 125S and 149S particles contained RNA while 90S particles contained none (data not shown).

DISCUSSION

Thermostabilization by WIN compounds: a valuable method for classifying drug-resistant mutants into exclusion and compensation classes. Exclusion mutants bound little or no drug and were characterized by substitution to bulkier

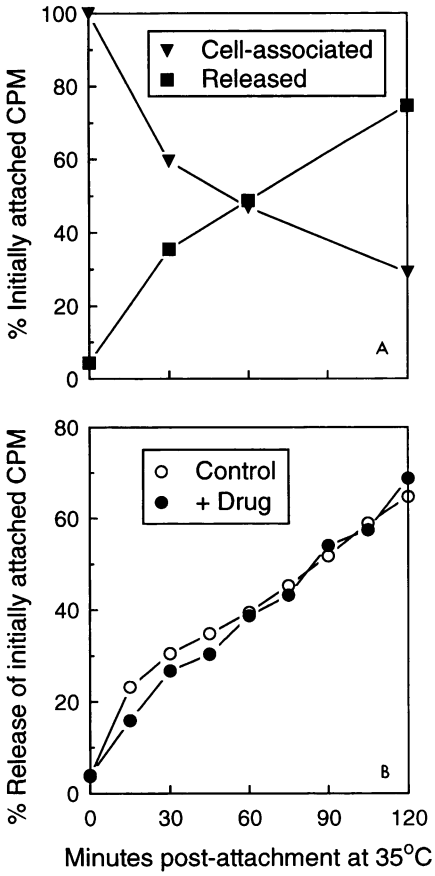

FIG. 6. Kinetics of elution of attached Val-1153→Ile particles. HeLa cells were infected with [^{35}S]methionine-labeled virus for 30 min at room temperature, unattached virus was removed, and the cell suspension was incubated at 35°C. (A) The proportions of initially attached ^{35}S cpm released into the medium and associated with cells were quantitated in the absence of drug. Cell-associated cpm were released by lysis with 0.01 N NaOH in scintillation vials. Values are expressed as percentage of the total cpm attached to cells prior to the 35°C incubation. (B) The effect of the drug on the extent and kinetics of elution was determined by measuring the accumulation of ^{35}S cpm released into the medium in the presence and absence of drug. Time point values are expressed as the percentage of the total cpm attached prior to the 35°C incubation.

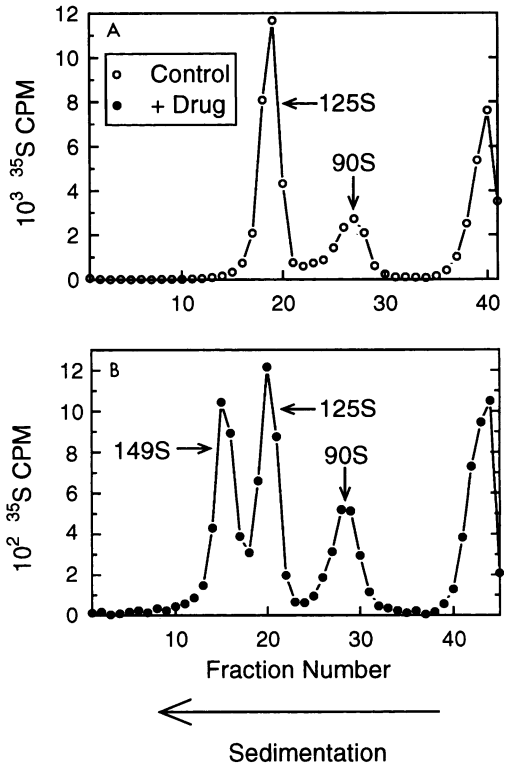

FIG. 7. Sedimentation profiles of eluted particles of the Val-1153→Ile mutant in the absence (A) and presence (B) of 7 μg of WIN 52035-2 per ml. HeLa cells were infected for 30 min at room temperature with [^{35}S]methionine-labeled virus that had been pretreated with or without drug. Unattached virus was removed by sedimentation, and the infected-cell suspension was incubated at 35°C for 1 h. The cells were then pelleted, and a sample of the supernatant was layered on a 5 to 20% sucrose gradient. Following sedimentation, the gradients were fractionated from the bottom.

amino acid side chains within the drug-binding pocket. Such mutants are of little utility in mapping capsid functions. The thermostabilization assay in the presence of drug provides a rapid screen for eliminating exclusion mutants before the more time-consuming sequencing process.

Compensation mutants provide a means of mapping regions of the viral capsid involved in drug-sensitive functions; this is reinforced by the location of compensation mutations in canyon regions that are distorted by drug binding (3, 35) and which ICAM-1 is believed to contact (27). For example, Ser-1223, which lies on the canyon floor, is known to be involved in binding of HRV14 to ICAM-1 (7); its substitution with glycine increases binding affinity (28). Mutants Val-1153→Ile (Table 2) and Asn-1100→Ser (33) also display enhanced binding to HeLa cells. Whether these substitutions alter contact with the receptor or flexibility of the drug-deformable regions remains to be determined. X-ray crystallographic studies of the compensation mutants are under way, and determination of their structures may clarify this question.

VP4 release: a second target of WIN compounds in HRV14. The delayed rise period in the growth curves of compensation mutants (Fig. 3B) signaled a second effect of WIN compounds, which was initially thought to be at the RNA release step, the second step in uncoating (Fig. 9). However, failure of these mutants to accumulate A particles in the presence of drug (Fig. 5) revealed that drug acts instead at the first step of uncoating, eclipse (release of VP4). Thus, the antiviral activity of WIN 52035-2 on HRV14 results from targeting two steps of infection, attachment and uncoating.

WIN compounds block uncoating but not attachment of polioviruses and the minor-group HRVs HRV2 and HRV1A (11, 16, 37). WIN 51711 (37) and arildone (9) are known to block the conversion of native poliovirus to A particles in infected cells. The step at which WIN compounds interfere with the uncoating of minor-group HRVs has not been determined, but the observation that the drugs thermostabilize HRV1A (28) suggests that this virus may also be stabilized in its native conformation by the drug. Thus, for both polioviruses and rhinoviruses, it appears that WIN compounds inhibit uncoating by delaying the release of VP4.

In studies with HRV14, HRV1A (33), and poliovirus types 1 (33) and 3 Sabin (25), the WIN compounds delayed but never completely prevented uncoating. It is not clear

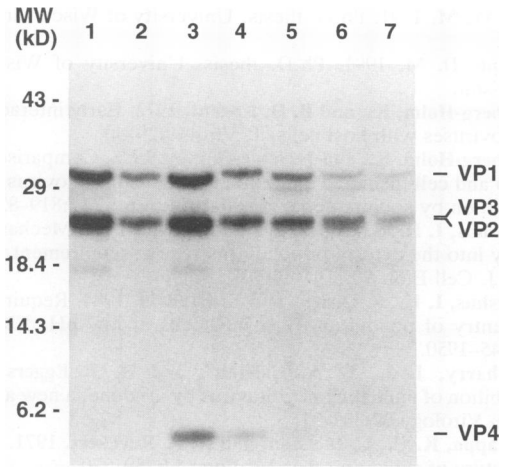

FIG. 8. Tricine-SDS-polyacrylamide gel electrophoresis of Val-1153→Ile native, eluted, and cell-associated particles. A [^{35}S]methionine-labeled sample was loaded in each lane and resolved by electrophoresis as described in Materials and Methods. The gel was soaked in Amplify, and the bands were visualized by fluorography. Lanes: 1, eluted 125S particles; 2, eluted 90S particles; 3, native virus; 4, cell-associated 149S particles immediately following attachment; 5, cell-associated 125S particles immediately following attachment; 6, cell-associated 125S particles after a 30-min incubation at 35°C; 7, cell-associated 90S particles after a 30-min incubation at 35°C. The positions of molecular mass markers are indicated on the left, and the positions of viral capsid proteins are indicated on the right.

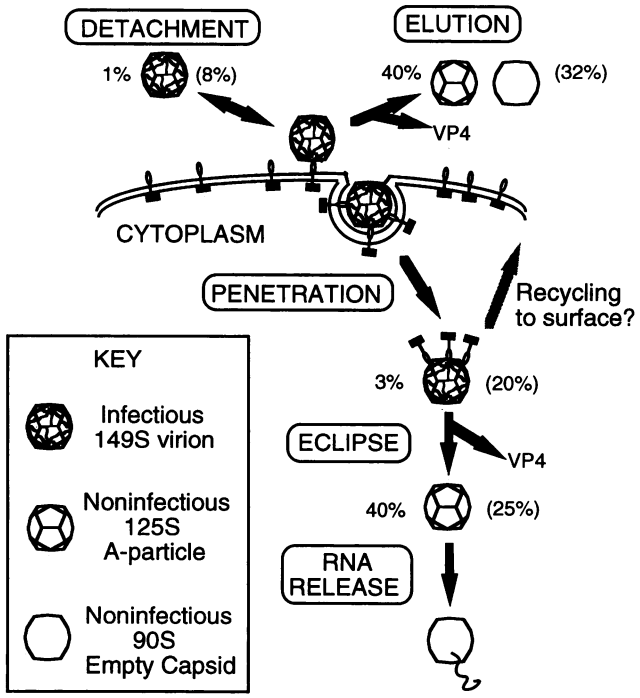

FIG. 9. Proposed (19, 20) pathway of infection by HRV14 following attachment to susceptible host cells. By 1 h, about 50% of the attached virus is released into the medium as infectious particles or as noninfectious eluted particles. The remaining virus particles undergo eclipse (release of VP4) in cellular vesicles or in association with plasma membranes (see text). The location of release of the viral genome to the cytosol is not known. Neither is it known whether native and altered particles are eluted directly from the surface, after internalization, or both. Numbers, derived from experiments described in the legends to Fig. 5 and 7, represent the redistribution of Val-1153→Ile virions after 1 h at 35°C in the absence and presence (parentheses) of WIN 52035-2. The numbers do not sum to 100%, because about 15% of the cell-associated virions were not released by detergent lysis.

whether this incomplete block in uncoating was due to low drug activity, subsaturating binding of drug to virus, or possibly even reversal of inhibition with progressive acidification of the virus in endosomes. A low pH may cause conformational changes in the virus that enable it to uncoat despite the presence of bound drug.

Lack of action of WIN compounds on abortive elution of HRV14, a pitfall in interpreting growth curves to pinpoint the action of drugs on the uncoating step. The single-step growth curve provided a useful tool for identifying the effect of WIN drugs on the attachment step. However, it proved misleading when applied to analysis of the uncoating block because at least 70% of the decline in infectivity following attachment was due to abortive processes upon which drug has no effect. This completely masked the inhibitory effect of drug on release of VP4 (eclipse).

Abortive elution, or sloughing, was first recognized with poliovirus (10, 15) and was later observed with HRV14 and HRV2 (1, 19, 26). In each case, the eluted particles were missing VP4, and it might therefore have been expected that a drug which inhibits the release of VP4 would also have inhibited the elution process. The observation that it did not suggests that elution is controlled not by loss of VP4 but by some other process, such as receptor metabolism (5). Whatever the mechanism, it must eventually account for the fact that elution is not confined to any one picornavirus receptor family since poliovirus, HRV2, and HRV14 all use different receptors.

Whereas WIN 52035-2 did not affect the fraction of cell-attached particles eventually released into the medium, it did alter the spectrum of particles observed (Fig. 9). After a 1-h incubation at 35°C, a larger proportion of attached particles was released as infectious virus (detachment) when drug was present (8%) than when it was absent (1%). This increased detachment in the presence of drug probably results from the attachment-inhibitory effect of the drug. Virus may attach more slowly and remain more loosely associated with the cell when the drug is present, thus, promoting its detachment from the cell.

Models of the early stages of picornavirus infection. The sequence of events in infection begins with attachment of virus to susceptible cells followed by recruitment of multiple receptors (Fig. 9). The attached virions uncoat by losing VP4 (eclipse) and then releasing the RNA genome to the cytosol at the plasma membrane (4) or in cellular vesicles involved in receptor-mediated endocytosis (21, 22).

In our studies, we have provided further evidence to support the role of VP4 release or eclipse in the pathway of picornavirus infection. However, the methods used here provide no information about the cellular location at which the uncoating process occurs.

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REFERENCES

1. **Abraham, G., and R. J. Colonno.** 1988. Characterization of human rhinoviruses displaced by an anti-receptor monoclonal antibody. J. Virol. **62:**2300–2306.
2. **Badger, J., S. Krishnaswamy, M. J. Kremer, M. A. Oliveira, M. G. Rossmann, B. A. Heinz, R. R. Rueckert, F. J. Dutko, and M. A. McKinlay.** 1989. Three-dimensional structures of drug-resistant mutants of human rhinovirus 14. J. Mol. Biol. **207:**163–174.
3. **Badger, J., I. Minor, M. J. Kremer, M. A. Oliveira, T. J. Smith, J. P. Griffith, D. M. A. Guerin, S. Krishnaswamy, M. Luo, M. G. Rossmann, M. A. McKinlay, G. D. Diana, F. J. Dutko, M. Fancher, R. R. Rueckert, and B. A. Heinz.** 1988. Structural analysis of a series of antiviral agents complexed with human rhinovirus 14. Proc. Natl. Acad. Sci. USA **85:**3304–3308.
4. **Boulanger, P., and K. Lonberg-Holm.** 1981. Components of non-enveloped viruses which recognize receptors, p. 21–46. *In* K. Lonberg-Holm and L. Philipson (ed.), Receptors and recognition, series B, vol. 8; virus receptors. Part 2. Animal viruses. Chapman & Hall, London.
5. **Brown, V. I., and M. I. Greene.** 1991. Molecular and cellular mechanisms of receptor-mediated endocytosis. DNA Cell Biol. **10:**399–409.
6. **Caliguiri, L. A., J. J. McSharry, and G. W. Lawrence.** 1980. Effect of arildone on modification of poliovirus in vitro. Virology **105:**86–93.
7. **Colonno, R. J., J. H. Condra, S. Mizutani, P. L. Callahan, M. E. Davies, and M. A. Murcko.** 1988. Evidence for the direct involvement of the rhinovirus canyon in receptor binding. Proc. Natl. Acad. Sci. USA **85:**5449–5453.
8. **Eggers, H. J.** 1977. Selective inhibition of uncoating of echovirus 12 by rhodanine. A study on early virus-cell interactions. Virology **78:**241–252.
9. **Everaert, L., R. Vrijsen, and A. Boeye.** 1989. Eclipse products of poliovirus after cold-synchronized infection of HeLa cells. Virology **171:**76–82.
10. **Fenwick, M. L., and P. D. Cooper.** 1962. Early interactions between poliovirus and ERK cells: some observations on the nature and significance of the rejected particles. Virology **18:** 212–223.
11. **Fox, M. P., M. J. Otto, and M. A. McKinlay.** 1986. The prevention of rhinovirus and poliovirus uncoating by WIN 51711: a new antiviral drug. Antimicrob. Agents Chemother. **30:**110–116.
12. **Greve, J. M., G. Davis, A. M. Meyer, C. P. Forte, S. C. Yost, C. W. Marlor, M. E. Kamarck, and A. McClelland.** 1989. The major human rhinovirus receptor is ICAM-1. Cell **56:**839–847.
13. **Heinz, B. A., R. R. Rueckert, D. A. Shepard, F. J. Dutko, M. A. McKinlay, M. Fancher, M. G. Rossmann, J. Badger, and T. J. Smith.** 1989. Genetic and molecular analyses of spontaneous mutants of human rhinovirus 14 that are resistant to an antiviral compound. J. Virol. **63:**2476–2485.
14. **Heinz, B. A., D. A. Shepard, and R. R. Rueckert.** 1990. Escape mutant analysis of a drug-binding site can be used to map functions in the rhinovirus capsid, p. 173–186. *In* G. Laver and G. Air (ed.), Use of x-ray crystallography in the design of antiviral agents. Academic Press, Inc., New York.
15. **Joklik, W. K., and J. E. Darnell.** 1961. The adsorption and early fate of purified poliovirus in HeLa cells. Virology **13:**439–447.
16. **Kim, S., T. J. Smith, M. S. Chapman, M. G. Rossmann, D. C. Pevear, F. J. Dutko, P. J. Felock, G. D. Diana, and M. A. McKinlay.** 1989. Crystal structure of human rhinovirus 1A (HRV1A). J. Mol. Biol. **210:**91–111.
17. **Lee, W. M.** 1991. Ph.D. thesis. University of Wisconsin, Madison.
18. **Leippe, D. M.** 1991. Ph.D. thesis. University of Wisconsin, Madison.
19. **Lonberg-Holm, K., and B. D. Korant.** 1972. Early interaction of rhinoviruses with host cells. J. Virol. **9:**29–40.
20. **Lonberg-Holm, K., and J. Noble-Harvey.** 1973. Comparison of in vitro and cell-mediated alteration of a human rhinovirus and its inhibition by sodium dodecyl sulfate. J. Virol. **12:**819–826.
21. **Madshus, I. H., S. Olsnes, and K. Sandvig.** 1984. Mechanism of entry into the cytosol of poliovirus type 1: requirement for low pH. J. Cell Biol. **98:**1194–1200.
22. **Madshus, I. H., S. Olsnes, and K. Sandvig.** 1984. Requirements for entry of poliovirus RNA into cells at low pH. EMBO J. **3:**1945–1950.
23. **McSharry, J. J., L. A. Caliguiri, and H. J. Eggers.** 1979. Inhibition of uncoating of poliovirus by arildone, a new antiviral drug. Virology **97:**307–315.
24. **Medappa, K. C., C. McLean, and R. R. Rueckert.** 1971. On the structure of rhinovirus 1A. Virology **44:**259–270.
25. **Mosser, A. G., and R. R. Rueckert.** 1993. WIN 51711-dependent mutants of poliovirus type 3: evidence that virions decay after release from cells unless drug is present. J. Virol. **67:**1246–1254.
26. **Noble, J., and K. Lonberg-Holm.** 1973. Interactions of components of human rhinovirus type 2 with HeLa cells. Virology **51:**270–278.
27. **Olson, N. H., P. R. Kolatkar, M. A. Oliveira, R. H. Cheng, J. M. Greve, A. McCelland, T. S. Baker, and M. G. Rossmann.** 1993. Structure of a human rhinovirus complexed with its receptor molecule. Proc. Natl. Acad. Sci. USA **90:**507–511.
28. **Pevear, D. C. (Sterling Winthrop Pharmaceuticals Research Division).** 1992. Personal communication.
29. **Pevear, D. C., M. J. Fancher, P. J. Felock, M. G. Rossmann, M. S. Miller, G. Diana, A. M. Treasurywala, M. A. McKinlay, and F. J. Dutko.** 1989. Conformational change in the floor of the human rhinovirus canyon blocks adsorption to HeLa cell receptors. J. Virol. **63:**2002–2007.
30. **Racaniello, V. R., and C. Meriam.** 1986. Poliovirus temperature-sensitive mutant containing a single nucleotide deletion in the 5′-noncoding region of the viral RNA. Virology **155:**498–507.
31. **Rueckert, R. R.** 1990. Picornaviridae and their replication, p. 507–548. *In* B. N. Fields, D. M. Knipe, R. M. Chanock, M. S. Hirsch, J. L. Melnick, T. P. Monath, and B. Roizman (ed.), Virology, 2nd ed. Raven Press, New York.
32. **Schagger, H., and G. Von Jagow.** 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem. **166:**368–379.
33. **Shepard, D. A.** Unpublished data.
34. **Sherry, B., and R. Rueckert.** 1985. Evidence for at least two dominant neutralization antigens on human rhinovirus 14. J. Virol. **53:**137–143.
35. **Smith, T. J., M. J. Kremer, M. Luo, G. Vriend, E. Arnold, G. Kamer, M. G. Rossmann, M. A. McKinlay, G. D. Diana, and M. J. Otto.** 1986. The site of attachment in human rhinovirus 14 for antiviral agents that inhibit uncoating. Science **233:**1286–1293.
36. **Staunton, D. E., V. J. Merluzzi, R. Rothlein, R. Barton, S. D. Marlin, and T. A. Springer.** 1989. A cell adhesion molecule, ICAM-1, is the major surface receptor for rhinoviruses. Cell **56:**849–853.
37. **Zeichhardt, H., M. J. Otto, M. A. McKinlay, P. Willingmann, and K. O. Habermehl.** 1987. Inhibition of poliovirus uncoating by disoxaril (WIN 51711). Virology **160:**281–285.