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Genetic and Molecular Analyses of Spontaneous Mutants of Human Rhinovirus 14 That Are Resistant to an Antiviral Compound

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Spontaneous mutants of human rhinovirus 14 resistant to WIN 52084, an antiviral compound that inhibits attachment to cells, were isolated by selecting plaques that developed when wild-type virus was plated in the presence of high (2 μg/ml) or low (0.1 to 0.4 μg/ml) concentrations of the compound. Two classes of drug resistance were observed: a high-resistance (HR) class with a frequency of about 4×10^{-5}, and a low-resistance (LR) class with a 10- to 30-fold-higher frequency. The RNA genomes of 56 HR mutants and 13 LR mutants were sequenced in regions encoding the drug-binding site. The HR mutations mapped to only 2 of the 16 amino acid residues that form the walls of the drug-binding pocket. The side chains of these two residues point directly into the pocket and were invariably replaced by bulkier groups. These findings, and patterns of resistance to related WIN compounds, support the concept that HR mutations may hinder the entry or seating of drug within the binding pocket. In contrast, all of the LR mutations mapped to portions of the polypeptide chain near the canyon floor that move when the drug is inserted. Because several LR mutations partially reverse the attachment-inhibiting effect of WIN compounds, these mutants provide useful tools for studying the regions of the capsid structure involved in attachment. This paper shows that the method of escape mutant analysis, previously used to identify antibody binding sites on human rhinovirus 14, is also applicable to analysis of antiviral drug activity.

The WIN compounds are a family of experimental drugs developed at the Sterling-Winthrop Research Institute, Rensselaer, N.Y., that inhibit the replication of picornaviruses. These drugs bind reversibly to the virus capsid and either block uncoating, as observed for poliovirus and human rhinovirus 2 (HRV2) (5, 28), or inhibit attachment to the cell receptor, as seen with HRV14 (19). Antiviral activity has been shown to occur in cell culture (15) as well as in localized and systemic infections in the mouse (12, 13). WIN 52084 (Fig. 1A) was selected for study because it is highly effective against our target virus, HRV14, whose capsid structure is known in atomic detail (21).

The protein shell of HRV14 (Fig. 1B) is composed of 60 protomers, each composed of three external polypeptides (VP1, VP2, and VP3) and one internal polypeptide (VP4). A 2.5-nm-deep depression known as the canyon encircles each icosahedral fivefold axis of symmetry (21). The canyon is postulated to contain the site that attaches to cell receptors (3, 21, 22). WIN compounds insert into a hydrophobic pocket within the β-barrel of VP1 (26) that lies just beneath the floor of the canyon; at saturation, approximately one drug molecule binds per protomer (26). Sixteen amino acid residues with side chains lying within 0.4 nm of the bound drug constitute the lining of the drug-binding pocket (J. Badger, S. Krishnaswamy, M. J. Kremer, M. A. Oliveira, M. G. Rossmann, B. A. Heinz, R. R. Rueckert, F. J. Dutko, and M. A. McKinlay, J. Mol. Biol., in press).

In HRV14, binding of the drug is accompanied by conformational changes in three regions of VP1 that comprise both the roof of the drug-binding pocket and the canyon floor (2). This deformation in the canyon floor is accompanied by inhibition of virus attachment to cells (19). All of the WIN compounds examined to date interact with the capsid at the same binding site (Fig. 1B), and all induce the same conformational changes. However, the orientation and position of each compound within the pocket differ (2). WIN 52084 enters the pocket with the isoxazole group pointing toward the viral fivefold axis.

To understand better the mode of action of these compounds and to begin to map the sites on the capsid that are involved in virus attachment to cells, we have located mutations in a collection of naturally occurring drug-resistant mutants of HRV14. In this report we describe the types of mutation responsible for conferring different degrees of resistance to the drug and identify the residues on the virus structure that can interfere with drug function.

MATERIALS AND METHODS

Cells and media. A cloned line of HeLa cells was propagated in suspension culture in medium B or as monolayers in medium A as previously described (14). Virus stocks were diluted in phosphate-buffered saline containing 0.1% bovine serum albumin (BSA) (25). For plaque assays we used medium P6 (25); the liquid overlay was supplemented with 2 mM glutamine, 1.2 mM pyruvate, 2 mM oxaloacetate, and 0.2% glucose. Plaque assays were incubated at 35°C for 48 h for PFU enumeration and 72 h for mutant selection. Plaques were visualized for selection by an additional 30- to 60-min incubation in the presence of 0.12 mg of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) per ml.

WIN 52084 was dissolved in dimethyl sulfoxide (DMSO) to 1,000× concentrations. These were diluted 1:1,000 into

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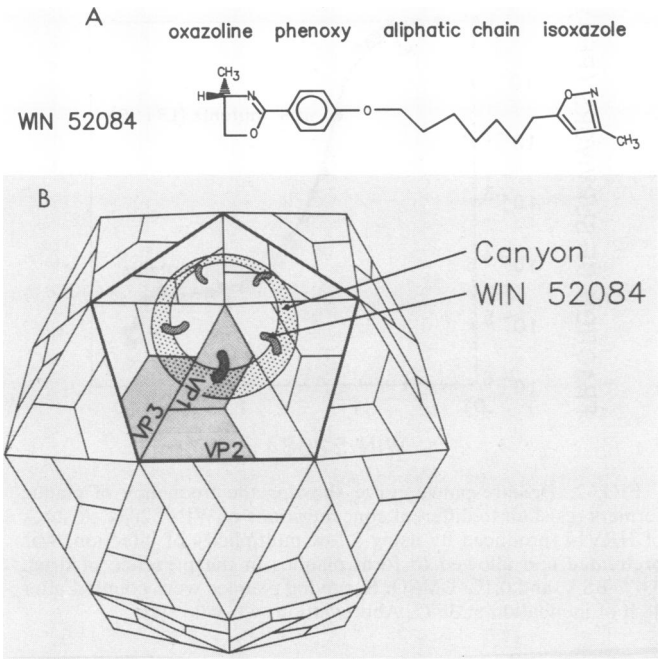

FIG. 1. (A) Structure of WIN 52084 [(S)-(−)-5-[7-[4-(4,5-dihydro-4-methyl-2-oxazolyl)phenoxy]heptyl]-3-methylisoxazole], the antiviral compound used for selection of mutants. (B) Schematic representation of the HRV14 capsid and the location of bound WIN 52084. A pentamer is outlined in a heavy line, and a biological protomer is shaded.

media or phosphate-buffered saline that had been supplemented with 0.4% BSA. The DMSO (final concentration, 0.1%) had no measurable effect on virus replication (data not shown).

Virus stocks. HRV14 (strain 1059) was originally obtained from V. V. Hamparian, Ohio State University. It was passaged several times in cell culture, and its identity was confirmed by antibody neutralization tests. Low-multiplicity-of-infection stocks were prepared by infecting cells in suspension culture with 0.5 PFU per cell. Progeny virus was harvested after an 8-h incubation (single cycle) at 35°C by sedimenting the cells, freeze-thawing three times in medium A containing 25 mM *N*-2-hydroxyethylpiperazine-*N*-2′-ethanesulfonic acid (HEPES), and centrifuging at low speed to remove cellular debris. Multiplication was limited to a single cycle at low multiplicity of infection to minimize the production of viral pseudotypes (i.e., mutant RNA surrounded by a wild-type capsid).

Dose-response curve. The fraction of drug-resistant mutants present in a population of HRV14 was examined by determining the frequency of survivors that could replicate in different concentrations of drug. Low-multiplicity-of-infection stocks of virus were diluted in phosphate-buffered saline containing 0.4% BSA and drug and allowed to interact at room temperature for 1 h. These solutions were then allowed to form plaques in medium containing drug at the same concentrations as during the pretreatment.

Selection and amplification of independent drug-resistant mutants. Wild-type plaques were individually amplified at 35°C for 8 h in monolayer cells to increase the number of resistant mutants they contained and to unmask viral mosaics that had been formed during plaque formation. Each amplified wild-type preparation was pretreated and allowed to form plaques in the presence of WIN 52084 and 0.4% BSA as described above. A total of 52 and 17 mutants were isolated in the presence of 2 and 0.1 to 0.4 μg of WIN 52084 per ml, respectively. To ensure that all of our mutants arose from independent events, only one resistant mutant was selected from each amplified wild-type plaque. Each mutant was further amplified in monolayer culture to a final titer of 1×10^9 to 5×10^9 PFU in 60 ml of medium containing drug and BSA. This preparation was supplemented with HEPES (pH 7.4) (final concentration, 25 mM), frozen and thawed three times to release virus, and clarified by low- and high-speed centrifugation.

Mutant infectivity was assayed by plaque titer determination in the presence and absence of 2 μg of WIN 52084 per ml to evaluate the degree of drug resistance and to detect drug dependence (none was observed). Mutants were diluted in phosphate-buffered saline with 0.4% BSA containing drug or 0.1% DMSO only and allowed to interact at room temperature for 1 h (permitting the drug to diffuse into or out of the binding pocket). Plaque assays were then conducted with media containing the same components as during pretreatment.

Viral RNA preparations. Amplified mutants were concentrated and purified in one step by immunoprecipitation with a monoclonal antibody that forms insoluble complexes with virus over a wide range of antibody-to-virion ratios (D. Leippe, unpublished results). This avoided the need for exact quantitation of each mutant. Use of a monoclonal antibody also served as a check on the mutant serotype. The optimum antibody-to-virion ratio was estimated to be approximately 120:1. Antibody was allowed to interact with virus for 1 h at room temperature and then overnight at 4°C. Immune complexes were precipitated by centrifugation (12,060 × *g* for 30 min at 4°C) and then suspended to a final volume of 2.5 ml in HENA buffer (50 mM HEPES, 0.1 M NaCl, 1 mM EDTA [pH 7.4]) containing 1% sodium dodecyl sulfate.

Viral RNA was extracted directly from immune complexes in HENA buffer by a procedure reported previously (24), modified as follows to accommodate larger amounts of protein. RNA was extracted three times with phenol-chloroform by high-speed centrifugation (12,060 × *g* for 10 to 40 min), extracted in chloroform-isoamyl alcohol, precipitated in ethanol, and stored in 50 μl of diethyl pyrocarbonate-treated deionized-distilled water at −70°C.

Dideoxy sequencing. Viral RNA was sequenced by second-strand synthesis by using reverse transcriptase, dideoxynucleotides, and a ^{32}P-labeled oligonucleotide primer (6). Four primers were used to sequence regions encoding most of VP1 (amino acids 70 to 232 [5′-AGGTTGAGATGCAGTG-3′, 5′-ATGCCAATCCTACAT-3′, and 5′-CTCTGATCTTG ACAAG-3′]) and the N-terminal end of VP3 (amino acids 22 to 26 [5′-GTTATGAACTTTCCC-3′]). Primers were prepared and purified by high-pressure liquid chromatography at the Biotechnology Center, University of Wisconsin-Madison. About 0.12 μg of primer was radiolabeled by heating 13 μl of a mixture containing 102 μCi of [γ-^{32}P]ATP (5,000 Ci/mmol), 70 mM Tris hydrochloride (pH 7.6), 10 mM $MgCl_2$, 5 mM dithiothreitol, and 10 U of polynucleotide kinase (New England BioLabs, Inc., Beverly, Mass.) at 37°C for 30 min. The solution was boiled for 5 min, and 7 μl of distilled water was added (final concentration of oligonucleotide, 5.7 ng/μl). This preparation was stored at −20°C.

A 1-μl portion of primer was annealed to the viral RNA template (0.5 to 1.0 μg) in a 10-μl volume containing 10 mM Tris hydrochloride (pH 8.3) and 250 mM KCl by heating at 70°C for 3 min. The mixture was allowed to cool slowly to

40°C. Primer extension was conducted with reaction mixtures containing 20 mM Tris hydrochloride (pH 8.3), 11 mM $MgCl_2$, 80 mM KCl, 5 mM dithiothreitol, 0.4 mM dATP, 0.4 mM dCTP, 0.4 mM dTTP, 0.8 mM dGTP, 0.5 U of reverse transcriptase (AMV-002; Life Sciences, Inc., St. Petersburg, Fla.) per ml, and one of the dideoxynucleotides (0.15 mM ddATP, 0.15 mM ddCTP, 0.15 mM ddGTP, or 0.3 mM ddTTP). These solutions were incubated at 50°C for 45 min. The reactions were stopped by the addition of formamide (24) and heated in a boiling-water bath for 5 min. Samples were electrophoresed on 6% polyacrylamide gels (24) at 45 W, fixed in 10% methanol-10% acetic acid, and dried for autoradiography.

Plaque reduction assays to determine MICs. HeLa cell monolayers in six-well plates (Costar, Cambridge, Mass.) were inoculated with approximately 100 PFU in 1.0 ml of phosphate-buffered saline and incubated for at least 1 h at 33°C in a 2% CO_2 atmosphere. The inoculum was removed, and the cells were overlaid with (per well) 3 ml of agarose overlay medium (M199, 5% newborn calf serum, 15 μg of DEAE-dextran per ml, 30 mM $MgCl_2$, 0.5% SeaKem Agarose [FMC Corp., Marine Colloids Division, Rockland, Maine]) containing WIN compound or DMSO only. Plates were incubated at 33°C for 3 to 4 days in a 2% CO_2 atmosphere, fixed with 5% glutaraldehyde, and stained with 0.25% crystal violet. The MIC was defined as the concentration of compound that inhibited the plaque titer by 50%.

Phenotypes of highly resistant mutants. To determine whether selection of highly resistant mutants was biased during plaque isolation, we examined three examples of each of the most resistant mutants (those involving a change from Cys to Trp, Arg, or Tyr at position 199 and those involving a change from Val to Met at position 188) for variation in plaque phenotype and relative replication efficiencies. Plaque size and clarity were visualized either in MTT or after fixation and staining in crystal violet. Single-cycle infections (8 h; multiplicity of infection of 3) were conducted for each mutant on triplicate monolayer cultures. This was done by using both 2 μg of drug per ml and 0.1% DMSO only (0.4% BSA). The progeny virus titer was determined under the same conditions as during replication.

Virus crystallography. Wild-type HRV14 crystals (21) were soaked with various WIN compounds including WIN 51711, WIN 52084(S/R), WIN 52084(R), and WIN 52035 (2, 26). The crystals are of space group $P2_13$, with $a = 44.51$ nm. Each of the four virions in the unit cell sits on a crystallographic threefold axis. Thus, there is a 20-fold noncrystallographic redundancy. X-ray diffraction data were collected at the Cornell High-Energy Synchrotron Source by using oscillation photographs. These gave partial data sets (Badger et al., in press) extending to 0.3-nm resolution. Phase angles associated with the HRV14-WIN data and estimates for missing data were obtained by iterative real-space averaging within a molecular envelope starting from the phase set for the native structure (1, 21). The resultant maps were displayed and fitted with the appropriate structure. The coordinates were refined by real space map fitting (9), followed by geometry idealization. Coordinates and structure factors have been deposited with the Brookhaven Protein Data Bank.

RESULTS

Use of the dose-response curve to measure the frequency of drug-resistant mutants. A preliminary indication that resistance to WIN 52084 might be acquired by a single mutational event was obtained by measuring the effect of drug concentration on plaque titer (Fig. 2). Under the conditions of the assay (legend to Fig. 2), the inhibitory effect of the compound against wild-type virus was first detectable at about 0.05 μg/ml. With further increases in drug concentration, the curve descended rapidly until it reached a plateau at about 1 μg/ml. This plateau, corresponding to a frequency of survivors of about 4×10^{-5}, was in the same range (10^{-4} to 10^{-5}) as the frequency previously observed for single-step mutations with antibody escape mutants of HRV14 (25). At drug concentrations exceeding 7 μg/ml, the plaque titer again declined. We attribute this last decline to cytotoxicity which was manifested by both detachment of cells from the monolayer and poor staining with crystal violet. This toxicity could be counteracted by increasing the concentration of BSA in the media (not shown), probably because the protein binds a substantial fraction of free drug. The drug-binding capacity of the protein was also manifested by decreased sensitivity of the virus to the drug when high concentrations of BSA were present (data not shown).

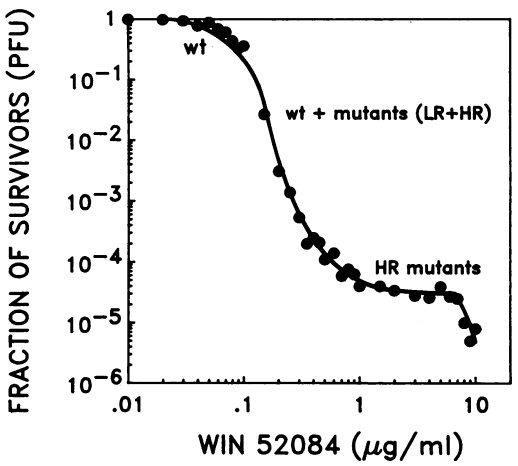

FIG. 2. Dose-response curve showing the frequency of plaque formers resistant to different concentrations of WIN 52084. A stock of HRV14 (produced by using a low multiplicity of infection) was pretreated and allowed to form plaques in the presence of drug, 0.4% BSA, and 0.1% DMSO. Surviving plaques were counted after 48 h of incubation at 35°C. Abbreviation: wt, wild type.

We confirmed that the plaques appearing on the lower plateau of the dose-response curve represented drug-resistant virus by selecting agar plugs from 40 plaques grown at 2 μg of drug per ml and replating the virus (roughly 100 PFU per plate) in the presence and absence of drug at 2 μg/ml, a concentration which totally inhibited the development of wild-type virus controls. All 40 drug-selected plaque isolates exhibited roughly the same infectivity titer in the presence of the drug as in its absence (data not shown); i.e., all 40 were high-resistance (HR) mutants. This experiment indicates that the fraction of survivors at the lower plateau region of the curve (4×10^{-5}) is a valid measure of the frequency of resistant mutants in the virus population.

Properties of mutants selected at low drug concentrations. Isolation of plaques at drug concentrations on the descending slope of the dose-response curve (0.1 to 0.4 μg/ml) revealed a second class of mutants able to form plaques at these low drug concentrations but not at a high (2 μg/ml) concentration; these were called low-resistance (LR) mutants. The relative proportions of HR and LR mutants found depended on the drug concentration used for selection.

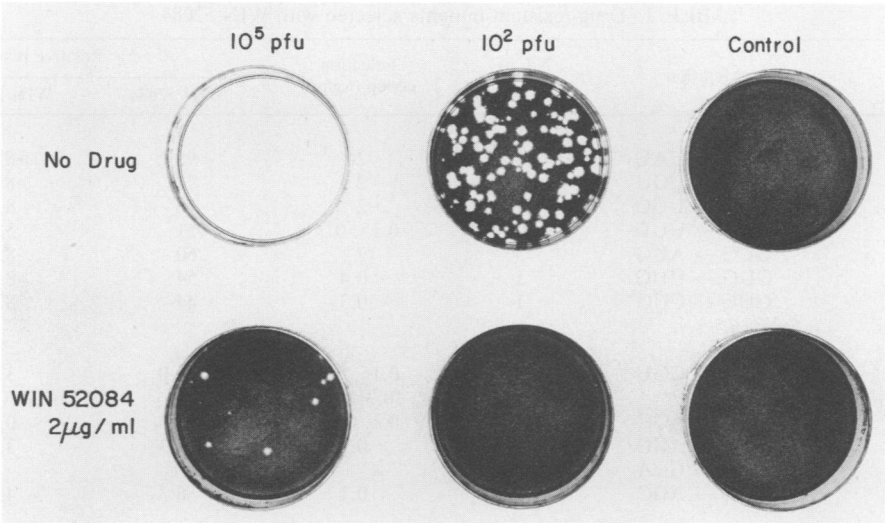

FIG. 3. Plaque method for selecting drug-resistant mutants. Each plate was inoculated with 10^5, 10^2, or 0 PFU that had been pretreated with 0 or 2 μg of WIN 52084 per ml in the presence of 0.4% BSA and 0.1% DMSO. Plates were incubated for 48 h at 35°C, fixed with Formalin, and stained with 0.1% crystal violet. Control plates show that the drug is not cytotoxic under these conditions.

Whereas about two-thirds of the plaques isolated at 0.15 μg/ml were LR mutants, about four-fifths of the plaques selected at 0.3 μg/ml were HR mutants (data not shown).

That the fraction of plaque survivors was not a valid measure of mutant frequency in the upper region of the dose-response curve was shown by increasing the plaque development time of wild-type virus (40 PFU) plated at 0, 0.05, 0.15 and 0.3 μg of WIN 52084 per ml. As expected, 0.05 μg of drug per ml showed no inhibitory effect on wild-type plaque formation by 48 or 72 h. When 0.15 μg of drug per ml was used, the plaque titer at 48 h (the standard plaque development time used in the dose-response curve) was about 10% of that for untreated controls and the plaques were significantly smaller and indistinct; by 72 h, however, nearly 50% of the inoculum scored as a plaque (with normal size and clarity). By contrast, no wild-type virus was able to form plaques after 48 or 72 h in 0.3 μg of drug per ml; that is, wild-type plaque formation was totally inhibited by the drug. Thus, it appears that the fraction of plaque survivors in the dose-response curve became a measure of mutant frequency at roughly 0.3 μg/ml, where the frequency (about 5×10^{-4}) resulted from the appearance of both LR and HR mutants. At lower drug concentrations, the frequency of plaques resulted from a combination of mutants plus wild-type survivors whose growth was slowed but not inhibited by the drug.

Selection of independent drug-resistant mutants. To ensure that each mutant represented an independent mutational event (i.e., that no two isolates were siblings), we isolated only one drug-resistant plaque from each individually amplified wild-type plaque. Mutants with a variety of plaque sizes were selected; the typical appearance of these plaques is shown in Fig. 3. Fifty-two HR mutants were isolated in 2 μg of WIN 52084 per ml; four additional HR mutants were selected in lower concentrations of the drug (Table 1). The LR mutants were isolated in low concentrations (0.1 to 0.4 μg/ml) of drug: two mutants were isolated at 0.1 μg/ml, three mutants were isolated at 0.15 μg/ml, three mutants were isolated at 0.2 μg/ml, three mutants were isolated at 0.3 μg/ml, and two mutants were isolated at 0.4 μg/ml.

Mutants resistant to WIN 52084. The RNA genomes of 69 independent mutants were sequenced by primer extension in regions encoding the drug-binding site. All of the mutations encoded amino acid substitutions. Moreover, the HR and LR classes of mutations mapped to different locations in the drug-binding site (Table 1). All 56 HR mutations mapped to just 2 of the 16 amino acids lining the drug-binding pocket: cysteine 199 of VP1, which lies at the base of the pore providing entry to the pocket, and valine 188 of VP1, which lies near the center of the pocket (Fig. 4 and 5). The side chains of both residues point into the drug-binding pocket (Fig. 4B), and, in each case, were replaced by larger ones (Cys-199 by Trp, Arg, Tyr, or Phe, and Val-188 by Leu or Met). Single LR mutations, on the other hand, were observed at three different amino acids (Table 1): Asn-105, Asn-219, and Ser-223. A double mutation at Asn-198 and Val-176 was also observed.

The mutation that induced HR most frequently (37 of 56 HR mutants) was substitution of Trp for Cys-199 (a transversion event, UGU to UGG). In an effort to explain this predominance, three examples of each of the most frequent HR mutants (containing the Cys-199-to-Trp, -Arg, and -Tyr, and the Val-188-to-Met mutations) were examined for plaque appearance and infectivity yield in both the presence and absence of the drug. We found no evidence of selection bias due to different plaque morphology, nor did the Cys-to-Trp mutant have a growth advantage either in the presence or absence of drug (data not shown). The selection of independent mutants ensures that these mutants were not siblings. Therefore, the predominance of Cys-199-to-Trp mutations may reflect a higher mutation rate at the third nucleotide relative to the other two, even though transversions are generally thought to be uncommon events.

The population of LR mutants was also dominated (9 of 13 LR mutants) by a particular mutation, Ser-223 to Gly (Table 1). Because the sample size is relatively small, however, the significance of this result is unclear.

Resistance of mutants to related WIN compounds. Although they are quite similar in structure, each WIN compound exhibits a unique spectrum of activity against different virus serotypes (4). Mutants selected with WIN 52084 were examined for resistance to two related WIN compounds (Fig. 6). WIN 51711 is identical to WIN 52084, except that it lacks the (*S*)-methyl substitution on the oxazoline ring, whereas WIN

TABLE 1. Drug-resistant mutants selected with WIN 52084

Amino acid[a]	Mutation	No. of mutants	Selection concn (μg/ml)	Relative resistance[b] to:		
				WIN 52084	WIN 51711	WIN 52035
HR mutants						
Cys-199 → Tyr	UGU → UAU	3	2	98	>8.8[c]	>9.1[c]
Cys-199 → Arg	UGU → CGU	7	2	77	>8.8	4.7
Cys-199 → Trp	UGU → UGG	37	2	76	8.6	2.9
Cys-199 → Phe	UGU → UUU	2	0.15, 0.3	51	5.1	1.4
Val-188 → Met	GUG → AUG	5	2	60	>8.8	>9.1
Val-188 → Leu[d]	GUG → UUG	1	0.4	54	>8.8	3.2
Val-188 → Leu	GUG → CUG	1	0.3	44	>8.8	3.5
LR mutants						
Ser-223 → Gly	AGU → GGU	9	0.15, 0.2 0.3, 0.4	5.0	3.4	2.0
Asn-105 → Ser	AAC → AGC	2	0.1, 0.15	7.4	0.4	6.8
{Asn-198 → Ser[e] {Val-176 → Ala	AAU → AGU GUA → GCA	1	0.2	4.3	3.1	3.2
Asn-219 → Ser	AAC → AGC	1	0.1	6.3	4.6	4.1
Wild type				1.0	1.0	1.0

[a] Residues of VP1 numbered 1 to 289 from amino end.
[b] Relative resistance measured as the MIC required to inhibit mutant plaque titer by 50% divided by MIC required to inhibit wild-type plaque titer by 50%; mean of three replicate assays of three or four mutants when available.
[c] Upper limit determined by drug solubility and low activity.
[d] Double mutant with Leu-112 to Phe (CUU → UUU); Leu-112 resides about 0.8 nm from the drug.
[e] Double mutant.

52035 differs from 51711 in that it has an aliphatic chain of five rather than seven methylenes.

In some cases, for example, the mutant containing the Cys-199-to-Tyr mutation, low activities and solubilities of WIN 51711 and WIN 52035 imposed an upper limit on relative drug resistance (Table 1). Therefore, this mutant is considered highly resistant to these two drugs because it could form plaques in the presence of the highest concentration of WIN 51711 and WIN 52035 that could be tested. A mutation that confers high resistance to one drug does not necessarily confer high resistance to other drugs (Table 1). For instance, the mutants containing the Cys-199-to-Arg, -Trp, or -Phe mutations, which were highly resistant to WIN 52084, were much less resistant to WIN 52035. Therefore, the degree of resistance must be defined relative to a particular compound.

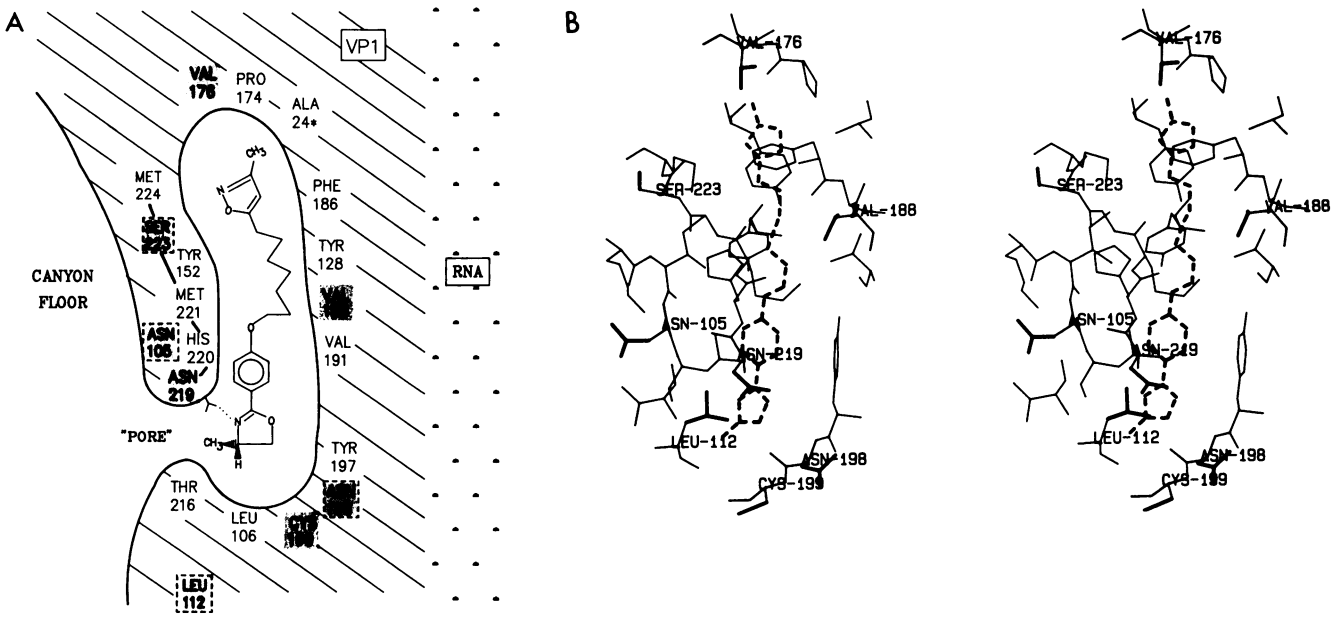

FIG. 4. Orientation of WIN 52084 and identity of the amino acid residues lining the wall of the drug-binding pocket in VP1. (A) Diagram showing the relative location of side chains in the stereo diagram shown below. Shaded residues mutated spontaneously to produce drug resistance. Residues with dashed borders lie outside the 0.4-nm boundary used to define the pocket wall. Ala-24* belongs to capsid chain VP3. (B) Molecular graphics stereo diagram showing orientation of amino acid side chains around the drug (- - -). Side chains which mutate to drug resistance are labeled and shown by heavy lines.

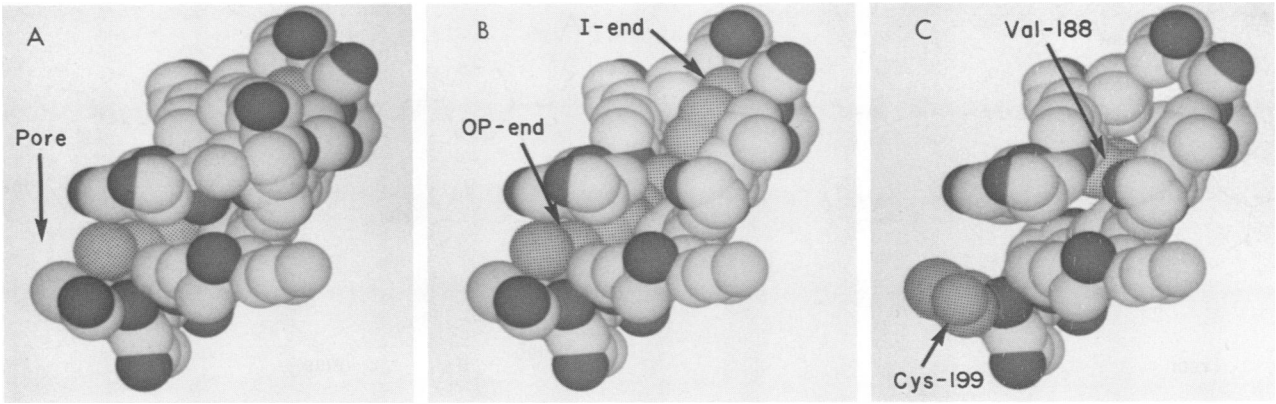

FIG. 5. (A) Space-filling model of WIN 52084 (stippled atoms) within the drug-binding pocket viewed directly down the z-axis. The promoter fivefold axis is up. The oxazoline end of the drug is visible near the pore at the floor of the canyon. The amino acids shown are as in Fig. 4, except for the omission of Leu-112. (B) Exposure of the drug by removal of four residues (Lys-103, Ile-104, Ser-223, and Met-224). (C) Structure of the pocket in its drug-deformed state with the drug removed. The stippled residues are the only ones that mutated spontaneously to confer high resistance.

Our results demonstrate that the inhibitory effect of the drug depends upon the length of the aliphatic chain and orientation of the drug within the pocket. Thus, most of the HR mutants to WIN 52084 were also HR mutants with respect to WIN 51711, the seven-methylene compound that binds within the pocket in the reverse orientation relative to WIN 52084 (2). These mutants were not equally resistant to the shorter (five-methylene) WIN 52035, however, although this drug is oriented within the pocket in a manner analogous to WIN 51711 (2). Because WIN 52035 is smaller than WIN 52084, it may shift position slightly within the mutated binding pocket to alleviate unfavorable bonding interactions. Two mutations (Cys-199 to Tyr and Val-188 to Met) conferred high resistance to WIN 52035; the other substitutions at these residues conferred intermediate or minimal resistance to this compound.

DISCUSSION

Relative frequencies of HR and LR mutants. We have shown that the dose-response curve is a true measure of mutant frequency at drug concentrations of about 0.3 μg/ml or above. This raises the question of why LR mutants were seen at a frequency at least 10-fold higher than HR mutants (Fig. 2). There are two possible explanations for this high frequency. First, the population of survivors at low drug concentrations was composed of both LR and HR mutants (HR mutants accounted for about one-third of the plaques selected at 0.15 μg/ml and about four-fifths of the plaques selected at 0.3 μg/ml). Second, and probably more important, there may be a larger number of mutational pathways to LR than to HR mutants. A total of 56 independent HR mutants resulted from substitutions at only two residues in the drug-binding pocket; by contrast, 13 LR mutants selected in WIN 52084 resulted from substitutions at at least three residues (Table 1). Two additional LR mutants (Asn-100 to Ser and Val-153 to Ile) were recently identified by isolating mutants in 7 μg of a different drug, WIN 52035, per ml (data not shown). Furthermore, substitutions in several other LR mutants have not yet been located, indicating that their mutations lie outside the region of viral RNA sequenced (amino acids within 0.5 nm of the drug).

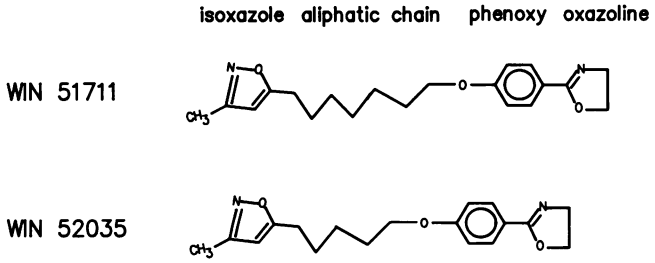

FIG. 6. Structures of the antiviral drugs WIN 51711 [5-[7-[4-(4,5-dihydro-2-oxazolyl)phenoxy]heptyl]-3-methylisoxazole] and WIN 52035 [5-[5-[4-(4,5-dihydro-2-oxazolyl)phenoxy]pentyl]-3-methylisoxazole] drawn in the reverse orientation relative to WIN 52084 to indicate their preferred binding orientation (with the isoxazole ring pointing away from the viral fivefold axis). These compounds were used to correlate the degree of resistance with the drug structure and orientation within the binding pocket.

Drug resistance results from single mutations. The primer extension method used to detect mutations scans only about 20% of the viral coat gene. However, two lines of evidence support the idea that resistance can be acquired by a single mutational event. First, the frequency of mutants was similar to that of single-step antibody-escape mutants (24), and second, crystallographic analyses of two mutants (Badger et al., in press) detected only one mutation in the crystallographically visible regions of the coat protein (all except 16 residues at the amino end of VP1 and 25 residues at the amino end of VP4 [21]). We plan to verify this conclusion by introducing single mutations into wild-type HRV14 by using site-directed mutagenesis.

Molecular basis for high resistance. The finding that the side chains of Cys-199 and Val-188, which point into the drug-binding pocket, are always replaced by bulkier side chains suggests that high resistance to WIN 52084 results from exclusion of the drug from its binding site. In theory, exclusion can occur in two ways: by blocking entrance to the pocket (by restricting entry of the drug through the pore at the floor of the canyon) or by steric interference with the usual seating of the drug within the pocket. Cys-199 lies just under the pore, where mutations to a larger side chain might be expected to block the entryway. Examination of the resistance pattern of the Cys-199-to-Tyr mutant to related

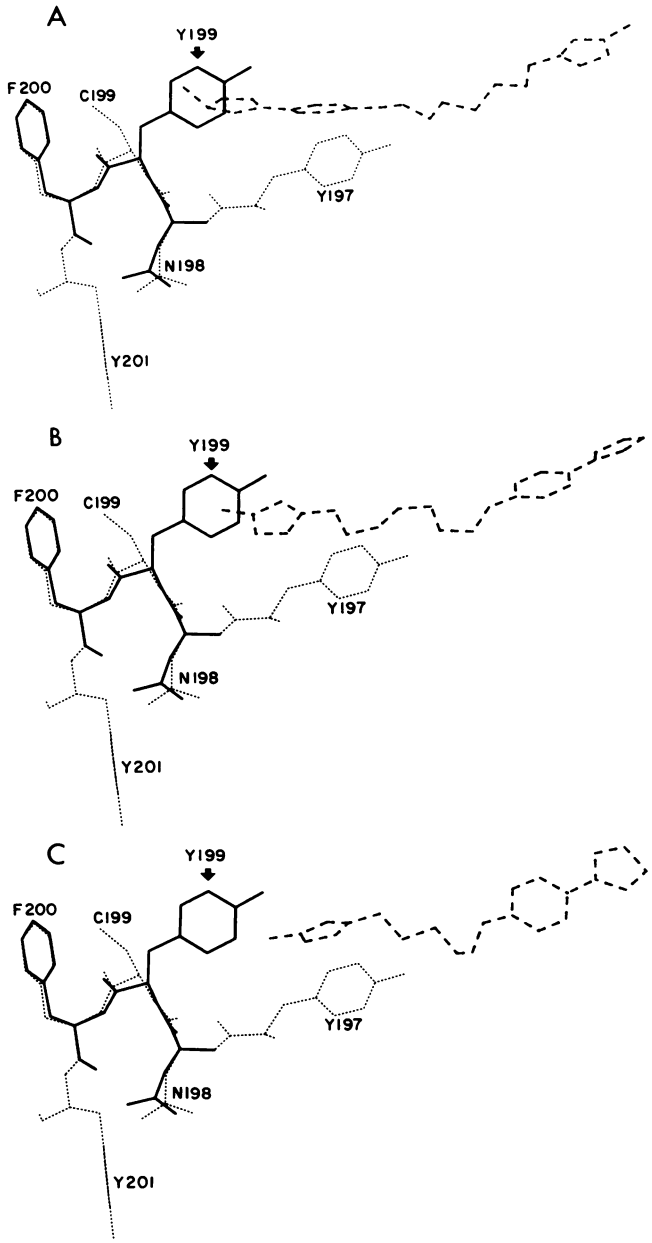

FIG. 7. Superpositions of WIN 52084 (A), WIN 51711 (B), or WIN 52035 (C) in the drug-binding pocket of the Cys-199-to-Tyr mutant, illustrating the unfavorable steric interaction between the seven-methylene compounds and Tyr-199. Note the shift in the peptide C backbone of the mutant. ----, Drug; . . . ., wild-type structure (amino acids 197 to 201 of VP1); ——, mutant structure (amino acids 198 to 200 of VP1).

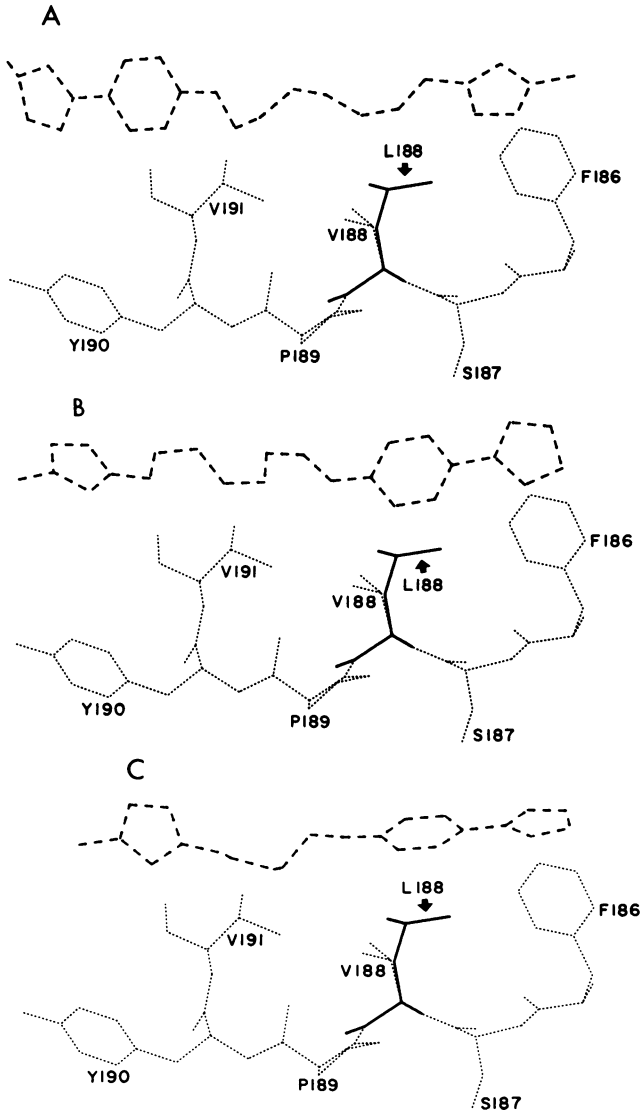

FIG. 8. Superpositions of WIN 52084 (A), WIN 51711 (B), or WIN 52035 (C) in the drug-binding pocket of the Val-188-to-Leu mutant. The degree of resistance to these drugs can be explained by the position and orientation of the phenyl ring relative to Leu-188. ----, Drug; . . . ., wild-type structure (amino acids 186 to 191 of VP1); ——, mutant structure (amino acid 188 of VP1).

WIN drugs (Table 1) and the atomic structure of this mutant (Badger et al., in press) (Fig. 7) supports this idea. The bulky Tyr side chain shortens the drug-binding pocket about 0.7 nm and would interfere with the predicted seating of the seven-methylene compounds WIN 52084 and WIN 51711 (see overlap between drug and Tyr in Fig. 7A and B); a lesser degree of interference is seen with the shorter compound, WIN 52035 (Fig. 7C). However, this mutant is highly resistant to WIN 52035 (Table 1). These results support crystallographic evidence (Badger et al., in press) that the Tyr side chain blocks the pore entryway. Replacing Cys-199 with Trp, Arg, or Phe, on the other hand, did not result in an HR mutant to WIN 52035 (Table 1). This is consistent with a molecular model of the Trp mutant (Badger et al., in press) which predicts more space within the binding pocket of this mutant than was found in the pocket of the Tyr mutant. However, this model also predicts that the pore will be partially blocked by the Trp side chain. This apparent contradiction raises the possibility that the roof of the pocket is not static; drugs may enter through a pore expanded by transient elevation of the roof.

The structure of the Val-188-to-Leu mutant (Badger et al., in press) (Fig. 8) and its resistance to related WIN drugs (Table 1) support the concept that high resistance in this mutant is due to steric interference with drug binding. The Leu-188 side chain, which projects into the binding pocket, is predicted (Badger et al., in press) to make close contacts

(0.23 nm) with both WIN 52084 and WIN 51711 in their expected orientations (Fig. 8A and B, respectively); interference is expected to be even greater in the Met-188 mutant. The Leu-188 mutant is still sensitive to WIN 52035, however (Table 1). This suggests that the shorter drug can relieve the unfavorable interaction with Leu-188 by shifting its position within the binding pocket. This shift is presumably not possible when Val-188 is replaced by the still bulkier Met residue.

Molecular basis for LR. Because the structures of LR mutants have not yet been solved, the basis for their drug resistance is still open to speculation. One explanation is that the mutations associated with these mutants alter virus drug-binding interactions. This might occur by decreasing the amount of space within the pocket, by interfering with the proposed hydrogen bond at Asn-219 (26), or by affecting the flexibility of the chains that move when the drug inserts, causing subtle changes in the three-dimensional contours of the binding pocket. We are using crystallographic analysis of each mutant in the presence and absence of drugs to investigate these hypotheses.

An alternate explanation for the molecular basis of LR is suggested by the fact that the primary mode of action of the WIN compounds on HRV14 is to block attachment to cells (19). In contrast to the HR mutations, all five of the single mutations found in LR mutants (Asn-105 to Ser, Asn-219 to Ser, Ser-223 to Gly, and the mutations forming the two additional mutants that were isolated in 7 μg of WIN 52035 per ml, Asn-100 to Ser and Val-153 to Ile) occurred in amino acids that reside on portions of the polypeptide chain that are deformed when the drug is inserted (Fig. 9A); these amino acids reside on or just below the canyon floor (22) (Fig. 9B). It is therefore likely that some of the LR mutants are altered at the receptor-binding site. On the other hand, the ability of high concentrations of WIN 52084 to inhibit the binding of HRV14 to cells does not necessarily mean that one or more of the loops which move on binding participate directly in binding to receptors. Instead, the drugs may inhibit a reversible conformational change required for tight binding to receptors. The role of Ser-223 in virus attachment has already been reported by Colonno et al. (3), who showed that substituting Ala for Ser-223 decreases the binding affinity of the virus for HeLa cells. We have preliminary evidence (not shown) that the Ser-223-to-Gly mutation reduces the attachment-inhibiting effect of the drug. Because attachment of this LR mutant is only weakly inhibited by a concentration of WIN 52084 that prevents plaque formation (2 μg/ml), the compound must be interfering with a different step in infection, possibly uncoating, in this mutant.

Our results suggest that there are two different mechanisms of drug resistance: several HR mutants appear to decrease the available space within the drug-binding pocket, whereas some LR mutants relieve the attachment-inhibiting effect of the drug. It is, however, unlikely that the mechanism of resistance will be determined by the mutant class. Recent studies involving the use of an assay in which bound drug is detected as enhanced viral thermostability (B. A. Heinz, D. A. Shepard, and R. R. Rueckert, *in* W. G. Laver and G. Air, ed., *The Use of X-Ray Crystallography in Design of Antiviral Agents*, in press) indicate that at least one HR mutant (containing the Cys-199-to-Phe mutation) also relieves the attachment-inhibiting effect of bound drug.

What determines the skewed distribution of HR mutants? The goal of these studies was to characterize many independent drug-resistant mutants and to identify most of the amino acid residues that influence drug activity. Mutants resistant

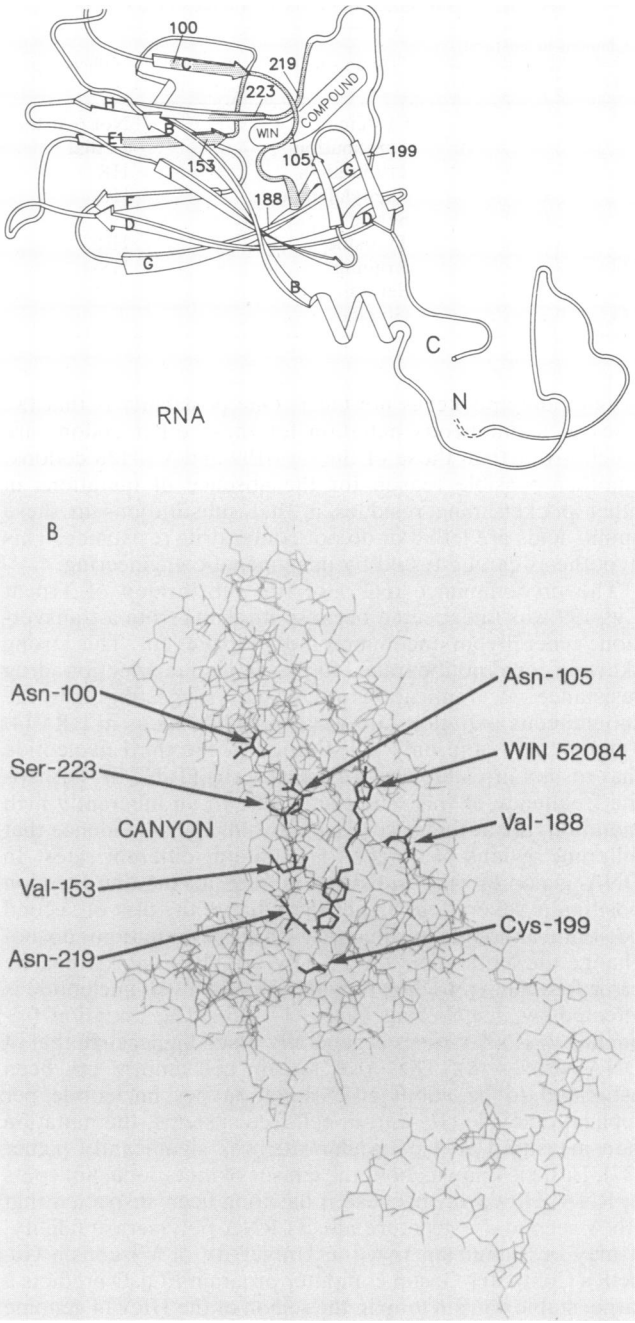

FIG. 9. (A) Ribbon drawing of VP1, showing the locations of mutations that confer drug resistance. Dotted regions of the polypeptide chain deform the canyon floor when the drug inserts. The position of bound WIN compound is indicated. (B) Molecular graphics diagram of VP1, showing the locations of HR and LR mutations relative to WIN 52084 and the canyon floor.

to high concentrations of drug revealed only two such residues: Cys-199 and Val-188. Nine other residues lining the drug-binding pocket are potential sites for HR mutations in the sense that their side chains point into the pocket and could be replaced by a larger amino acid through a single nucleotide change: Ile-104, Leu-106, Ser-107, Leu-116, Pro-174, Val-176, Val-191, and Asn-219 of VP1, and Ala-24 of VP3 (Table 1 in Badger et al., in press). Why no mutations were observed at any of the other amino acids lining the

TABLE 2. Possible single-base changes at Cys-199

Changes at position:			Amino acid	Observed	Phenotype
1 (U)	2 (G)	3 (U)			
		C	Cysteine	−	Not resistant
		A	Termination	−	Lethal
		G	Tryptophan	+++	HR
	U		Phenylalanine	+	HR
	C		Serine	−	?
	A		Tyrosine	+	HR
C			Arginine	+	HR
A			Serine	−	?
G			Glycine	−	?

drug-binding pocket is not clear. One possibility is that the rates of spontaneous mutation for these other codons are much lower than those of the Cys-199 and Val-188 codons. Another possible reason for the absence of mutations in other pocket-lining residues is that substitutions in these amino acids are lethal or do not confer drug resistance. This hypothesis can be tested by using genetic engineering.

The predominance (66%) of the substitution of Trp at Cys-199 was unexpected because it results from a transversion, generally an uncommon mutational event. This strong skewing could not be traced to bias in plaque selection, drug resistance, or replication advantage, suggesting that the spontaneous mutation rate varies with the locus in HRV14. Tryptophan is the only substitution at the third nucleotide that results in viable drug-resistant virus (Table 2). Perhaps the frequency of this mutation is due to an inherently high mutation rate at this base. There is substantial evidence that different regions of a genome mutate at different rates. In DNA genomes, the substitution rate at the third codon position is severalfold higher than that at the first or second position, although the majority of these mutations do not change the amino acid (10). There are also reports that the error frequency of DNA polymerase at a given nucleotide is affected by neighboring bases (17, 18). The mutation frequency of RNA viruses is generally much higher than that of DNA viruses (8). The error rate in cell culture has been estimated to be about 10^{-6} mutation per nucleotide per replication cycle (16, 23); in cell-free systems, the mutation rate measured at a particular site was significantly higher (27). Little is known about the causes of mutational hot spots in RNA. However, because it has long been suspected that RNA secondary structure affects RNA polymerase fidelity, it may be significant that the University of Wisconsin Genetics Computer Group computer program FOLD predicts a large, stable hairpin loop in the region of the HRV14 genome encoding the drug-binding pocket, with Cys-199 situated nearly at the apex.

Single-step mutations at either of the first two bases in the cysteine codon would produce two mutants not observed, substituting either serine or glycine for cysteine (Table 2). These mutants, if viable, may well prove sensitive to the drug, because the substituted residues have small side chains. We plan to produce these mutants by genetic engineering to test this hypothesis.

Speculations on the natural function of the drug-binding pocket. The repeating protein subunits of icosahedral RNA plant and animal viruses all appear to consist of eight-stranded antiparallel β-barrels, with the interior forming a hydrophobic pocket (20). The size and shape of the pocket vary with the virus serotype. For example, the drug-binding pocket of HRV1A has greater similarity to that of HRV14 complexed with drug than to that of native HRV14 (S. Kim, T. J. Smith, M. G. Rossmann, D. Pevear, F. J. Dutko, P. J. Felock, and M. A. McKinlay, submitted for publication). Furthermore, some hydrophobic pockets, such as those in mengovirus (11) and those within VP2 and VP3 of HRV14 (20), are not accessible for binding of drugs such as the WIN compounds.

The ubiquitous presence of these hydrophobic pockets suggests that they perform a natural role in virus replication. This role is likely to involve viral uncoating: it has been proposed (20, 26) that loose packing of polypeptide chains making up an empty drug-binding pocket is needed to accommodate conformational changes during disassembly. Moreover, the primary effect of the WIN compounds on viruses other than HRV14, such as HRV2 and poliovirus (5, 28), is to block uncoating. Thus, the inhibition of attachment seen in HRV14 may be a fortuitous side effect of drug insertion. This hypothesis is supported by the observation that the mutant containing the Ser-223-to-Gly mutation is inhibited by WIN 52084 at a step other than attachment. The fact that structurally different WIN compounds cause the same deformation at the HRV14 binding site suggests that this region of the capsid can exist in at least two conformations. The WIN compounds may function by stabilizing the capsid in one of its conformations.

An alternate hypothesis is suggested by the detection by Hogle et al. (7) of some electron density that could be a long-chain aliphatic molecule in the hydrophobic pockets of poliovirus type 1 Mahoney. This raises the possibility that the WIN compounds exert their inhibitory effect by displacing a pocket factor vital for replication. On the other hand, this pocket factor may simply diffuse out of poliovirus at the appropriate stage during infection. Finally, Smith et al. (26) have proposed that the presence of these molecules in the virion could be an artifact of virus purification. Thus, it remains unclear whether the pocket factor in poliovirus is relevant to the natural function of the picornavirus drug-binding pocket.

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