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Mutations in the herpes simplex virus DNA polymerase gene conferring hypersensitivity to aphidicolin

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ABSTRACT

Fourteen mutants known or likely to contain mutations in the herpes simplex virus DNA polymerase gene were examined for their sensitivity to aphidicolin in plaque reduction assays. Eleven of these exhibited some degree of hypersensitivity to the drug; altered aphidicolin-sensitivity correlated with altered sensitivity to the pyrophosphate analog, phosphonoacetic acid. The DNA polymerase specified by one of these mutants, PAA^r5, required roughly seven-fold less aphidicolin to inhibit its activity by 50% than did polymerase specified by its parental strain. Mutations responsible for the aphidicolin-hypersensitivity phenotype of PAA^r5 were mapped to an 0.8 kbp region in the herpes simplex virus DNA polymerase locus. These data taken together indicate that 1) mutations in the herpes simplex virus DNA polymerase gene can confer altered sensitivity to aphidicolin, 2) that the HSV polymerase is sensitive to aphidicolin *in vivo*, and 3) that amino acid alterations which affect aphidicolin binding may affect the pyrophosphate exchange-release site as well, suggesting that aphidicolin binds in close proximity to this site.

INTRODUCTION

Sensitivity to aphidicolin is a hallmark of the principal cellular DNA polymerase required for eukaryotic DNA replication, DNA polymerase α[1-7]. Aphidicolin inhibition of DNA polymerase *in vitro* correlates with its ability to inhibit DNA replication *in vivo*. More convincingly, mutants of Drosophila and mammalian cells which are aphidicolin-resistant specify aphidicolin-resistant α-polymerases[5-7].

The mechanism of aphidicolin inhibition remains poorly understood[6]. Aphidicolin bears no obvious resemblance to known polymerase substrates, yet it appears to inhibit polymerases competitively with dCTP[3,4,8-11], and perhaps other dNTP's[9-11]. Because of this it has been proposed that aphidicolin binds at or very near the dCTP binding site[3,4,8-10]. Alternative models favoring longer range interactions have also been presented[5,6,9].

Herpes simplex virus (HSV) induces a novel DNA polymerase which resembles cellular α-polymerases in several respects including resistance to dideoxythy-

midine triphosphate and sensitivity to aphidicolin[4,11-13]. Studies of HSV DNA polymerase have been aided considerably by the use of mutants which exhibit altered polymerase activity (pol mutants) - in particular mutants resistant to antiviral drugs[14-23]. These studies have enabled correlation of polymerase properties with mutant phenotypes and fine mapping of the region of HSV DNA specifying polymerase, the pol locus[19,24-26].

To investigate further the interaction between aphidicolin and DNA polymerase, we examined the aphidicolin-sensitivity of fourteen known or putative HSV pol mutants. Unexpectedly, eleven mutants exhibited hypersensitivity to aphidicolin. We present data herein demonstrating for one of these mutants, PAA^r5, that aphidicolin-hypersensitivity is due to a pol mutation and for all the mutants studied, that altered aphidicolin-sensitivity correlates with altered sensitivity to the pyrophosphate analog, phosphonoacetic acid (PAA).

MATERIALS AND METHODS

Cells and viruses: Vero cells used for growing virus stocks and plaque reduction assays and primary kidney cells used for marker rescue experiments were propagated and maintained as described[25]. Relevant properties of wild-type strain KOS and Patton and mutants derived from them are summarized and referenced in Table 1. The construction by marker transfer of recombinant viruses containing the drug-resistance phenotypes of PAA^r5 will be presented elsewhere[26], and is summarized briefly in the text.

Aphidicolin-sensitivity assays: Viruses were titered by plaque assays utilizing a 2% methylcellulose overlay[27] containing no drug or the indicated concentration of aphidicolin. Aphidicolin was kindly provided by Dr. A.H. Todd (ICI, Macclesfield, UK) and was dissolved in DMSO and diluted with water to yield a 100 microgram/ml stock in 10% DMSO. This stock was filter sterilized and stored at -20°C.

DNA polymerase assays: HSV DNA polymerase was prepared from KOS- and PAA^r5-infected HeLa cells as described[22]. Polymerase was assayed as described[22] with the indicated concentration of aphidicolin, and substrate concentrations of 100 μM dGTP, 100 μM dATP, 100 μM dTTP, and 3 μM dCTP[4]. ^3H dTTP was present at a specific activity of 192 cpm/pmol. Criteria for the viral specificity of polymerase activity included elution profiles during column chromatography purification and stimulation by 100 mM ammonium sulfate. Additionally, the KOS polymerase exhibited inhibition by PAA and acyclovir-triphosphate as expected for wild-type viral, but not cellular enzyme. Similar rates of incorporation of ^3H dTTP into TCA-insoluble material were observed

Table 1

Plating efficiencies in aphidicolin of strains KOS and Patton and mutants derived from them.

Virus	Evidence for pol mutation[b]	Efficiency of Plating in Aphidicolin[a] 0.5 µg/ml x 100%	1 µg/ml x 100%	Phenotype[c]	PAA- (ref) Phenotype[d]
KOS (wt)	--	81	23	s	s
tsD9	14,15,24-26,30	0.15	0.15	hs	r (15,30)
tsC4	14,22,26	0.05	0.05	hs	hs (26)
tsC7	14,17,24-26	96	19	s	s (26)
ts022	14,25	70	19	s	s (unpub.)
PAA^r5	21,22,26,31	<0.02	<0.02	hs	r (21)
PAA^r3	*	3.7	<3.0	hs	r (30)
$AraA^r6$	*	46	1.0	hs	r (22)
$AraA^r7$	*	0.02	0.02	hs	r (22)
$AraA^r8$	*	47	2.0	hs	r (22)
$AraA^r9$	*	68	6.0	hs/s	r/s (22)
$AraA^r12$	*	<0.01	<0.01	hs	r (22)
$AraA^r13$	*	0.07	<0.07	hs	r (22)
ACG^r4	21,31	0.15	<0.15	hs	r (21,31)
Patton (wt)	--	95	72	s	s
BW^r	21,unpublished	100	78	s	s (21)

[a]Mutants tsD9, tsC7, and ts022 were titered at 34°; all other mutants were titered at 37°. The EOPs in aphidicolin of KOS were similar at both temperatures. $\text{EOPs} = \frac{\text{titer of virus in indicated concentration of drug}}{\text{titer of virus in no drug}}$

[b]The references listed present biochemical and genetic evidence that a given mutant is a pol mutant; i.e. data indicates altered polymerase activity and data indicating that the ts or drug-resistance mutation of each mutant resides in the pol locus. Asterisks (*) denote putative pol mutants. Although biochemical and genetic evidence that these mutants are indeed pol mutants is lacking, they all exhibit PAA-resistance[22,30]. All PAA-resistant mutants which have been examined to date have been shown to be pol mutants[15,16,18,19,21,23].

[c]Mutants were designated sensitive (s), hypersensitive (hs), or marginally hypersensitive (hs/s) based on the data presented in this table and in Figure 1 in which the sensitivity of each mutant is compared with its wild-type parent. Differences in EOP of less than two-fold are not considered significant. Although values for aphidicolin-sensitivity vary from assay to assay, the results presented reflect the sensitivities of mutant viruses relative to their wild-type parents in any given assay. The values presented represent the average of two or more assays of each virus.

[d]Mutants were designated sensitive (s), hypersensitive (hs), resistant (r) or marginally resistant (r/s) on the basis of experiments reported in the indicated references.

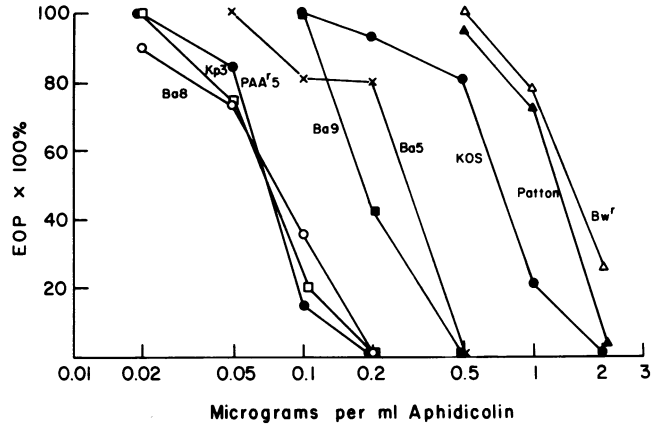

Figure 1. Plaque-reduction curves of Patton, BW^r, KOS, PAA^r5, and recombinant viruses. Plaque-reduction assays were performed utilizing a 2% methylcellulose overlay containing the indicated concentrations of aphidicolin. Recombinant viruses Ba5, Ba8, Ba9 and Kp3 were constructed as described in Coen et al.[26] from marker transfer using a Bam HI fragment and a Kpn I fragment, respectively, of PAA^r5 DNA (see Figure 3). Similar results were obtained using recombinant viruses plaque-purified a single time or plaque-purified an additional time and kindly provided by J.D. Hall.

for the preparations of both KOS and PAA^r5 polymerase in the absence of aphidicolin.

Marker rescue: Marker rescue was performed by co-transfecting primary rabbit kidney cells[22,25,26] with infectious PAA^r5 DNA prepared as described[22] and isolated restriction fragments of pSG17 DNA[26] which contains the HSV-1 strain KOS Eco RI "M" fragment[28]. Plasmid pSG17 was kindly provided by R. Sandri-Goldin and M. Levine (University of Michigan, Ann Arbor, MI). Progeny virus derived from each transfection were titered in the presence or absence of 0.5 μg/ml aphidicolin.

RESULTS

Aphidicolin-sensitivities of pol mutants

Table 1 and Figure 1 present plaque-reduction experiments comparing the sensitivities to aphidicolin of wild-type HSV-1 strains KOS and Patton, and fourteen known or putative pol mutants derived from them. Known pol mutants are those which have been shown to specify altered DNA polymerase and to contain mutations at the pol locus by complementation, recombination, and/or physical mapping analysis. Putative pol mutants are those which, in the ab-

sence of additional evidence, exhibit PAA-resistance. Importantly, all PAA-resistant mutants which have been examined to date have been shown to be pol mutants[15,16,18,19,21,23,24].

Table 1 summarizes some of the relevant properties of the mutants. Notably, none of these mutants was selected on the basis of sensitivity or resistance to aphidicolin. Of four temperature-sensitive pol mutants, tsD9, tsC4, tsC7 and ts022, two, tsD9 and tsC4[14,15,24,26,29] proved to be considerably more sensitive to aphidicolin than their wild-type parent, KOS. We term this phenotype, aphidicolin-hypersensitivity. On the other hand, tsC7 and ts022[14,17,24-26,29] exhibit wild-type levels of sensitivity (Table 1). Mutants PAA^r5 and PAA^r3, selected for resistance to PAA[30], were also aphidicolin-hypersensitive (Table 1 and Figure 1). Six of six mutants selected for resistance to arabinosyladenine (araA), $AraA^r6,7,8,9,12$ and 13[22], also exhibited some degree of aphidicolin-hypersensitivity (Table 1).

Of two pol mutants selected for resistance to acyclovir (ACG), ACG^r4[21,31] and BW^r[21], ACG^r4 was aphidicolin-hypersensitive (Table 1). Both BW^r and its parental strain Patton were slightly more resistant to aphidicolin than strain KOS. BW^r, however, appeared no more or less aphidicolin-sensitive than Patton (Table 1 and Figure 1). The finding that four known and seven putative pol mutants exhibit aphidicolin-hypersensitivity strongly suggests that pol mutations confer this phenotype.

Correlation between altered sensitivity to aphidicolin and PAA

An interesting property of ten of the eleven mutants which exhibit aphidicolin-hypersensitivity is that they also exhibit some degree of PAA-resistance[15,21,22,26,30,31]. Of these ten, $AraA^r9$ exhibited both the least PAA-resistance[22] (and unpublished results) and the least aphidicolin-hypersensitivity (Table 1). Although the eleventh aphidicolin-hypersensitive mutant, tsC4, is not PAA-resistant, it is PAA-hypersensitive[26]. In contrast, pol mutants tsC7, ts022, and BW^r exhibit little if any alteration in either aphidicolin- or PAA-sensitivity compared with their wild-type parents[21,26] (and unpublished results). Further strengthening the correlation between alterations in PAA- and aphidicolin-sensitivities is the observation that strain Patton is both more aphidicolin-resistant and PAA-resistant[21] than strain KOS.

In contrast, none of the other mutant phenotypes exhibited by these viruses correlate with altered aphidicolin-sensitivity. For example, although all mutants which were aphidicolin-hypersensitive also exhibit some degree of ACG-resistance[21,26,31] (and unpublished results), BW^r, which is ACG-resistant

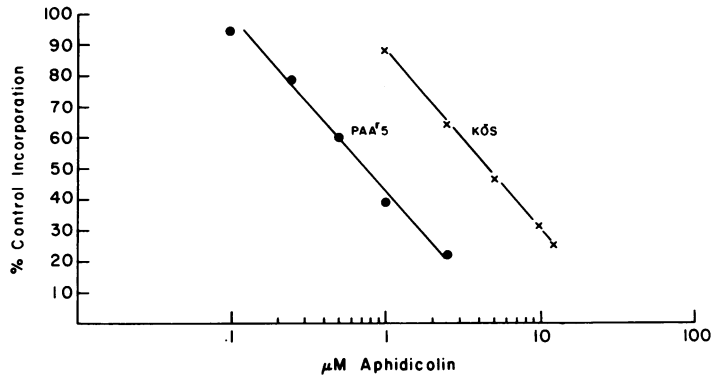

Figure 2. Inhibition of purified HSV DNA polymerases from KOS- and PAA^{r}5-infected cells by aphidicolin. The enzymes were assayed as described in the text with the indicated concentrations of aphidicolin (1 μM aphidicolin = .33 μg/ml).

due to a pol mutation[21] does not exhibit altered aphidicolin-sensitivity.

Aphidicolin-hypersensitivity of PAA^{r}5 is due to a pol mutation

Biochemical studies: We sought to determine if the aphidicolin-hypersensitivity phenotype of mutant PAA^{r}5 is due to a pol mutation. To this end, both biochemical and genetic tests were performed. Figure 2 shows that HSV DNA polymerase purified from PAA^{r}5-infected cells was considerably more sensitive to aphidicolin than the corresponding enzyme purified from KOS-infected cells. Analysis of the data presented in Figure 2 yields 50% inhibition values of 4.5 μM for the KOS-specified polymerase and 0.7 μM for PAA^{r}5-specified polymerase, nearly a seven-fold difference. This difference is similar to the roughly ten-fold difference seen in the 50% plaque reduction values in the presence of aphidicolin for these two viruses (Figure 1). The concentrations required to obtain 50% inhibition of polymerase activity are fairly similar to those for 50% plaque reduction (KOS 0.8 μg/ml, 2.4 μm; PAA^{r}5 0.08 μg/ml, 0.24 μm).

Genetic studies: Two types of experiments were performed to map the aphidicolin-hypersensitivity phenotype of PAA^{r}5. The first experiment employed thirteen recombinant viruses containing the PAA-, ACG-, and araA-resistance phenotypes of PAA^{r}5 which were constructed by marker transfer from restriction fragments of PAA^{r}5 DNA into an otherwise wild-type background. These marker transfer experiments have mapped the three drug-resistance markers to a 1.1 kbp region within the HSV pol locus[22,26]. Three recombinant viruses were derived using the HpaI B fragment[22], three using the cloned Bgl II D fragment of PAA^{r}5 DNA in plasmid pPA22[26], three using the Kpn I d fragment of pPA22[26] and

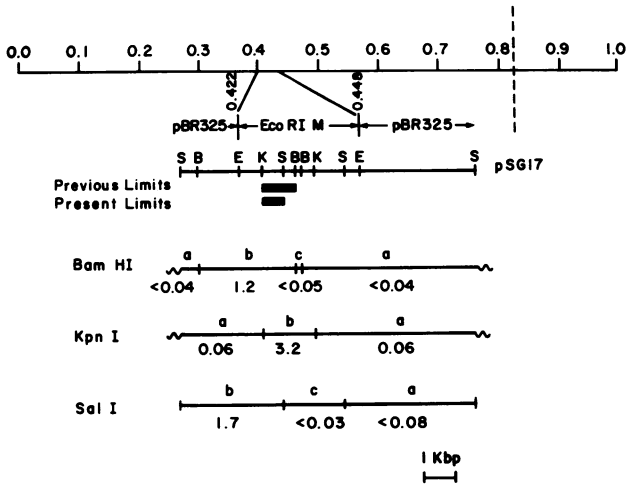

Figure 3. Marker rescue of aphidicolin-hypersensitivity of PAA^{r}5. The numbers on the top line are physical map co-ordinates of the HSV-1 strain KOS genome. The second line depicts plasmid pSG17 which contains the strain KOS Eco RI M fragment inserted into pBR325[28] as a linear molecule beginning and ending at the Sal I site contained within pBR325. Sequences derived from pBR325 and Eco RI M are specified by double-headed arrows. The lines from the top line to the second line indicate the approximate position of the Eco RI M fragment on the HSV-1 genome; the numbers indicate the map co-ordinates of the Eco RI M fragment[25]. The third line is a restriction map of pSG17[26]. Sites for restriction enzymes Sal I, Bam HI, Eco RI, and Kpn I are designated by S, B, E, and K, respectively. The larger bar below the third line shows the 1.1 kbp map limits for aphidicolin-hypersensitivity (and other drug resistance markers)[26] of PAA^{r}5 derived from the data presented in Figure 1. The smaller bar indicates the map limits derived from the experiment depicted in this figure. The bottom three lines show restriction maps of pSG17 for each of the enzymes, Bam HI, Kpn I, and Sal I. Fragments are designated with small case letters corresponding to the size of the fragment. The squiggly lines indicate that "end fragments" (e.g. Bam HI a) are actually one fragment traversing the Sal I site within pBR325 which forms the ends of the depicted linear molecule. Below each of the three bottom lines are marker rescue efficiencies obtained with each fragment. Marker rescue efficiencies are expressed as EOP x 10^3 ($\text{EOP} = \frac{\text{titer with drug}}{\text{titer without drug}}$).

four using the Bam HI d fragment of pPA22[26] (Figure 3). These recombinant viruses were tested for aphidicolin-hypersensitivity by plaque reduction assays. Eleven of thirteen were as aphidicolin-hypersensitive as PAA^{r}5. The plaque reduction data for two of these eleven, Kp3 and Ba8, are shown in Figure 1. The DNA restriction fragments used in constructing these recombinant viruses overlap by 1.1 kbp in the *pol* locus; thus, these data map the aphidicolin-hypersensitivity of PAA^{r}5 to this region (see Figure 3, "previous

limits").

The two remaining recombinant viruses, both derived from the Bam HI d fragment of pPA22, Ba5 and Ba9, were aphidicolin-hypersensitive, but less so than PAA^{r}5. The data for Ba5 and Ba9 suggest that more than one mutation within the 1.1 kbp region may be responsible for aphidicolin-hypersensitivity and that these mutations may have segregated during the construction of the recombinant viruses. Consistent with this suggestion are data obtained with a partial revertant of PAA^{r}5 selected for its ability to form plaques in 0.2 μg/ml aphidicolin. This revertant, like Ba5 and Ba9, is more sensitive to aphidicolin than KOS but less sensitive than PAA^{r}5. Moreover, it retains considerable PAA-resistance (unpublished results). Clearly then, more than one mutational site can affect aphidicolin-sensitivity.

The aphidicolin-hypersensitivity phenotype of PAA^{r}5 was mapped more finely in the marker rescue experiment depicted in Figure 3. In this experiment, cells were co-transfected with infectious mutant PAA^{r}5 DNA and restriction fragments of wild-type strain KOS DNA. The progeny from these transfections were tested for their ability to form plaques in 0.5 μg/ml aphidicolin. As shown in Figure 3, the Bam HI b, Kpn I b, and Sal I b fragment of pSG17[26,28] restored wild-type levels of aphidicolin-sensitivity. These data narrow the map limits of the phenotype to an 0.8 kbp region between the indicated Kpn I and Sal I restriction sites (Figure 3, "present limits"). Progeny which formed plaques in 0.5 μg/ml aphidicolin derived from this marker rescue experiment were as PAA-sensitive as wild-type strain KOS (not shown). Thus, the aphidicolin-hypersensitivity marker of PAA^{r}5 is closely linked to its PAA-resistance marker within the region of HSV DNA containing the pol locus[19,24,26].

DISCUSSION

Both biochemical and genetic experiments show then that the aphidicolin-hypersensitivity of PAA^{r}5 is due to a pol mutation. It seems likely that the aphidicolin-hypersensitivities exhibited by tsD9, tsC4, PAA^{r}3, AraA^{r}6-9,12,13, and ACG^{r}4 are due to pol mutations as well. These results imply that HSV DNA polymerase is a target of aphidicolin not only *in vitro*, but *in vivo*.

PAA is a pyrophosphate analog which appears to bind to the pyrophosphate exchange-release site of herpes virus DNA polymerases[23,32]. The correlation of altered aphidicolin-sensitivity with altered PAA-sensitivity would seem in simplest terms to indicate that amino acid alterations which affect this site affect aphidicolin binding as well. This suggests that aphidicolin binds in

close proximity to this site. An alternative, but more complicated model is that the amino acid alterations in the different mutants may be affecting both binding sites indirectly through changes in protein folding. A third possibility which cannot be excluded at present is that the aphidicolin binding site is apart from the pyrophosphate exchange-release site but that aphidicolin and PAA somehow share a common ultimate mechanism of inhibition as has been suggested for phosphonoformic acid and acyclovir[23]. Consistent with all three suggestions is the finding that aphidicolin inhibits pyrophosphate exchange catalyzed by HeLa cell DNA polymerase α[33]. Examination of additional mutants for coordinate alterations in aphidicolin- and PAA-sensitivity, and comparison of the activities of mutant and wild-type polymerases in the presence of varying concentrations of these two drugs should shed light on these issues.

Because aphidicolin appears to inhibit DNA polymerases competitively with dCTP, it has been proposed that it binds at or near the dCTP binding site[3,4,8-11]. Interestingly, PAA^{r}5-specified DNA polymerase which is substantially aphidicolin-hypersensitive (Figure 2) exhibits only a two-fold higher apparent K_m for dCTP than wild-type enzyme. A similar small increase in K_m was found for the other three dNTP's for PAA^{r}5-specified polymerase (P.A.F. and M.H. St. Clair, unpublished) and for dGTP and dTTP for the polymerases of five other PAA-resistant mutants[23]. Sugino and Nakayama found no change in the apparent K_m for dCTP for an aphidicolin-resistant DNA polymerase α of Drosophilia[5]. If aphidicolin binds at the dCTP binding site, then these mutations must affect that site to alter aphidicolin binding greatly while altering dCTP binding only minimally. Because DNA sequences containing the mutation(s) conferring aphidicolin-hypersensitivity in PAA^{r}5 have been cloned[26], it should be possible to determine the amino acid alterations associated with this phenotype.

Recently, Liu *et al.* have described an aphidicolin-resistant mammalian α-polymerase which exhibits a *decrease* in its apparent K_m for dCTP[7]. The cell line specifying this mutant polymerase exhibits a higher spontaneous mutation rate which may be due to the increased affinity for dCTP. Interestingly, several of the aphidicolin-hypersensitive mutants described here, including PAA^{r}5, exhibit lower spontaneous mutation rates[34]. In the case of PAA^{r}5, this antimutator phenotype maps to the *pol* locus[34]. This correlation is not absolute, however, as AraA^{r}9 which is only marginally aphidicolin-hypersensitive (Table 1) is an antimutator[34] and mutant PAA^{r}C which is *not* an antimutator[34] is aphidicolin-hypersensitive (H. Chiou and D.M.C., unpublished).

Since modified HSV polymerase can be hypersensitive to aphidicolin, it may be that a modified form of aphidicolin could effectively inhibit HSV without major deleterious effects on cells and thus function as an effective antiviral.

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After this manuscript was submitted, it was reported[35] that five other PAA-resistant mutants[23] are hypersensitive to aphidicolin.

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