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Aphidicolin Resistance in Herpes Simplex Virus Type 1 Appears To Alter Substrate Specificity in the DNA Polymerase

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We describe novel mutants of herpes simplex virus which are resistant to aphidicolin. Their mutant phenotypes suggest that they encode DNA polymerases with altered substrate recognition. This conclusion is based on their abnormal sensitivity to polymerase inhibitors and to the abnormal mutation rates exhibited by two of the mutants.

Aphidicolin is a potent inhibitor of many DNA polymerases, including DNA polymerase alpha from eucaryotic cells and DNA polymerases from herpesviruses (6, 9, 13, 17, 21, 22, 24). This drug appears useful for studying interactions between DNA polymerases and deoxyribonucleotide triphosphate (dNTP) substrates, since aphidicolin interferes with dNTP binding (5, 7, 13). Consequently, a subset of polymerase residues which interact with aphidicolin should also bind dNTPs, and mutants with altered sensitivity to this drug should help identify such residues.

In accord with this prediction, several previously characterized aphidicolin mutants encode DNA polymerases which exhibit altered substrate interactions (i.e., altered affinities for dNTPs and for certain substrate analogs; 8, 10, 12, 16, 18, 23). Some mutants also exhibit mutator or antimutator phenotypes, suggesting alterations in their abilities to select correctly base-paired substrates (1, 11, 16, 19). Among the mutants from herpes simplex virus type 1 (HSV-1), several are hypersensitive to aphidicolin (8), but only two resistant viral mutants known to carry polymerase changes have been reported, one moderately resistant mutant from HSV-1 (14) and one from the closely related HSV-2 (18).

Several other polymerase inhibitors have been useful for isolating mutants from HSV-1 which affect substrate recognition. These inhibitors include substrate analogs with altered ribose rings [e.g., acycloguanosine, arabinosyladenine (araA), and 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG)] and phosphonoacetic acid. The analogs are phosphorylated in herpesvirus-infected cells. Once utilized by the polymerase as substrates, they inhibit further replication (2–4, 20). Since these analogs mimic normal substrates, mutants with altered sensitivity to these compounds appear to carry changes which affect substrate recognition. Phosphonoacetic acid has also been used to isolate substrate-specificity mutants, since mutants resistant to this drug also exhibit altered sensitivity to the substrate analogs (8, 15).

This report describes our studies of new aphidicolin-resistant derivatives of HSV-1. Because these mutants show changes in sensitivity to both aphidicolin and to substrate analogs with altered ribose rings, they appear to carry mutations within the DNA polymerase gene at the dNTP-binding site. Our mutants differ from previously reported aphidicolin-resistant viral mutants, suggesting that they carry novel changes and should provide new information about the aphidicolin- and substrate-binding sites.

Isolation of mutants. Mutants were obtained by growing virus sequentially in increasing concentrations of aphidicolin. TC7 cells (11) were inoculated with the KOS strain of HSV-1 at 0.1 PFU per cell in the presence of 0.12 μg of drug per ml. Plaque-purified stocks (11) were used to assure independent isolation of mutants from different infections. Progeny virus were then grown sequentially in 0.25, 0.5, and 0.6 μg of aphidicolin per ml. Drug-resistant plaques were selected and plaque purified in the absence of drug. Mutants Aph^{r}10 and Aph^{r}12 were isolated from the same stock, but both were studied since they exhibit different phenotypes. Mutants Aph^{r}14 and Aph^{r}16 were isolated from separate stocks.

Mapping of the mutations. We have cloned regions of the DNA polymerase gene from each mutant and shown by marker rescue experiments that recombinants which incorporate the cloned sequences acquire resistance to aphidicolin (J. Hall, S. Woodward, and M. Berlin, manuscript in preparation). Two such recombinants were recovered from each mapping experiment, and their phenotypes were characterized as described below (i.e., drug sensitivities, spontaneous mutation rates). In each case, the recombinants showed the same phenotypes as the original mutants (data not shown; Table 1). Consequently, the phenotypes described below for the original mutants most likely result from the aphidicolin resistance mutations.

Sensitivity to DNA polymerase inhibitors. The resistance of these mutants to aphidicolin is shown in Fig. 1A. Drug

TABLE 1. Spontaneous mutation levels, measured by production of progeny resistant to ICdR[a]

Strain or mutant	Avg fraction of ICdR-resistant progeny	Relative fraction of ICdR-resistant progeny	Phenotype
KOS (wild type)	2.1×10^{-4}	1.00	Normal
Aph^{r}12	$<8.0 \times 10^{-6}$	<0.038	Antimutator
Aph^{r}14	$<5.7 \times 10^{-6}$	<0.027	Antimutator
KOS (wild type)	2.6×10^{-4}	1.00	Normal
Aph^{r}10	6.9×10^{-4}	2.7	Near normal
Aph^{r}10-R1[b]	1.7×10^{-4}	0.7	Normal
Aph^{r}10-R2[b]	3.0×10^{-4}	1.2	Normal
Aph^{r}16	2.7×10^{-4}	1.0	Normal

[a] Virus was grown through several replication cycles from approximately 20 PFU (11). Each result represents the average of 3 to 18 separate infections.
[b] R1 and R2 derivatives of the Aph^{r}10 mutant are recombinants from marker rescue experiments described in the text.

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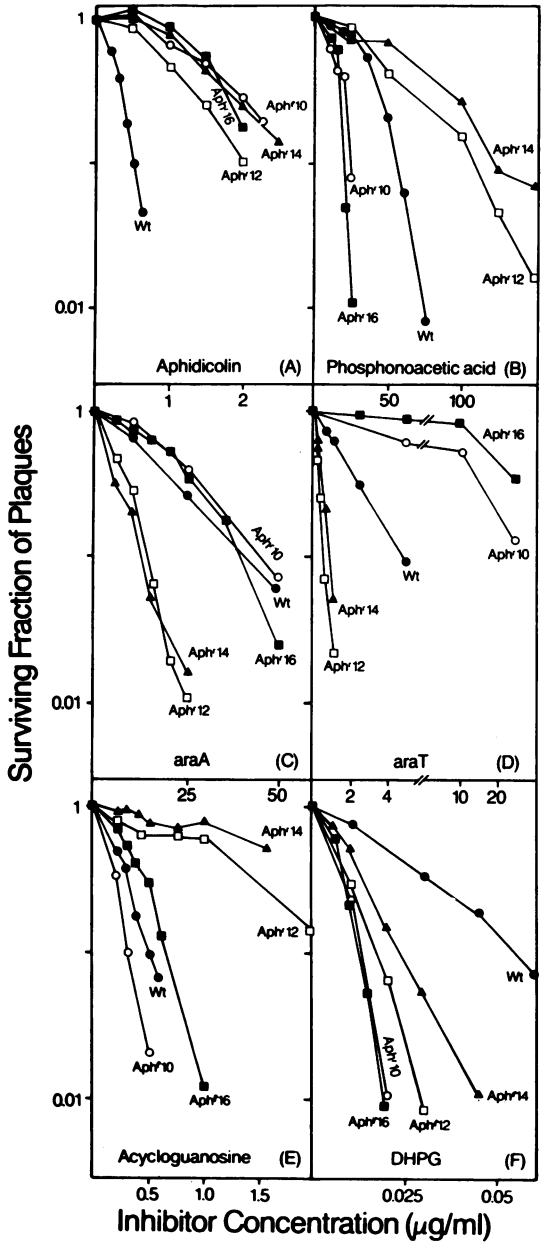

FIG. 1. Survival of plaque formation of aphidicolin-resistant (Aph^r) and wild-type (Wt) viruses in the presence of various DNA polymerase inhibitors. The results represent the averages of two or more experiments.

sensitivity was determined from the survival of plaque formation when 200 to 500 PFU of virus was plated onto TC7 monolayers in the presence and absence of drug. Clearly, the mutants are substantially more drug resistant than their wild-type parent.

The effects of other inhibitors on plaque survival are shown in Fig. 1B through F and are summarized in Table 2. Although all the mutants are more sensitive to DHPG than is the wild-type virus (Fig. 1F), they fall into two distinct phenotypic classes on the basis of their sensitivity to the other drugs. Aph^{r}10 and Aph^{r}16 are hypersensitive to phosphonoacetic acid and are resistant to arabinosylthymine (araT), whereas Aph^{r}12 and Aph^{r}14 are resistant to phosphonoacetic acid and are hypersensitive to araT. Aph^{r}10 and Aph^{r}16 show near-normal sensitivities to araA and acycloguanosine, whereas Aph^{r}12 and Aph^{r}14 are hypersensitive to araA and are resistant to acycloguanosine.

TABLE 2. Summary of phenotypes of mutant viruses[a]

Mutant	Drug sensitivity relative to wild type[b]						Mutagenesis level
	Aphidicolin	Phosphonoacetic acid	araA	araT	Acycloguanosine	DHPG	
Aph^{r}10	R	HS	S	R	S	HS	Normal
Aph^{r}16	R	HS	S	R	S	HS	Normal
Aph^{r}12	R	R	HS	HS	R	HS	Antimutator
Aph^{r}14	R	R	HS	HS	R	HS	Antimutator

[a] Summary of data from Fig. 1 and Table 1.
[b] R, Resistance; HS, hypersensitivity; S, similar sensitivity to wild type.

Because these mutants exhibit altered sensitivities to the substrate analog inhibitors (DHPG, araA, araT, and acycloguanosine), we conclude that they probably carry mutations which affect substrate recognition. The contrasting phenotypes of these mutants further suggest that at least two different mutations within the aphidicolin-binding site are involved.

Mutation frequencies. Since previously characterized aphidicolin-resistant mutants (from HSV-2 and rodent cells) are associated with a mutator phenotype (1, 16, 19), we measured the mutation frequencies for our mutants. The results are shown in Table 1 and were determined from the fractions of spontaneous viral mutants resistant to iododeoxycytidine (ICdR) in several parallel infections (11).

Although Aph^{r}10 and Aph^{r}16 are similar in their inhibitor sensitivities to the aphidicolin-resistant, mutator derivative from HSV-2 (19), they fail to exhibit this mutator phenotype. Aph^{r}16 showed normal levels of ICdR-resistant mutant production. Although the average level for Aph^{r}10 was slightly higher than that for the wild type, most of the values fell within the wild-type range (data not shown), and recombinants from marker rescue experiments with Aph^{r}10 sequences showed normal mutation frequencies. Aph^{r}12 and Aph^{r}14 produce substantially fewer mutants than does wild type. However, they differ from previously studied antimutators from HSV-1 which are hypersensitive to aphidicolin (8, 11). These observations provide support for the uniqueness of our mutants and suggest that the mutations within Aph^{r}12 and Aph^{r}14 (the antimutators) will identify new residues involved in substrate selection.

Conclusions. Our aphidicolin-resistant mutants show altered sensitivities to substrate analogs, suggesting that they carry DNA polymerase mutations which affect substrate recognition. These mutants differ from other herpesvirus drug-resistant mutants (8, 14, 18) and, hence, should provide novel information about residues required for substrate binding. Sequence analysis (in progress) should reveal at least two new polymerase mutations, since the mutants exhibit two distinct phenotypes.

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LITERATURE CITED

1. **Aizawa, S., L. A. Loeb, and G. M. Martin.** 1987. Aphidicolin-resistant mutator strains of mouse teratocarcinoma. Mol. Gen.

Genet. **208**:342–348.
2. **Brigden, D., P. Fiddian, A. E. Rosling, and T. Ravenscroft.** 1981. Acyclovir—a review of the preclinical and early clinical data of a new antiherpes drug. Antiviral Res. **1**:203–212.
3. **Cheng, Y.-C., S. P. Grill, G. E. Dutschman, K. Nakayama, and K. F. Bastow.** 1983. Metabolism of 9-(1,3-dihydroxy-2-propoxymethyl)guanine, a new antiherpes virus compound, in herpes simplex virus-infected cells. J. Biol. Chem. **258**:12460–12464.
4. **Derse, D., and Y.-C. Cheng.** 1981. Herpes simplex virus type 1 DNA polymerase. Kinetic properties of the associated 3′-5′ exonuclease activity and its role in araAMP incorporation. J. Biol. Chem. **256**:8525–8530.
5. **Dicioccio, R. A., K. Chadha, and B. I. S. Srivastava.** 1980. Inhibition of herpes simplex virus-induced DNA polymerase, cellular DNA polymerase alpha, and virus production by aphidicolin. Biochim. Biophys. Acta **609**:224–231.
6. **Edgar, L. G., and J. D. McGhee.** 1988. DNA synthesis and the control of embryonic gene expression in *C. elegans*. Cell **53**:589–599.
7. **Frank, K. B., D. D. Derse, K. F. Bastow, and Y.-C. Cheng.** 1984. Novel interaction of aphidicolin with herpes simplex virus DNA polymerase and polymerase-associated exonuclease. J. Biol. Chem. **21**:13282–13286.
8. **Gibbs, J. S., H. C. Chiou, K. F. Bastow, Y.-C. Cheng, and D. M. Coen.** 1988. Identification of amino acids in herpes simplex virus DNA polymerase involved in substrate and drug recognition. Proc. Natl. Acad. Sci. USA **85**:6672–6676.
9. **Habará, A., K. Kano, H. Nagano, Y. Mano, S. Ikegami, and T. Yamashita.** 1980. Inhibition of DNA synthesis in the adenovirus DNA replication complex by aphidicolin and 2′,3′-dideoxythymidine triphosphate. Biochem. Biophys. Res. Commun. **92**: 8–12.
10. **Hall, J. D.** 1988. Modeling functional sites in DNA polymerases. Trends Genet. **4**:42–46.
11. **Hall, J. D., D. M. Coen, B. L. Fisher, M. Weisslitz, S. Randall, R. E. Almy, P. T. Gelep, and P. A. Schaffer.** 1984. Generation of genetic diversity in herpes simplex virus: an antimutator phenotype maps to the DNA polymerase locus. Virology **132**:26–37.
12. **Hall, J. D., P. A. Furman, M. H. St. Clair, and C. W. Knopf.** 1985. Reduced *in vivo* mutagenesis by mutant herpes simplex DNA polymerase involves improved nucleotide selection. Proc. Natl. Acad. Sci. USA **82**:3889–3893.
13. **Huberman, J. A.** 1981. New views of the biochemistry of eucaryotic DNA replication revealed by aphidicolin, an unusual inhibitor of DNA polymerase alpha. Cell **23**:647–648.
14. **Larder, B. A., and G. Darby.** 1986. Susceptibility to other antiherpes drugs of pathogenic variants of herpes simplex virus selected for resistance to acyclovir. Antimicrob. Agents Chemother. **29**:894–898.
15. **Larder, B. A., S. D. Kemp, and G. Darby.** 1987. Related functional domains in virus DNA polymerases. EMBO J. **6**: 169–175.
16. **Liu, P. K., C.-C. Chang, J. E. Trosko, D. K. Dube, G. M. Martin, and L. A. Loeb.** 1983. Mammalian mutator mutant with an aphidicolin-resistant DNA polymerase alpha. Proc. Natl. Acad. Sci. USA **80**:797–801.
17. **Longiaru, M., J.-E. Ikeda, Z. Jarkovsky, S. B. Horwitz, and M. S. Horwitz.** 1979. The effect of aphidicolin on adenovirus DNA synthesis. Nucleic Acids Res. **6**:3369–3386.
18. **Nishiyama, Y., S. Suzuki, M. Yamauchi, K. Maeno, and S. Yoshida.** 1984. Characterization of an aphidicolin-resistant mutant of herpes simplex virus type 2 which induced an altered viral DNA polymerase. Virology **135**:87–96.
19. **Nishiyama, Y., S. Yoshida, T. Tsurumi, N. Yamamoto, and K. Maeno.** 1985. Drug-resistant mutants of herpes simplex virus type 2 with a mutator or antimutator phenotype. Microbiol. Immunol. **29**:377–381.
20. **North, T. W., and S. S. Cohen.** 1979. Aranucleosides and aranucleotides in viral chemotherapy. Pharmacol. Ther. **4**: 81–108.
21. **Oguro, M., C. Suzuki-Hori, H. Nagano, Y. Mano, and S. Ikegami.** 1979. The mode of inhibitory action by aphidicolin on eukaryotic DNA polymerase alpha. Eur. J. Biochem. **97**:603–607.
22. **Ohashi, M., T. Taguchi, and S. Ikegami.** 1978. Aphidicolin: a specific inhibitor of DNA polymerases in the cytosol of rat liver. Biochem. Biophys. Res. Commun. **82**:1084–1090.
23. **Tsurumi, T., K. Maeno, and Y. Nishiyama.** 1987. A single-base change within the DNA polymerase locus of herpes simplex virus type 2 can confer resistance to aphidicolin. J. Virol. **61**:388–394.
24. **Wist, E., and H. Prydz.** 1979. The effect of aphidicolin on DNA synthesis in isolated HeLa cell nuclei. Nucleic Acids Res. **6**:1583–1590.