JOURNAL OF VIROLOGY, Feb. 1997, p. 1697–1702 Vol. 71, No. 2
0022-538X/97/$04.00+0

Rhodanine Resistance and Dependence of Echovirus 12: a Possible Consequence of Capsid Flexibility

WERNER KRAUS, HOLGER ZIMMERMANN, HANS J. EGGERS, AND BIRGIT NELSEN-SALZ*

Institut für Virologie der Universität zu Köln, 50935 Cologne, Germany

Received 5 August 1996/Accepted 25 October 1996

Recombinant viruses of echovirus 12 carrying mutations of a rhodanine-resistant or -dependent variant, were investigated, and five single mutations each inducing a rhodanine-resistant or -dependent phenotype were defined. Four mutations are localized in the capsid protein VP1, and the fifth exchange is in VP4. All original and recombinant viruses were shown to be stabilized by the antiviral drug rhodanine against heat inactivation. Hence, resistant and dependent variants still seem able to bind rhodanine, and apparently none of the exchanges affects the putative drug binding site. We hypothesize that drug resistance and dependence are consequences of an increased flexibility of the virus capsid.

Echoviruses are the largest subgroup of enteroviruses, one of the five genera of the family *Picornaviridae*. Although serious clinical syndromes are associated with many of the 32 different serotypes known, e.g., aseptic meningitis, myocarditis, or encephalitis, early events in the viral infectious cycle are not yet sufficiently understood. Antiviral compounds interacting with the capsid and stabilizing the virus turned out to be helpful to investigate initial steps of virus infection. Early experiments in this field were performed with rhodanine and arildone, specific inhibitors of echovirus type 12 and poliovirus, respectively (4, 5, 9). The organic compound rhodanine (2-thio-4-oxothiazolidine) selectively inhibits uncoating of echovirus 12, but other processes of the viral infectious cycle are not affected (5, 6). It has been shown with labelled compound that rhodanine binds to the virus capsid (data not shown) and prevents formation of noninfectious "A particles" thereby maintaining intact, infectious virions associated with the host cell (5, 14).

Isolation and cloning of a rhodanine-dependent variant of echovirus 12. Echovirus 12 strain Travis 2-85 (supplied by A. B. Sabin) was isolated from the human intestine and may cause diseases such as diarrhea or aseptic meningitis. Individually amplified wild-type plaques were serially passaged in the presence of 100 μg of rhodanine/ml of medium and without the compound, respectively, as described for the resistant variant (8). Virus mutants growing exclusively in the presence of the antiviral drug were again plaque purified, and after five passages in cell culture a drug-dependent variant was selected which grows in the presence of the drug to an infectious titer of 10^8 PFU/ml, whereas in the absence of rhodanine only 10^3 PFU/ml is produced. Since early events in the virus infectious cycle are influenced by rhodanine and steps beyond uncoating of RNA are not affected, crucial mutations leading to the dependent phenotype are expected to be positioned in the P1 region coding for the capsid proteins. Hence, cDNA synthesis was performed by using an internal echovirus 12-specific oligonucleotide (positions 4432 to 4452), and the longest clone obtained spans the genome from position 528 to 4452. The sequence of this fragment was determined and compared to that of the wild type. Seven mutations causing amino acid exchanges were found, i.e., one localized in VP4, four in VP2, and two in VP1, respectively (Table 1). No mutation was detected within the approx. 1.2-kbp P2 portion of the cDNA fragment.

Antiviral activity of rhodanine on the recombinant echovirus 12 wild type. Prior to exchanging restriction fragments of the wild-type clone pT7E12 wt (described previously [8]) by corresponding fragments of resistant clones (8) as well as of the dependent clone, the antiviral activity of rhodanine on pT7E12 wt had to be verified. It was shown that the recombinant responds to rhodanine as sensitively as the original counterpart (8). To study whether the uncoating step of the recombinant virus is affected, remaining infectivity after a 4-h incubation at 37°C in the presence and absence of rhodanine was determined by plaque tests. If uncoating has taken place, infectivity should be significantly reduced, since free viral RNA does not measurably induce plaques under these conditions. The experiments revealed that the uncoating of both viruses, the original and the recombinant wild type, were inhibited to the same extent (Fig. 1). In each case, in the absence of rhodanine only about 15% infectivity could be recovered as compared to that in the presence of the compound.

Rhodanine sensitivity assays of resistant clones. The rhodanine sensitivity of recombinant viruses generated by introducing single or various mutations from the resistant clones described earlier into the wild-type construct pT7E12 wt (8) was tested in the presence of 0 to 150 μg of the compound per ml (Fig. 2 and 3A).

No effect is noted if the mutation $C_{3D\text{-}363}R$ in the 3D gene is introduced into the wild-type genome alone (clone 11) or in combination with the exchanges in VP2/VP3 (clone 10). Likewise, no influence on the resistant phenotype of clone 1 is noticeable after the exchange $C_{3D\text{-}363}R$ is removed (clone 4). On the other hand, the resistant and intermediate characters of clones 2 and 3, respectively, become clearly drug dependent by removing the mutation $C_{3D\text{-}363}R$ (clones 13 and 14). This result is surprising, since the 3D gene product—the RNA polymerase—is expected to be involved only in later processes of the viral infectious cycle, and former experiments revealed no effect of rhodanine on steps beyond uncoating (5). On the other hand, RNA polymerase could be detected in highly purified preparations of foot-and-mouth disease virus as well as

* Corresponding author. Mailing address: Institut für Virologie der Universität zu Köln, Fürst-Pückler-Str. 56, 50935 Köln, Germany. Phone: (49) 221-4784482. Fax: (49) 221-407490. E-mail: akv13@rrz.uni-koeln.de.

TABLE 1. Positions of the mutations in the variants

Exchange	No.	Position
Resistant[a]		
$H_{2154}Y$	1[b]	VP2
$G_{2159}S$	2	VP2
$I_{3046}M$	3	VP3
$L_{1068}F$	4a[c]	VP1
$Y_{1075}C$	5	VP1
$V_{1101}A$	5a[d]	VP1
$V_{1157}A$	6	VP1
$C_{3D\text{-}363}R$	7	3D
Dependent		
$F_{4053}Y$	8	VP4
$T_{2037}M$	8a[e]	VP2
$A_{2138}E$	9	VP2
$N_{2142}S$	10	VP2
$H_{2254}Y$	1[b]	VP2
$N_{1176}S$	11	VP1
$Y_{1230}H$	12	VP1

[a] Cloning and sequencing as described before (8).
[b] The same exchange was found in the resistant and dependent variant.
[c] Found in one of six clones.
[d] Found in four of six clones.
[e] Found in one of two clones.

of poliovirus (11a, 12). Enclosed into the viral capsid, an influence of the polymerase on uncoating is conceivable.

The mutations in VP2 ($H_{2254}Y$, $G_{2159}S$) as well as in VP3 ($I_{3046}M$) do not influence the rhodanine sensitivity of the resulting recombinants, since neither the rhodanine sensitivity of the wild type is affected by introducing these mutations (clone 12) nor is the phenotype altered after removing these exchanges (clones 4→5; 10→11; 13→15; 14→16). Hence, the mutations found in the VP1 gene were further analyzed.

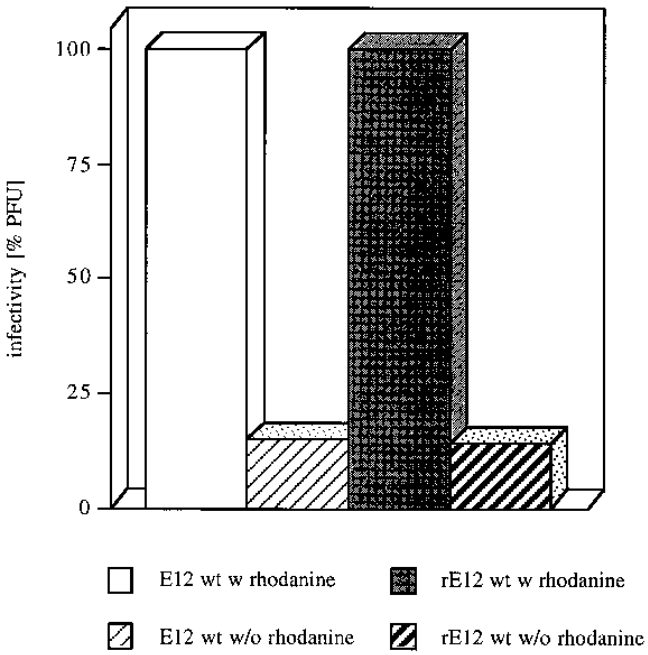

FIG. 1. Percent infectious echovirus 12 wild-type virus (E12 wt) and recombinant wild-type virus (rE12 wt) determined after adsorption and 4-h incubation at 37°C in the presence and absence of rhodanine, respectively. Infectivity after incubation in the presence of rhodanine equals 100%.

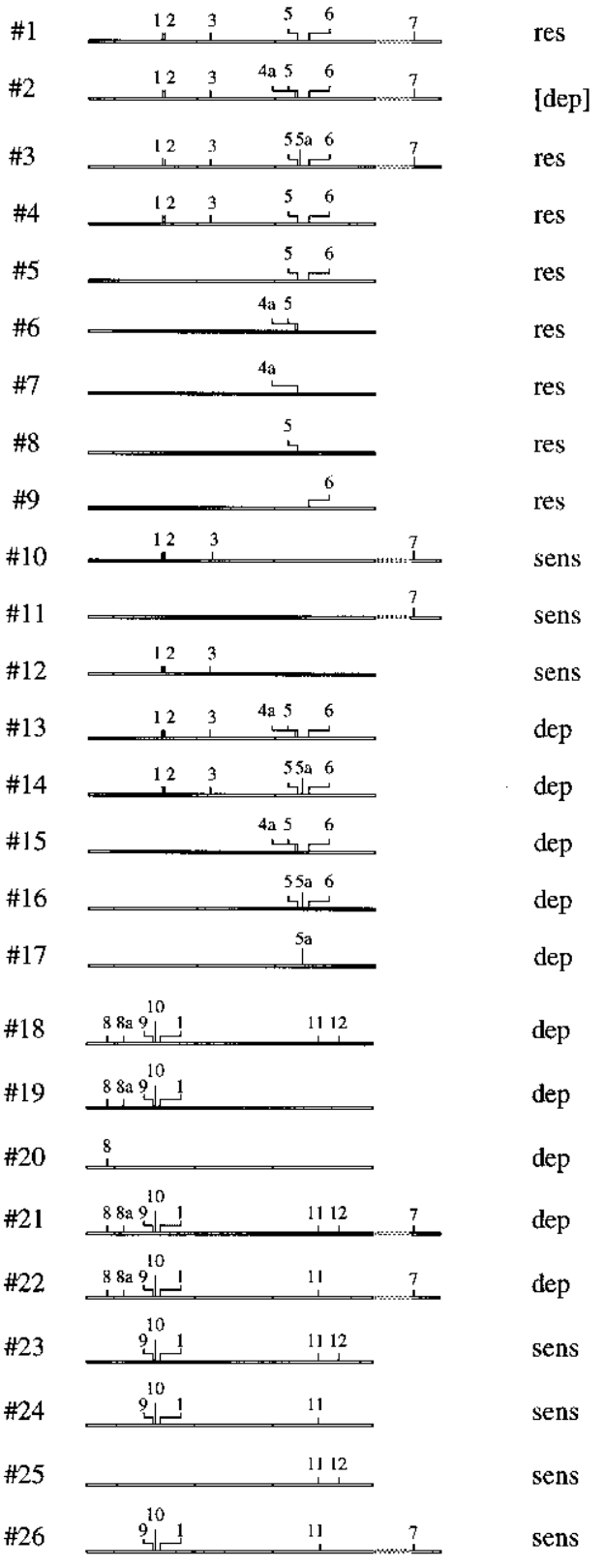

FIG. 2. Genomes and growth characteristics of recombinant viruses containing mutations found in the resistant (#1 to #17) and dependent (#18 to #26) variants. Construction of variants 1 to 3 is described in reference 8. The right-hand column shows sensitivity to rhodanine: res, resistant; sens, sensitive; dep, dependent; [dep], intermediate character (without rhodanine, virus-induced cell damage is retarded but not completely abolished [8]).

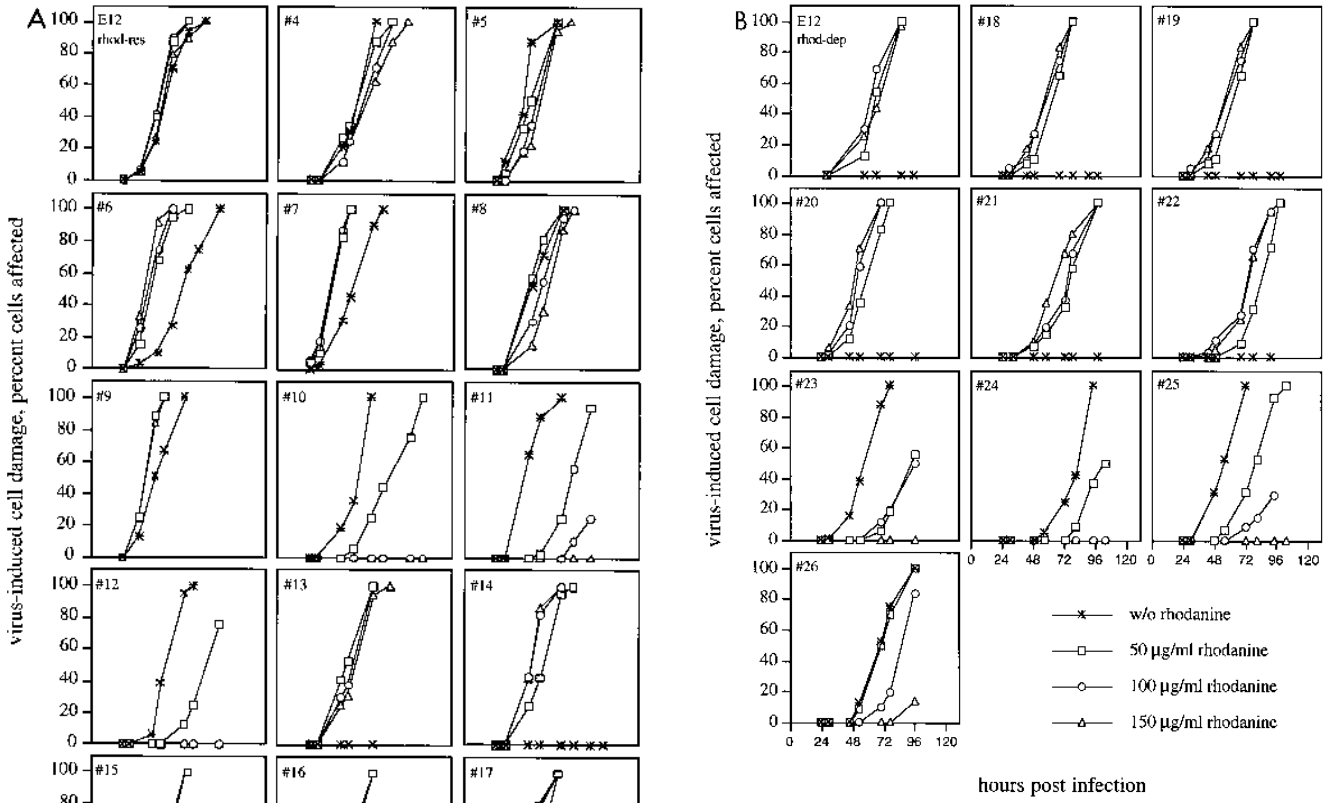

FIG. 3. Rhodanine sensitivity assays of the rhodanine-resistant variant (E12 rhod-res) (A) and rhodanine-dependent variant (E12 rhod-dep) (B) as well as of the recombinant viruses containing mutations found in the resistant and dependent variants, respectively. Numbering of the clones is done as in Fig. 2. Cells were infected with 100 50% tissue culture infective doses of the above mentioned viruses in the presence of 0, 50, 100, and 150 µg of rhodanine per ml, respectively. The percentage of cells exhibiting cytopathic effects was determined at indicated times.

By studying the effect of single exchanges it could be shown that three of four mutations found in the VP1 gene of the resistant variant independently lead to drug-resistant viruses (clone 7, $L_{1068}F$; clone 8, $Y_{1075}C$; and clone 9, $V_{1157}A$). Combinations of two of these exchanges do not alter the resistant phenotype (clones 5 and 6), whereas all three mutations together result in dependent viruses (clones 13 and 15). The fourth exchange ($V_{1101}A$) induces—separate and in combination with other exchanges—a rhodanine-dependent character (clones 14, 16, and 17). Only in combination with the exchange $C_{3D\text{-}363}R$ in the 3D gene, the substitution $V_{1101}A$ is found to generate a resistant virus (clone 3). These findings underscore the importance of VP1 as well as VP4 for viral uncoating and support the early concept that rhodanine stabilizes the virion.

Rhodanine sensitivity assays of "dependent" clones. The seven mutations found in the dependent clone were analyzed as were the resistant ones (Fig. 2). All recombinant viruses containing the mutation located in VP4 ($F_{4053}Y$) exhibited the dependent phenotype, both in the presence or absence of any other of the remaining six exchanges (Fig. 3B). On the other hand, all recombinants missing the exchange $F_{4053}Y$ (clones 23 to 26) are sensitive to the antiviral drug (Fig. 3B). Consequently, the mutation $F_{4053}Y$ in VP4 is sufficient to induce drug dependence.

It is noteworthy that the exchange in $F_{4053}Y$ in VP4 is positioned just in front to an T→A exchange found in the genomes of 5 of 14 WIN 51711-dependent mutants of poliovirus type 3 strain Sabin (P3/Sabin) (10). Since the three-dimensional structure of P3/Sabin is known, the position of this and six further amino acid exchanges found in the proteins of the dependent poliovirus variants could be determined. All mutations are clustered on or near the inner surface of the capsid close to the threefold axis of symmetry (10). VP4 and the N termini of the remaining three capsid proteins form a kind of network on the inner surface of the protein shell which probably plays an important role for virion stability (3, 7). For poliovirus it is proposed that mutations in this area may destabilize the virion and facilitate transition to intermediate particles substantial for viral uncoating (16).

Thermostability of echovirus 12 and the recombinants. Rhodanine has been shown to protect the wild-type echovirus 12 against inactivation by alkaline treatment or heat, presumably by binding of the compound to the capsid surface (4, 5, 14). To investigate the effect of the drug on the resistant and dependent variants as well as on the recombinants, diluted virus was heated in the absence or presence of rhodanine, respectively, and the remaining infectivity titers were determined by plaque tests.

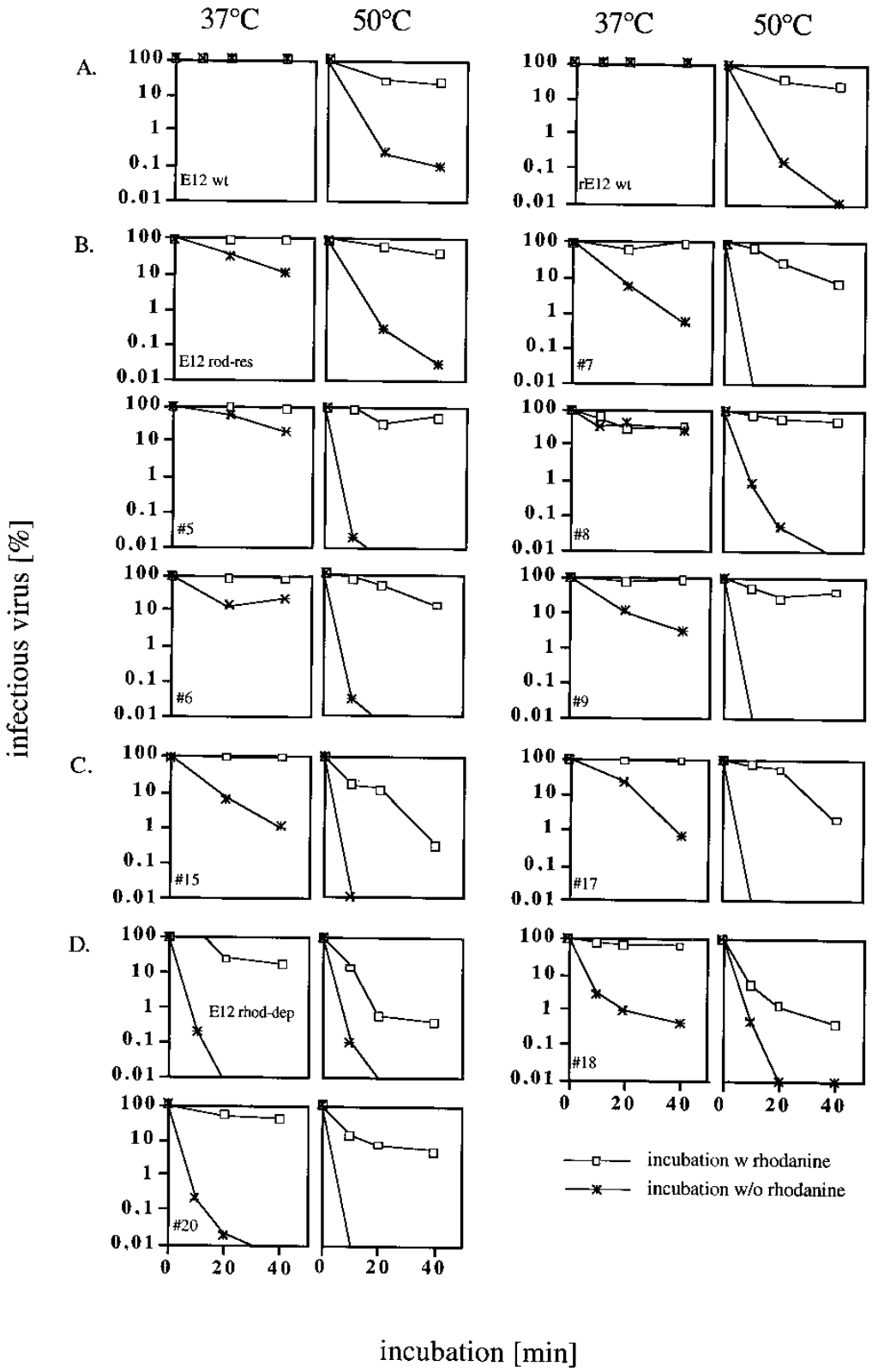

FIG. 4. Determination of thermostability of echovirus 12 wildtype virus (E12 wt) (A), the resistant echovirus 12 variant (E12 rhod-res) (B and C), and the dependent echovirus 12 variant (E12 rhod-dep) and the corresponding recombinants (D). The recombinant viruses containing mutations found in the resistant variant exhibit either the resistant (B) or the dependent (C) phenotype (for details, see text). For this analysis, virus was incubated at 37 or 50°C for the indicated time intervals in the presence or absence of rhodanine, respectively, and the remaining infectivity was determined by plaque test. Numbering of the viruses is as shown in Fig. 2.

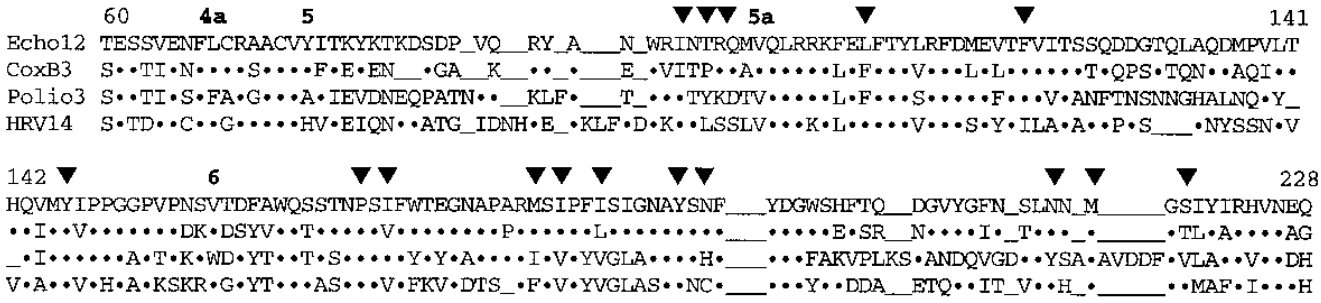

FIG. 5. Alignment of the amino acid sequence of VP1 fragments from related picornaviruses. The mutations found in the echovirus 12 resistant or dependent variants are marked by boldface numbers as shown in Table 1. Filled triangles mark the residues postulated to line the hydrophobic pocket of coxsackievirus B3 (1). Numbers indicate the position of the corresponding amino acid in echovirus 12. Identical amino acids are shown by dots; dashes are introduced to optimize the alignment.

Both the original and the recombinant wild-type viruses are stable at 37°C for 40 min in the presence as well as in the absence of the drug, but at 50°C they show a significant decrease in thermostability in the absence of the drug (Fig. 4A). However, in the presence of rhodanine the proportion of survivors in the original and recombinant viruses after incubation at 50°C is increased by a factor of 100 and 1,000, respectively.

Compared to the wild type, the original resistant variant is slightly more unstable at both temperatures tested (Fig. 4B). The single mutations $L_{1068}F$ (clone 7), $Y_{1075}C$ (clone 8), or $V_{1157}A$ (clone 9) leading independently to the resistant phenotype decrease the thermostability of the recombinant wild-type clone (Fig. 4B). Combination of two exchanges (clones 5 and 6) does not strikingly alter thermostability. Both viruses with mutations found in the original resistant variant leading to drug dependence (clones 15 and 17) show a further decreased stability at 37°C in the absence of rhodanine (Fig. 4C).

The original drug-dependent virus shows a dramatic reduction of thermostability (Fig. 4D). When incubated at 37 as well as at 50°C, after 20 min no remaining infectivity is measurable, but, again, this inactivation can be diminished by incubation in the presence of rhodanine. The dependent recombinant containing the exchange $F_{4053}Y$ (clone 20) exhibits roughly the same features as the original dependent isolate. The recombinant comprising all seven mutations found within the sequenced part of the dependent variant (clone 18), however, at 37°C appears slightly more stable compared to both dependent strains described before.

Conclusions. Because no X-ray crystallographic studies with echoviruses have as yet been performed, the amino acid exchanges introduced by the mutations in the genes for VP1 or VP4 of echovirus 12 can not be correlated with distinct positions of the three-dimensional structure of the virus capsid. Besides, it is not known whether a hydrophobic pocket of the VP1 beta barrel described, e.g., for rhinovirus 14 (15), poliovirus (7), and coxsackievirus B3 (11) holds also for VP1 of echoviruses. Nevertheless, with about 65% amino acid identity between VP1 of coxsackievirus B3 and echovirus 12, the presence of such a pocket appears likely. Ten of 16 amino acids residues lining the wall of this putative drug binding pocket of coxsackievirus B3 can also be found at the corresponding sites of echovirus 12, and four additional positions are occupied by related amino acids (Fig. 5). Hence, we propose a very similar pocket in the capsid of echovirus 12 serving as a rhodanine binding site.

The amino acids supposedly facing the binding site are not affected by the mutations found in the genomes of the echovirus 12 variants analyzed—a first hint that the putative drug binding site may not be altered in the variants. Furthermore, heat inactivation experiments—in the presence or absence of rhodanine—strongly suggest that all recombinants tested are still able to bind rhodanine, since the thermostability of all viruses is increased in the presence of rhodanine (Fig. 4). These findings may indicate that resistance and dependence are not simply consequences of an altered drug binding capacity.

The fact that rhodanine is of low molecular weight and does not affect adsorption of echovirus 12 wild-type virus (5) or of the dependent variant (data not shown) indicates that binding of the drug does not cause, e.g., deformation of the canyon floor as described for several WIN compounds and human rhinovirus 14 (2, 13). More likely, binding of rhodanine does not induce measurable conformational alterations analogous to the binding of the uncoating inhibitor WIN 66393 to coxsackievirus B3 (11). Instead of inducing major structural changes, the effect of rhodanine binding to the wild-type echovirus 12 may be inhibition of conformational movements. The assumed pocket beneath the canyon may act as a kind of hinge, and if this space is occupied by a substance, structural modifications required for uncoating are blocked. The mutations found in VP1 and VP4 which are responsible for a modified sensitivity to rhodanine may allow such movements of the viral capsid proteins necessary for release of the viral RNA, even in the presence of the inhibitor. A similar model, viz., destabilizing mutations apart from the drug binding site lower the energy barrier built up by bound antiviral drugs, is proposed for poliovirus (16).

This theory is supported by the observation that, compared to the wild type, the stability of the virus capsid is decreased in the resistant and—even more pronounced—in the dependent variant (Fig. 4). The wild-type capsid is stabilized by binding of rhodanine, thus preventing uncoating. A slight destabilization could allow uncoating in the presence as well as in the absence of rhodanine and might lead to a resistant phenotype. If the flexibility of the capsid is further increased, the virus apparently needs the stabilizing effect of rhodanine to perform a productive infection. This model allows intermediate forms of viral stability. Hence, we speculate that resistance and dependence of echovirus 12 are effects of a similar mechanism, namely, an increase in flexibility of the capsid.

We gratefully acknowledge the excellent technical assistance of Christa Schuchardt.

REFERENCES

1. **Andries, K., B. Dewindt, J. Snoeks, L. Wouters, H. Moereels, P. J. Lewi, and P. A. Janssen.** 1990. Two groups of rhinoviruses revealed by a panel of

antiviral compounds present sequence divergence and differential pathogenicity. J. Virol. **64:**1117–1123.
2. **Badger, J., I. Minor, M. J. 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.
3. **Basavappa, R., R. Syed, O. Flore, J. P. Icenogle, D. J. Filman, and J. M. Hogle.** 1994. Role and mechanism of the maturation cleavage of VP0 in poliovirus assembly: structure of the empty capsid assembly intermediate at 2.9 Å resolution. Protein Sci. **3:**1651–1669.
4. **Eggers, H. J.** 1970. Inhibition of early stages of virus-cell interactions. Ann. N. Y. Acad. Sci. **173:**417–419.
5. **Eggers, H. J.** 1977. Selective inhibition of uncoating of echovirus 12 by rhodanine. Virology. **78:**241–252.
6. **Eggers, H. J., M. A. Koch, A. Furst, G. D. Daves, Jr., J. J. Wilczynski, and K. Folkers.** 1970. Rhodanine: A selective inhibitor of the multiplication of echovirus 12. Science **167:**294–297.
7. **Grant, R. A., C. N. Hiremath, D. J. Filman, R. Syed, K. Andries, and J. M. Hogle.** 1994. Structures of poliovirus complexes with anti-viral drugs: implications for viral stability and drug design. Curr. Biol. **4:**784–797.
8. **Kraus, W., H. Zimmermann, A. Zimmermann, H. J. Eggers, and B. Nelsen-Salz.** 1995. Infectious cDNA clones of echovirus 12 and a variant resistant against the uncoating inhibitor rhodanine differ in seven amino acids. J. Virol. **69:**5853–5858.
9. **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.
10. **Mosser, A. G., J. Y. Sgro, and R. R. Rueckert.** 1994. Distribution of drug resistance mutations in type 3 poliovirus identifies three regions involved in uncoating functions. J. Virol. **68:**8193–8201.
11. **Muckelbauer, J. K., M. Kremer, I. Minor, G. Diana, F. J. Dutko, J. Groarke, D. Pevear, and M. G. Rossmann.** 1995. The structure of coxsackievirus B3 at 3.5 Å resolution. Structure **3:**653–667.

11a. **Newman, J. F. E.** Personal communication.

12. **Newman, J. F. E., P. G. Piatti, B. M. Gorman, T. G. Burrage, M. D. Ryan, M. Flint, and F. Brown.** 1994. Foot-and-mouth disease virus particles contain replicase protein 3D. Proc. Natl. Acad. Sci. USA **91:**733–737.
13. **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.
14. **Rosenwirth, B., and H. J. Eggers.** 1979. Early processes of echovirus 12-infection: elution, penetration, and uncoating under the influence of rhodanine. Virology **97:**241–255.
15. **Smith, T. J., M. J. Kremer, M. Luo, G. Vriend, E. Arnold, G. Kamer, M. G. Rossman, 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.
16. **Wien, M. W., M. Chow, and J. M. Hogle.** 1996. Poliovirus: new insight from an old paradigm. Structure **4:**763–767.