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Sequence Determinants of 3A-Mediated Resistance to Enviroxime in Rhinoviruses and Enteroviruses

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Using site-directed mutagenesis of the 3A coding region of rhinovirus 14, we have expanded our analysis of resistance to enviroxime. We have observed that high and low levels of drug resistance involve two domains within 3A and that the amino acid at position 30 is critical in determining resistance.

The *Picornaviridae* are a large family of viruses which include many significant human pathogens, notably the human rhinoviruses (RV) and the enteroviruses (whose prototype is poliovirus). These viruses have a positive-sense, single-stranded RNA genome that encodes seven proteins required for RNA replication and/or protein processing. The specific roles of several of the proteins involved in viral RNA replication remain poorly understood. The production of single-stranded RNA occurs within virus-induced lipid-containing cytoplasmic vesicles known as replication complexes (1, 2). The 3B protein, also known as VPg, serves as a primer for plus-strand RNA synthesis (10, 11, 14–16). Because 3B is a highly polar polypeptide, however, it is thought to require a hydrophobic carrier to facilitate attachment to membranes. This attachment is most likely via the conserved hydrophobic domain of the 3AB precursor protein (4, 5, 13); this domain is membrane associated in virus-infected cells (16). In addition to its role in membrane association, recent in vitro studies have demonstrated a physical interaction between 3AB and the 3D polymerase (7, 12); this association appears to function in vivo as well (9). The interaction between 3AB and 3D probably involves the charged amino acids that lie upstream of the hydrophobic domain of 3A (3, 17). Thus, 3A may be involved in linking one or more of the viral replication proteins to the intracellular membranes, thereby functioning as a scaffolding protein for the replication complex.

The analysis of resistance to antiviral drugs has proven to be an extremely useful approach for investigating the natural role of picornavirus proteins. We previously reported that mutants of RV14 and poliovirus 1 that demonstrate resistance to the antiviral compound enviroxime carry mutations which map to the 3A coding region (6). Our data suggested that two regions of the 3A protein might play a role in determining drug resistance. In this study, we have characterized more spontaneous mutants and used oligonucleotide-directed mutagenesis of RV14 to enhance our understanding of the sequence determinants of 3A-mediated drug resistance. In addition, we have analyzed resistance in two enteroviruses, coxsackievirus B3 (CB3) and CA21.

HeLa cell cultures, selection and analysis of spontaneous viral mutants, and enviroxime stocks were prepared as described previously (6). Identified mutations were confirmed and new mutations were introduced by site-directed mutagenesis using techniques previously reported (6). Because the mutants are not sufficiently resistant to form plaques in the presence of drug, we analyzed viral drug resistance by comparing virus yields following an extended infection (24 h for RV14, 18 h for CB3, and 21 h for CA21) at a multiplicity of infection (MOI) of 1 in the presence or absence of drug; harvested virus was quantitated by plaque assay in the absence of drug.

On the basis of results observed with spontaneous RV14 drug-resistant mutants (6), we selected two regions of the 3A protein to explore more fully. First, we had observed that the Glu-30 residue seemed to be particularly important for determining drug resistance. Similar levels of resistance resulted when Glu-30 was replaced by Gln, Asp, or Val, suggesting that the character of the substituted amino acid was not critical. To test this theory, we constructed and analyzed RV14 mutants containing all 20 possible amino acids at the Glu-30 site. Surprisingly, all of the constructs except that containing Pro-30 resulted in viable virus. Moreover, all of the viable substitutions conferred a drug-resistant phenotype (Fig. 1). Resistance varied from 1.2 to 10.3% survivors (compared with 0.06% for wild-type virus). Only three substitutions (Gly, Arg, and Tyr) resulted in virus manifesting a small-plaque phenotype. The highest level of resistance resulted from Gly, Tyr, and Trp substitutions, none of which had been observed in spontaneous mutants. This is probably because these mutations either produced small-plaque-forming variants (and thus were less likely to be selected) or required more than one nucleotide mutation to occur. The tolerance of amino acid structure at the Glu-30 site suggests that it may lie on the outside of the folded 3A protein (or, perhaps, that the side chain points into a pocket). Furthermore, since most substitutions at this site conferred resistance, it is plausible that the drug interacts directly with this region of the protein.

Second, comparisons between sites conferring resistance in RV14 and poliovirus 1 had suggested that both the hydrophobic domain (specifically, Ser and Thr residues at positions 68 to 70) and the N-terminal charged patches of 3A might be involved in determining resistance to enviroxime (6). To test this idea, we analyzed the resistance of RV14 mutants that were altered in one or both of these regions (Fig. 2). RV14 mutants that contained an Ala substitution at Ser-69 were viable but not resistant, regardless of whether they also contained an N-terminal mutation (either Glu-30 to Gln or Met-54 to Ile). The addition of a third hydroxyl-containing residue in this region of the hydrophobic domain (i.e., by substitution of a Thr for Val at position 68) was not sufficient to confer drug resistance, nor could it substitute for the role played by Ser-69. Interestingly, the finding that Val-68 and Ser-69 are not interchangeable supports the computer-generated prediction that the hydrophobic domain of 3AB forms an amphipathic alpha-helix (8).

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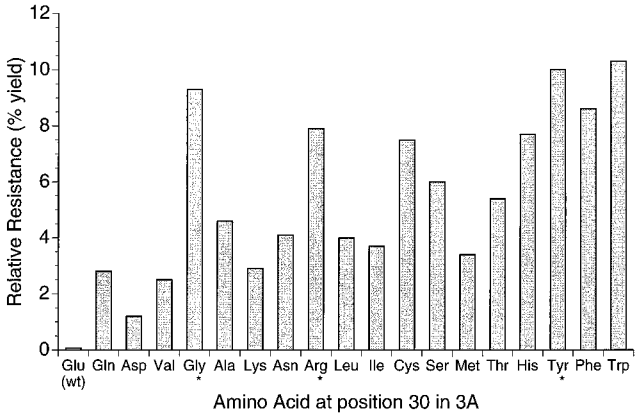

FIG. 1. Resistance of RV14 mutants containing all viable amino acids at position 30 introduced into a cDNA clone by oligonucleotide-directed mutagenesis. Asterisks indicate substitutions conferring a small-plaque phenotype. Resistance was measured as virus yields in PFU following one 24-h infection in monolayer cultures at an MOI of 1 in the presence of 1 μg of enviroxime per ml. Numbers are percent virus yields relative to no-drug controls. wt, wild type.

Using three viral mutants (carrying Ala-69, Ala-69 plus Gln-30, or Ala-69 plus Ile-54) as the parental inocula, we selected and analyzed independent isolates that arose during growth in 1 μg of enviroxime per ml (10 per parental stock). Because these parental stocks were growth compromised (Fig. 2A), this procedure selected for vigorous growth as well as drug resistance. For the double mutants (Ala-69 with either Gln-30 or Ile-54), we anticipated that drug resistance would result exclusively from mutations at the Ala-69, either by a reversion back to Ser or by a Thr substitution (the substitution preferred by resistant poliovirus mutants [6]); either amino acid substitution could occur via a single nucleotide change. This was observed for the Gln-30-containing double mutant: all isolates produced large plaques, showed a high level of resistance to enviroxime, and contained the reversion to Ser-69. In contrast, the Ile-54 plus Ala-69 double mutant reverted in only 1 of 10 cases. Moreover, the only other drug-resistant isolate showed a very low level of resistance. This mutant had a third mutation introduced into the 3A coding region (Asp-28 to Ala) and retained its intermediate plaque size. We confirmed the resistance phenotype of the triple mutant and the corresponding single and double mutants containing Asp-28 to Ala by site-directed mutagenesis (Table 1). It is interesting that Asp-28 lies in close proximity to the critical Glu-30 residue and that Ala-30 also confers a high level of resistance.

In order to understand why the two Ala-69 double mutants had given rise to different populations during selection in drug, we compared their levels of resistance to enviroxime at five drug concentrations: 0.2, 0.4, 0.6, 0.8, and 1 μg/ml (Table 2). Since 0.2 μg/ml represents a concentration 10-fold over the 50% inhibitory concentration (and thus drug excess), these values reflect the population of resistant variants at each drug level. All of the viruses showed similar levels of resistance to 1

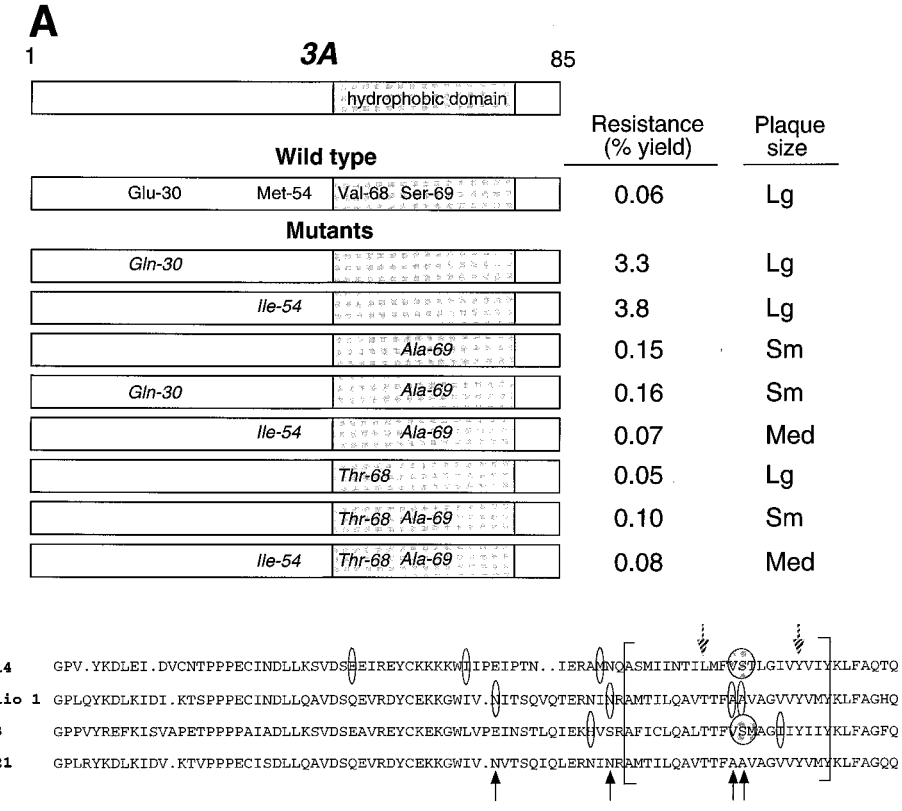

FIG. 2. (A) Schematic diagram illustrating the locations of mutations introduced into the 3A coding sequence of a cDNA clone of RV14. Relative resistance was measured as virus yields in PFU following one 24-h infection in monolayer cultures at an MOI of 1 in the presence of 1 μg of enviroxime per ml. Plaque sizes were determined in the absence of drug. Lg, large; Sm, small; Med, medium. (B) Sequence alignments of the 3A coding sequences of four viruses showing the locations of mutations conferring resistance to enviroxime (open ovals). Brackets indicate the hydrophobic domain; stippled circles highlight the hydroxyl-containing residues postulated to play a role in drug resistance. Filled arrows indicate residues of CA21 that are analogous to sites conferring drug resistance in poliovirus; striped arrows indicate mutations in RV14 which, when coupled with the Ala-69 mutation, conferred a relatively low level of drug resistance.

TABLE 1. Analysis of RV14 mutants containing multiple mutations in 3A[a]

Amino acid substitution(s)	Relative resistance (% yield)[b]
Wild type	0.06
Asp-28 to Ala plus Ser-69 to Ala plus Met-54 to Ile	0.47
Asp-28 to Ala plus Met-54 to Ile	7.6
Asp-28 to Ala	4.8
Ser-69 to Ala plus Tyr-75 to His	0.30
Ser-69 to Ala plus Leu-65 to Met	0.26

[a] Amino acid substitutions were introduced into a cDNA clone by oligonucleotide-directed mutagenesis.

[b] Because mutants grow poorly in the presence of drug, resistance was measured as PFU following one three-cycle (24-h) infection in monolayer cultures at an MOI of 1 in the presence of 1 μg of enviroxime per ml. Numbers are percent virus yields relative to no-drug controls.

μg of enviroxime per ml, the concentration used during the selection procedure. For the Ala-69 plus Gln-30 double mutant, the level of resistance was nearly the same at 0.2 μg of drug per ml. This result suggests a relatively homogeneous population of resistant mutants perhaps due, in part, to the strong selective pressure caused by compromised growth. On the other hand, wild-type virus and the Ala-69 plus Ile-54 double mutant did not reach their final population of mutants until about 0.6 μg of drug per ml was present. This result suggests that that these two virus populations may permit a greater variety of mutations conferring resistance (though perhaps low levels of resistance) than does the Gln-30 double mutant. Thus, an inherent lack of resistance of the Ile-54-containing double mutant does not explain our inability to isolate drug-resistant virus. It is possible that the presence of a mutation at Met-54 alters the mutation frequency at the Ser-69 residue, perhaps by changing the stability of the folded RNA. Alternatively, the mutation frequency might be affected by the presence of the drug.

It was also surprising that all 10 of the isolates selected from the parental stock containing the single substitution at Ala-69 showed drug resistance (at levels ranging from 6- to 53-fold higher than that shown by wild-type virus). In several cases, the resulting virus population was partially reverted back to Ser at position 69, suggesting that the new mutants were genetically unstable. We chose to explore three mutants more closely: one that showed a complete reversion of Ala-69 to Ser in the absence of other mutations in 3A (its level of resistance was 16-fold higher than that of the wild type), one that had a second mutation (to His) introduced at Tyr-75, and one that had a second mutation (to Met) introduced at Leu-65. The Ala-to-Ser revertant is presumed to contain a mutation in a coding region other than 3A which is responsible for the resistance phenotype. Because of reports linking the 3D polymerase to 3AB (7, 12), we chose to sequence this region; however, no additional mutations were found in 3D. The low-level-resistance phenotypes of the Ala-69 double mutants were confirmed by site-directed mutagenesis (Table 1). It is not obvious why these substitutions compensate even slightly for the loss of the critical Ser residue at position 69.

In order to enhance our understanding of sequence determinants conferring resistance to enviroxime, we characterized 9 isolates of CB3 and 10 isolates of CA21 selected in the presence of drug. Wild-type stocks of CB3 and CA21 showed levels of sensitivity to enviroxime sufficient for mutant selection, with 0.1 and 0.01% yields after selection in 1 μg/ml,

TABLE 2. Dose-response relationship for selected RV14 mutants

Amino acid substitutions	Relative resistance (% yield) in the presence of drug at the following concn (μg/ml)[a]:				
	0.2	0.4	0.6	0.8	1.0
Wild type	9.6	0.5	0.08	0.06	0.06
Ser-69→A + E-30→Q	0.34	0.30	0.28	0.28	0.20
Ser-69→A + Met-54→I	15.4	0.74	0.17	0.16	0.12

[a] Resistance was measured as PFU following one 24-h infection in monolayer cultures at an MOI of 1. Numbers are percent virus yields relative to no-drug controls.

respectively. As expected, the nine CB3 mutants had a resistant phenotype and contained single amino acid substitutions in the 3A coding region: eight (to Tyr) mapped to His-57 and one (to Thr) mapped to Ile-77. The predominance of the Tyr-57 mutation could be explained by its higher level of resistance, i.e., 18 to 34% survivors in contrast to 0.5% survivors for the Thr-77 mutant. It may be significant that the Thr-77 substitution adds a second hydroxyl-containing residue near the critical region within the hydrophobic domain. Despite the fact that wild-type CA21 is more sensitive to enviroxime than wild-type CB3 is, however, we were unable to isolate any resistant mutants of CA21 (that is, all 10 isolates selected showed resistance levels equivalent to that of wild-type virus). Alignment of the 3A coding sequences for RV14, poliovirus 1, CB3, and CA21 (Fig. 2B) does not suggest any explanation for the inability to produce drug-resistant variants; in the critical regions of 3A, the CA21 sequence is very similar to that of poliovirus 1. Thus, we must again conclude that resistance to enviroxime is influenced by factors other than the primary sequence of 3A. These factors might include the three-dimensional conformation of 3A or the conformation of another protein(s) which must function in association with 3A. Alternatively, as for the Ala-69 plus Gln-30 double mutant, the mutation frequency might be affected by the presence of the drug.

In conclusion, the unique role of 3A in viral replication and its relative intolerance of mutational variability (4) make it an excellent target for the development of antiviral agents. Thus, we are continuing to use enviroxime to probe the structure and function of the 3A protein and to help to identify other viral proteins that might function in association with 3A. In addition, studies designed to determine the actual binding site of enviroxime are under way.

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