Molecular Analysis of the Pyrimidine Deoxyribonucleoside Kinase Gene of Wild-type and Acyclovir-resistant Strains of Varicella-Zoster Virus

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SUMMARY

The pyrimidine deoxyribonucleoside kinase (dPK) genes from five wild-type and four acyclovir-resistant varicella-zoster virus (VZV) strains were studied. One of the acyclovir-resistant strains was isolated from a patient receiving chronic acyclovir therapy. Acyclovir-resistant strains expressed the 1.8 kb VZV dPK transcript but lacked dPK activity. To determine the basis for the lack of enzyme activity the dPK gene from each strain was cloned and its DNA sequence determined. The VZV dPK gene was found to be highly conserved among strains, with greater than 99% nucleotide and amino acid homology. Each acyclovir-resistant VZV strain differed from its wild-type parent in only a single amino acid. The dPK genes from the acyclovir-resistant strains contained either point mutations near the putative thymidine-binding site of the enzyme or ones that resulted in the premature termination of protein synthesis. Single point mutations were sufficient to render these strains dPK-negative and highly resistant to acyclovir. The molecular basis for acyclovir resistance at the dPK locus of VZV is similar to that previously noted to render herpes simplex viruses resistant to acyclovir.

INTRODUCTION

The varicella-zoster virus (VZV) genome encodes an M, 35K pyrimidine deoxyribonucleoside kinase (dPK) (Lopetegui et al., 1983; Shiraki et al., 1985). The VZV dPK gene has been mapped to the middle of the unique long (U1) segment of the VZV genome (map units 0.50 to 0.52) and shown to encode a 1.8 kb transcript (Sawyer et al., 1986; Davison & Scott, 1986). The herpes simplex virus (HSV) thymidine kinase (TK) shares approx. 28% amino acid sequence homology with the VZV dPK, and neither enzyme is essential for virus replication in vitro. These enzymes are, however, important for the activation of certain antiviral drugs (Elion et al., 1977) and may be important in the pathogenesis of infection (Field & Wildy, 1978; Tenser & Dunstan, 1979). The most effective antiviral agent currently available for VZV infections is the guanosine analogue acyclovir, which is phosphorylated preferentially by the VZV dPK. Although acyclovir-resistant VZV strains are readily isolated in vitro, the first clinically derived drug-resistant strain of VZV has only recently been recovered from a patient being treated with acyclovir. The majority of laboratory-selected acyclovir-resistant strains have alterations in expression of the viral dPK (Biron et al., 1982). Most of these possess no detectable dPK activity, but strains with altered dPK substrate specificity do occur (Shiraki et al., 1986).

Acyclovir resistance in HSV is also usually mediated by mutations in its TK gene (Coen & Schaffer, 1980; Schnipper & Crumpacker, 1980; Darby et al., 1981; Field et al., 1980). The
introduction of single point mutations within the TK coding region are sufficient to render strains unable to phosphorylate thymidine (Darby et al., 1986). Analysis of these HSV mutants helped to define the active domains of the HSV TK and has provided an understanding of one mechanism of acyclovir resistance.

In order to determine the molecular basis for acyclovir resistance in VZV, three acyclovir-resistant VZV strains were selected in vitro from wild-type (wt) parental strains. These strains were examined for their levels of dPK activity and for their abilities to synthesize the 1.8 kb VZV dPK transcript. The dPK gene was cloned from each wt and drug-resistant strain and its DNA sequence was determined. An additional acyclovir-resistant VZV strain has recently been isolated from a patient with chronic zoster who was receiving long-term acyclovir therapy; the DNA sequence of the dPK gene from this strain was also determined. These studies allowed us to determine a basis for acyclovir resistance at the VZV dPK locus and to compare the active domains of the VZV dPK with those of other herpesvirus TKs.

**METHODS**

**Virus strains.** Wild-type VZV strains studied include the high-passage VZV Ellen (American Type Culture Collection no. VR-586), VZV Oka, the vaccine strain (American Type Culture Collection no. VR-795), and VZV ppIIa. Also analysed was a low-passage clinical isolate, VZV GK. Acyclovir-resistant mutants were selected in vitro by passage of virus in the presence of acyclovir (25 to 100 μM). Isolates were plaque-purified at least twice before study. Acyclovir-resistant mutant Clone 3 (3-5-2) (KB3) was derived from VZV Ellen in Research Triangle Park, N.C., U.S.A. The mutant VZV 101 was derived from VZV Ellen at the National Institute of Allergy and Infectious Diseases, Bethesda, Md., U.S.A. The mutant VZV 40a2 was derived from VZV ppIIa. The clinically derived acyclovir-resistant VZV strain 10623 was shown to be a mixture of a wt strain (5-1-1) and an acyclovir-resistant strain (7-1-3). These subpopulations were plaque-purified and analysed separately.

**Preparation and cloning of viral DNA.** VZV was grown in human diploid fibroblasts (Flow 5000 cell line, Flow Laboratories; or MRC-5 cells, Whittaker MA Bioproducts, Walkersville, Md., U.S.A.) in a 1:1 mixture of Eagle's MEM and medium 199 (Quality Biologicals, Gaithersburg, Md., U.S.A.) supplemented with 10% foetal calf serum, penicillin G (100 units/ml), streptomycin (100 μg/ml) and 2 mM-glutamine. Viral DNA was purified from nucleocapsids as previously described (Straus et al., 1982), digested with the restriction enzyme PstI (Bethesda Research Laboratories), and cloned into the PstI site of the plasmid pUC8 (Bethesda Research Laboratories). Recombinant clones containing the 2.6 kb VZV PstI P DNA restriction fragments were isolated. For the majority of sequencing reactions cloned DNA was prepared by the method of Birnboim & Doly (1979) and further purified with two isopycnic bandings in caesium chloride followed by ethanol precipitation. For a few samples, DNA was prepared from overnight cultures of plasmid-containing bacteria by the method of Hattori & Sakaki (1986).

**dPK assay and acyclovir sensitivity.** For assaying dPK activity, each virus strain was grown in 150 cm² tissue culture flasks of Flow 5000 or MRC-5 cells until 50 to 75% of the cells showed c.p.e. Infected cells were washed three times with cold phosphate-buffered saline pH 7.4 and harvested by scraping. Five pellets were prepared from each flask and stored at -70 °C until assayed. The dPK activity in cell lysates was determined by measuring the in vitro phosphorylation of [3H]thymidine and [3H]deoxyctydine as previously described (Sawyer et al., 1986). The enzyme activity was normalized for the amount of protein in each extract. Protein concentrations were determined by the method of Bradford (1976).

The concentration of acyclovir required to inhibit virus growth was determined by a plaque reduction assay. Confluent monolayers of Flow 5000 or MRC-5 cells in 60 mm dishes were infected with cell-associated virus to yield approximately 50 to 100 plaques per dish. Virus was grown in the presence of a range of acyclovir concentrations (0 to 400 μM) for 5 to 7 days until plaques were clearly seen. Each drug concentration was tested in duplicate. The monolayers were fixed in 10% formalin in phosphate-buffered saline pH 7.4 for 30 min and then stained with 0.8% crystal violet in 50% ethanol for 1 h. Plaques were then counted. An inhibitory dose was calculated to be the concentration of acyclovir that reduced the plaque number by 50% (IC50).

**Northern blot analysis.** Total cellular RNA was isolated from VZV-infected and uninfected MRC-5 cells by the methods of Chirgwin et al. (1979) and Glisen et al. (1974) as modified by Ostrove et al. (1985). The RNAs were separated by electrophoresis through 6% formaldehyde-1.5% agarose horizontal slab gels (Rave et al., 1979), transferred to nitrocellulose paper and hybridized to a nick-translated [32P]dATP-labelled pUC8 plasmid (sp. act. 8.5 x 10⁶ c.p.m./μg) containing a 700 bp AvaI VZV DNA subfragment of PstI that lies within the dPK gene (Fig. 1).

**DNA sequencing.** Double-stranded plasmids containing VZV PstI P were used as templates for DNA sequencing by the dideoxy chain termination method (Sanger et al., 1977). Oligonucleotide DNA primers of 17 or 18 bp were prepared according to the published VZV DNA sequence (Davison & Scott, 1986) using an Applied
Acyclovir-resistant VZV

Acyclovir-resistant VZV strains were selected in vitro from two wt parent strains by growth in the presence of the drug. Their in vitro susceptibilities to acyclovir are shown in Table 1. Each strain had an ID_{50} that was similar to those of previously described wt or acyclovir-resistant VZV strains. Repeated passage in the absence of acyclovir did not alter the ID_{50}. Resistant strains exhibited a 12.9- to 85-fold increase in ID_{50} relative to their parental strains.

The acyclovir-resistant strains expressed very low levels of dPK activity, as measured by in vitro phosphorylation of [3H]thymidine and [3H]deoxycytidine (Table 2). As previously noted, the VZV dPK exhibits greater deoxycytidine kinase activity than thymidine kinase activity (Sawyer et al., 1986). Strain KB3 was also shown to exhibit acyclovir-phosphorylating activity that was less than 2% of that of its wt parent, VZV Ellen (data not shown).

A detailed restriction endonuclease map of the region of the VZV genome containing the dPK gene is shown in Fig. 1. The VZV dPK transcript has been characterized and is 1.8 kb in size (Sawyer et al., 1986; Davison & Scott, 1986). Northern blot analyses, in which infected cellular RNAs were probed with the 700 bp AvaI VZV DNA subfragment that lies completely within the dPK gene, demonstrated that wt and acyclovir-resistant mutants all produced the 1.8 kb

Table 1. Acyclovir sensitivity of virus strains

<table>
<thead>
<tr>
<th>Virus</th>
<th>ID_{50} (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type strains</td>
<td></td>
</tr>
<tr>
<td>VZV Ellen</td>
<td>5.0</td>
</tr>
<tr>
<td>VZV GK</td>
<td>6.7</td>
</tr>
<tr>
<td>VZV Oka</td>
<td>6.3</td>
</tr>
<tr>
<td>VZV ppIIa</td>
<td>6.6</td>
</tr>
<tr>
<td>Acyclovir-resistant strains</td>
<td></td>
</tr>
<tr>
<td>VZV KB3</td>
<td>425</td>
</tr>
<tr>
<td>VZV 40a2</td>
<td>214</td>
</tr>
<tr>
<td>VZV 101</td>
<td>176</td>
</tr>
</tbody>
</table>
Fig. 2. Northern blot analysis of the dPK mRNA from VZV-infected cells. The in vitro $^{32}$P-labelled AvaI 700 bp fragment from within the dPK gene was used as the probe. Lanes contain 10 μg of total cellular RNA from: uninfected cells (lane 1), cells infected with wt VZV strains Ellen (lane 2), GK (lane 3), Oka (lane 4) or ppIIa (lane 5), or acyclovir-resistant strains KB3 (lane 6), 40a2 (lane 7) or 101 (lane 8). Autoradiography was for 5 days at $-70\, ^\circ\mathrm{C}$.

Table 2. dPK activity of infected cell extracts

<table>
<thead>
<tr>
<th>Virus</th>
<th>Thymidine kinase*</th>
<th>Deoxycytidine kinase*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Wild-type strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSV</td>
<td>39</td>
<td>2</td>
</tr>
<tr>
<td>VZV Ellen</td>
<td>16·3</td>
<td>46</td>
</tr>
<tr>
<td>VZV GK</td>
<td>16</td>
<td>18·5</td>
</tr>
<tr>
<td>VZV Oka</td>
<td>16</td>
<td>29</td>
</tr>
<tr>
<td>VZV ppIIa</td>
<td>12·1</td>
<td>35</td>
</tr>
<tr>
<td>Acyclovir-resistant strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VZV KB3</td>
<td>1·0</td>
<td>0·7</td>
</tr>
<tr>
<td>VZV 40a2</td>
<td>1·5</td>
<td>0·9</td>
</tr>
<tr>
<td>VZV 101</td>
<td>0·54</td>
<td>1·7</td>
</tr>
</tbody>
</table>

* Activity is expressed relative to that of uninfected cells.

VZV dPK transcript (Fig. 2). The differences in the quantity of this transcript seen among strains appears to reflect different stages of cell culture infection at the time of RNA preparation, a problem that cannot be eliminated because of the inability to infect cell cultures synchronously. We cannot exclude the possibility of altered levels of transcription between the various strains. No transcripts were detected in uninfected cells.
Acyclovir-resistant VZV

The PstI P DNA fragments from these strains as well as the wt (5-1-1) and acyclovir-resistant (7-1-3) subpopulations of the clinically derived strain 10623 were cloned, and the nucleotide sequence of the VZV dPK gene was determined from both strands. This DNA fragment contains the dPK coding sequence as shown by its ability to transform successfully thymidine kinase-deficient mouse L (LTK⁻) cells to the LTK⁺ phenotype (unpublished observations). The dPK amino acid sequence was predicted from the DNA sequence (Fig. 3).

The sequence analysis for each strain showed the VZV dPK open reading frame to be highly conserved, having greater than 99% nucleotide and amino acid homology. A summary of the individual base changes noted between strains is given in Table 3. The amino acid leucine at residue 288 was shared by all strains examined, but differed from the published sequence of strain Dumas which has a serine in this position (Davison & Scott, 1986). Strain VZV GK has an alanine to valine substitution at amino acid 319 which did not alter dPK activity. VZV Ellen and the drug-resistant strains derived from it (KB3, 101) share an adenine to guanine transition at nucleotide +78 (relative to the first base of the open reading frame), one that does not result in a change in amino acid sequence. Strains ppIIa and 5-1-1 are identical to VZV Ellen except for this same transition at nucleotide +78. Each of the amino acid sequences of the acyclovir-resistant strains differs from that of its wt parent in only one location. VZV strain 40a2 differs from its wt parent (VZV ppIIa) in a thymine to cytosine transition at nucleotide +461, one that

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**Fig. 3.** The predicted amino acid sequence of the VZV dPK from VZV Ellen is shown in single-letter code. The putative nucleotide (residues 12 to 29)- and nucleoside (residues 129 to 145)-binding sites are underlined. The dPKs of the wt strains Oka, ppIIa and 5-1-1 had amino acid sequences identical to that of VZV Ellen. Differences noted in amino acid sequences from that of VZV Ellen are shown above the line and the strains having these changes shown underneath. Dum is VZV Dumas, the sequence of which was published by Davison & Scott (1986).
results in a leucine to proline substitution at residue 154. This residue is nine amino acids downstream from the end of the putative nucleoside-binding site of the enzyme (Kit, 1985). Strain 7-1-3 differs from the wt subpopulation isolated from the same patient in a guanine to adenine transition at nucleotide +389, one that results in an arginine to glutamine substitution at residue 130. This change occurs within the putative nucleoside-binding site. The two acyclovir-resistant strains derived from VZV Ellen (KB3, 101) share a guanine to adenine transition at nucleotide +675 which results in a premature stop codon at amino acid residue 225 and presumably leads to the synthesis of truncated proteins.

A third plaque-purified acyclovir-resistant mutant was derived from VZV Ellen in the same laboratory as KB3. The sequence of this strain proved to be identical to that of KB3 in its dPK gene.

**DISCUSSION**

We studied the dPK gene from four wt strains of VZV and three acyclovir-resistant mutants derived from them in the laboratory. These mutant strains were readily selected in the presence of acyclovir and had alterations in dPK expression. Wild-type and acyclovir-resistant VZV subpopulations isolated from a patient were also studied. The VZV dPK was not essential for virus replication *in vitro*, in that dPK-negative strains appeared to grow normally in tissue culture. DNA polymerase mutations also confer acyclovir resistance but occur much less frequently than dPK mutations.

The wt VZV strains examined all had normal levels of dPK activity as determined by *in vitro* phosphorylation of thymidine and deoxycytidine. Acyclovir-resistant strains had very low dPK activity. All strains studied produced the 1.8 kb dPK transcript, suggesting that the defects in dPK activity in these drug-resistant strains are post-transcriptional.

The precise mapping of the VZV dPK gene (Sawyer *et al*., 1986; Davison & Scott, 1986) allowed us to isolate, clone and sequence the gene from both drug-sensitive and drug-resistant VZV strains and to identify the mutations that render them acyclovir-resistant. The VZV dPK open reading frame consists of 1023 bp and encodes a protein of 341 amino acids (Davison & Scott, 1986). The protein has a predicted *M*, of 37 815, which is consistent with the previously reported size of the VZV dPK (Lopetegui *et al*., 1983; Shiraki *et al*., 1985).
The DNA sequence analyses showed the VZV dPK gene to be highly conserved among strains. The wt strains studied had geographically diverse origins and dPK-negative mutants were derived in three independent laboratories. These strains of diverse origin shared greater than 99% amino acid homology. Such a high level of homology suggests that the dPK gene in VZV is evolutionarily conserved. Since the dPK is not essential for virus replication in tissue culture, this conservation suggests that the VZV dPK gene may be important in aspects of infection in vivo. Studies of animal models of HSV infection suggest that the HSV TK may be involved in neurovirulence and also may be important in the establishment of latency (Field, 1982; Field & Darby, 1980; Price & Khan, 1981; Tenser & Dunstan, 1979; Tenser & Edris, 1987; Tenser et al., 1979, 1981). The high level of VZV dPK conservation also facilitated the recognition of mutations that were likely to be responsible for the loss of dPK activity in the acyclovir-resistant strains studied, since there were single changes between wt parents and drug-resistant mutants derived from them.

By comparing the amino acid sequence of kinase proteins from a number of herpesvirus and other eukaryotic kinases, Kit (1985) proposed nucleotide (ATP)- and nucleoside (thymidine)-binding site consensus sequences (Fig. 4). His predictions were supported by the finding that single base changes in the predicted nucleoside-binding site were noted in HSV strains with altered affinity for thymidine (Darby et al., 1986). Site-directed mutagenesis has confirmed the importance of the putative ATP-binding domain in HSV (Liu & Summers, 1988). Analysis of the VZV dPK sequence showed two areas that share significant homology with these putative binding sites. Amino acid residues 12 to 29 share homology with the ATP-binding regions and residues 129 to 145 share homology with the thymidine-binding regions. These regions share the same relative orientation and spacing in the VZV dPK as found in other herpesviruses (Fig. 4).

The drug-resistant strain VZV 40a2 differs from its wt parent in only one amino acid, a leucine to proline substitution at residue 154. This substitution falls near the putative nucleoside-binding site of the dPK gene. The insertion of a proline at this location might be expected to alter the secondary structure of the protein near the binding site sufficiently to render the enzyme inactive. Acyclovir-resistant (7-1-3) and drug-sensitive (5-1-1) isolates from the same patient differ only in an arginine to glutamine substitution at amino acid residue 130, which falls within the proposed nucleoside-binding site. These two mutants demonstrate the importance of this domain in the VZV dPK and support the suggestion that it constitutes a nucleoside-binding site.

The dPK-negative mutants selected from VZV Ellen (KB3, 101) share the same mutation; a guanine to adenine transition that results in a premature stop codon at amino acid residue 225.

Fig. 4. Putative (a) ATP- and (b) thymidine-binding regions of herpesvirus thymidine kinases. Portions of the thymidine kinase amino acid sequences from the virus strains identified on the left are shown in single-letter code. The location of these regions within each protein is shown by the amino acid residue numbers in parentheses. Sequences are aligned to show maximum homology. Virus strains represented include pseudorabies virus (BVK-F), HSV-1 (SC16), HSV-2 (333), marmoset herpes virus (HV) (K) and Epstein-Barr (EBV) (B95-8). Modified from Kit (1985).
These dPK-negative VZV strains are otherwise identical to their wt parent. Such a truncated protein would be expected to lack activity. Strain KB3 was shown to lack acyclovir-phosphorylating activity (unpublished observations), as well as dPK activity. This loss of kinase activity for several substrates is consistent with an alteration in protein structure, such as that caused by premature termination. Similar nonsense mutations have been identified in TK-deficient HSV strains (Summers et al., 1975; Cremer et al., 1979). It was surprising that these VZV mutants independently derived in separate laboratories contained the same base substitution. Since they were derived from the same parent strain, VZV Ellen, the most likely explanation of this finding is that the parental population is a mixed one that contains both wt and acyclovir-resistant subpopulations; in the presence of acyclovir the drug-resistant population is selected. Such mixed populations have been identified in HSV isolates from patients (Parris & Harrington, 1982). The fact that our acyclovir-resistant, VZV Ellen-derived strains were isolated more than 2 years apart suggests that such mixed populations may be stable over many passages in vitro and that neither wt nor dPK-negative viruses have a growth advantage in tissue culture.

The VZV dPK has an unusually long untranslated leader sequence of approximately 420 bp (Davison & Scott, 1986). This sequence contains two translation initiation (AUG) codons in reading frames different from the one actually used for initiation of translation. It has been proposed that such upstream initiation signals may influence the efficiency of translation (Kozak, 1984). This region was also found to be highly conserved among VZV strains, suggesting that it, too, is evolutionarily conserved because of some as yet unspecified function.

We have shown that single point mutations in the VZV dPK confer acyclovir resistance, as has been previously demonstrated in HSV (Darby et al., 1986). Alteration of TK activity has been shown to be an important mechanism of drug resistance in clinically derived HSV mutants in man (Burns et al., 1982; Crumpacker et al., 1982; Sibrack et al., 1982; Wade et al., 1982). VZV and HSV share significant homology in their dPK and TK nucleoside- and nucleotide-binding domains and mutations within the nucleoside-binding region can eliminate dPK activity. To date, drug-resistant herpesviruses have been of little clinical consequence in humans. Experience with HSV suggests that dPK-negative strains are less virulent than wt virus; however, there is no suitable animal model in which to address the potential virulence of VZV dPK-negative mutants for humans.

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REFERENCES


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Ayclovir-resistant VZV