Antiviral properties of a dominant negative mutant of the herpes simplex virus type 1 regulatory protein ICP0

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Dominant negative or trans-dominant mutants of viral proteins represent a new and exciting potential approach to antiviral therapy. Unfortunately, the extreme specificity of a given dominant negative mutant limits its general utility in treating a broad spectrum of viral diseases, since it can typically interfere with the activity of only a single viral polypeptide encoded by a single virus. However, it seems likely that dominant negative mutants of promiscuous viral trans-activator proteins, which by definition would repress rather than activate gene expression, should be able to inhibit infectious virus production for a number of different viruses. One such dominant negative mutant, derived from the herpes simplex virus type 1 (HSV-1) regulatory protein ICP0, was found previously to behave as a powerful repressor of gene expression from an assortment of HSV-1 and non-HSV-1 promoters in transient expression assays.

In the present study, this ICP0 mutant was found to be capable of inhibiting the replication of both HSV-1 and a completely unrelated virus, human immunodeficiency virus, in cell culture. The properties of this dominant negative mutant indicate that it may have potential as a means of treating diseases caused by a number of DNA and RNA viruses. Moreover, a truncated form of ICP0 which can hypothetically be created by alternative splicing was found to possess similar inhibitory capabilities, suggesting that a virus-encoded version of this dominant negative mutant may play a role in down-regulating HSV-1 gene expression during infection in vivo.

Introduction

The existence of a class of mutant proteins which could exert a dominant negative effect on the activity of their wild-type parents was first postulated several years ago (Herskowitz, 1987). Demonstrations that mutant viral proteins with these properties could act as antiviral agents in herpes simplex virus type 1 (HSV-1) (Friedman et al., 1988) and human immunodeficiency virus (HIV) (Malim et al., 1989; Green et al., 1989) infections followed soon thereafter. Interference by such dominant negative or trans-dominant mutants can be effected by a variety of mechanisms. For example, the mutant and wild-type proteins may interact directly to form a non-functional heterodimer. Alternatively, the targeted viral protein may have to interact with a cellular protein for activity, so that any mutant that associates with this host cell factor in a non-functional manner will mediate its sequestration from the wild-type protein. The latter situation has been observed for a truncated version of the HSV-1 trans-activator protein VP16, which was shown to inhibit virus replication by titrating out a host cell transcription factor, OTF-1, required for wild-type VP16 activity (Friedman et al., 1988).

The hallmark of the dominant negative mutants of viral proteins isolated to date is their specificity: each can interfere with the activity of only a single viral polypeptide encoded by a single virus. Thus, a mutant protein which may strongly inhibit the replication of one particular virus will have no effect on the infectious cycles of other viruses. It has not yet been possible to design a protein that could inhibit the replication of a wide variety of viruses in a trans-dominant manner. However, one class of viral polypeptides which might exhibit broad spectrum antiviral activity are the promiscuous trans-activator proteins found in DNA viruses. Dominant negative mutants of these proteins which would be able to inhibit promiscuously rather than stimulate gene expression should theoretically be able to reduce infectious virus production for a number of unrelated viruses.

One well studied promiscuous trans-activator protein is the HSV-1 immediate early polypeptide ICP0 (infected cell polypeptide 0), which has been shown to
increase gene expression dramatically from a variety of HSV-1 and non-HSV-1 promoters by a mechanism which is independent of specific promoter sequences (Everett, 1984; Gelman & Silverstein, 1985; O'Hare & Hayward, 1985; O'Hare et al., 1986; Quinlan & Knipe, 1985; Mosca et al., 1987; Ostrove et al., 1987; Shapira et al., 1987; Nabel et al., 1988; Sekulovich et al., 1988). It is hypothesized that this generalized transcriptional boost which ICP0 provides may play a role in the process of HSV-1 reactivation from latent infections in vivo (Cai & Schaffer, 1989; Clements & Stow, 1989; Harris et al., 1989; Leib et al., 1989a; Zhu et al., 1990). The means by which ICP0 mediates its stimulatory effects in such a promiscuous manner are far from clear; several possibilities include interactions with the basic transcriptional machinery of the host cell, reassembly of host cell chromatin into a more activated form, or recompartmentalization of transcription complexes in the nucleus (reviewed by Everett et al., 1991).

In a previous mutational analysis of the ICP0 gene, a powerful dominant negative mutant was isolated which possessed the ability not only to interfere with the activity of the wild-type ICP0 protein, but also that of other trans-activator proteins and non-HSV-1 viral promoters (Weber & Wigdahl, 1992). Thus, this mutation had converted the ICP0 protein from a promiscuous trans-activator into a promiscuous repressor, since presumably it was able to undergo non-productive interactions with the same host cell factor(s) utilized by ICP0 (and other regulatory proteins) during trans-activation. In this study, this ICP0 mutant was found to inhibit the replication of both HSV-1 and a heterologous virus, HIV. It therefore represents the first dominant negative mutant of a viral protein for which broad spectrum antiviral properties have been demonstrated.

Methods

Cell culture, transfection procedures and virus propagation. Vero cells were used in all chloramphenicol acetyltransferase (CAT) assay experiments and were grown in minimum essential medium supplemented with 5% calf serum. Transfection of plasmid DNA was performed using calcium phosphate precipitation as described by Shapira et al. (1987), except that the transfected cells were not subjected to glycerol or dimethyl sulphoxide shock, in accordance with the procedure of Everett (1988b). Equimolar amounts (approximately 2 µg) of each plasmid were transfected and all concentrations were verified by agarose gel electrophoresis prior to transfection. For experiments utilizing infectious HSV-1 DNA, Vero cells were cotransfected with 2 µg plasmid DNA and 2 µg gradient-purified HSV-1 (KOS) genomic DNA prepared as described by Homa et al. (1986). A 15% glycerol shock was performed 4 h after the precipitated DNAs were added, medium containing methylcellulose was added 24 h later and plaques were counted after 5 days. For experiments employing infectious HIV proviral DNA, 0.1 µg plasmid DNA and 0.2 µg pNL4-3, which contains a cloned HIV proviral genome (Adachi et al., 1986), were cotransfected into HeLa cells using Lipofectin reagent (Bethesda Research Laboratories). Culture supernatants were collected from the transfected cells after 24 and 48 h and were analysed for production of the HIV capsid protein p24 using an antigen capture ELISA kit (NEN-DuPont).

CAT assays. Forty-eight hours after transfection, cells were lysed and extracts prepared by a Triton X-100 detergent extraction procedure (Weber & Wigdahl, 1992). CAT assays were then performed on the extracts, and their butyrylated chloramphenicol products were extracted with xylene and quantified by a liquid scintillation counting assay (Weber & Wigdahl, 1992).

Construction of plasmids. The reporter construct employed in all transient expression assays was pgC-CAT, which contains the promoter for the HSV-1 late gene encoding glycoprotein C fused to the CAT gene (Shapira et al., 1987). All carboxy-terminal truncating mutations of HSV-1 trans-activator proteins were made using the vector pSG424 (Sadowski & Ptashne, 1989), which contains a polynucleotid with numerous cloning sites, an adjacent region containing stop codons in all three reading frames that truncates any inserted protein-encoding sequences and a simian virus 40 polyadenylation signal. The construction of pSG424-ICP0, which contains the wild-type ICP0 gene cloned into pSG424, and pD19T, pHKT and pHXT, which contain three carboxy-terminal truncating mutations of ICP0, was detailed in a previous paper (Weber & Wigdahl, 1992). pKST contains amino acids 1 to 241 of ICP0 as well as 21 amino acids derived from the beginning of the second intron of ICP0. It was constructed in two steps. First, the 0.2 kb KpnI–Sau3AI fragment of p111 (Everett, 1987) was inserted into the KpnI and BamHI sites of pGEM7 (Promega). Second, this insert was removed as a KpnI–SstI fragment and ligated into the KpnI and SstI sites of pHKT. The Sau3AI site which serves as the endpoint of the 3' deletion in pKST maps 23 bp upstream of the splice acceptor site of intron 2. pVP16T encodes amino acids 1 to 424 of VP16 and was constructed by inserting the 2.9 kb BglII–SstI fragment of pSG22 (Göldin et al., 1981) into the BglII and SstI sites of pSG424. This derivative has lost the powerful acidic transcriptional activation domain encoded by the last 66 amino acids of the wild-type VP16 protein, enabling VP16T to possess a dominant negative phenotype (unpublished results) analogous to that of the mutant described by Friedman et al. (1988). In all manipulations using pSG424, coding sequences of the GAL4 gene were replaced by ICP0 or VP16 DNA.

Results

The dominant negative mutant of ICP0 which was characterized in this work is contained in plasmid pD19T and was originally obtained in a mutational analysis of the wild-type protein (Weber & Wigdahl, 1992). It contains a carboxy-terminal truncation of the ICP0 protein, so that it retains amino acids 1 to 245 but has lost amino acids 246 to 775 (Fig. 1). Two other plasmids containing ICP0-encoding sequences were used as controls in the studies presented below (Fig. 1). pSG424-ICP0 encodes all 775 amino acids of ICP0 and was used as a wild-type construct. pHKT, however, encoded only amino acids 1 to 105 of ICP0 and the resultant polypeptide completely lacked both the trans-activation properties of the wild-type protein and the repression properties of the pD19T mutant; it was
Antiviral activity of a mutant ICP0 protein

Fig. 1. Plasmids encoding wild-type and dominant negative mutant derivatives of the HSV-1 immediate early protein ICP0. All plasmids were constructed as described in Methods. pSG424-ICP0 contains the wild-type ICP0 gene, and pD19T, pKST and pHXT encode truncated versions of ICP0 (sizes indicated; aa, amino acids). Open boxes represent the three protein-encoding exons of ICP0 (and the protein-encoding region of intron 2 in pKST); lines represent transcripts and introns.

Employed as a negative control in all of the experiments presented.

The ability of the pD19T mutant to interfere with the activity of the wild-type ICP0 protein is evident in transient expression assay experiments employing co-transfection of equimolar amounts of both plasmids (Weber & Wigdahl, 1992; Table 1). pD19T was found to behave as a dominant negative mutant in these co-transfection assays, since the level of trans-activation of the promoter for the HSV-1 late gene encoding glycoprotein C reflected that of the mutant protein rather than that of the wild-type protein. The strength of this inhibition was apparent in transfection assays which employed dilutions of the pD19T plasmid relative to a fixed concentration of the wild-type ICP0 protein. ICP0 trans-activation was typically reduced to below basal levels in the presence of an equimolar concentration of the pD19T mutant, and was restored to greater than 50% only after dilution of the pD19T mutant to a 1/20 molar concentration (Table 1). Cotransfection with equimolar levels of the ICP0 null mutant pHXt was found to have no inhibitory effects on ICP0 trans-activation (Table 1), demonstrating that the interference observed was a specific effect mediated by pD19T and was not simply due to a promoter competition phenomenon. The pD19T construct was examined previously for its ability to inhibit a wide variety of HSV-1 promoters, non-HSV-1 promoters and HSV-1 trans-activator proteins in transient expression assays. In every experiment carried out to date, this mutant protein has behaved as a powerful repressor of gene expression (Weber & Wigdahl, 1992). Thus, just as the wild-type ICP0 protein behaves as a promiscuous trans-activator of gene expression in transient assays, the dominant negative mutant of ICP0 in pD19T behaved as a promiscuous repressor.

The pronounced ability of the pD19T mutant to interfere with promoter activation by three different HSV-1 trans-activator proteins (Weber & Wigdahl, 1992) suggested that it might manifest antiviral activity if expressed in HSV-1-infected cells. This possibility was tested using a transfection assay approach (Gao & Knipe, 1991) in which infectious HSV-1 genomic DNA was cotransfected into Vero cells with individual ICP0 or VP16 plasmids; the resulting infections were then characterized with respect to plaque formation (Table 2). pD19T and pSG424-ICP0 gave results which paralleled the findings from the transient assay experiments: the former was found to act as a powerful inhibitor of HSV-1 replication in that it nearly abolished plaque formation.

Table 1. Characterization of ICP0 plasmids in transient expression assays

<table>
<thead>
<tr>
<th>Transfected plasmids</th>
<th>CAT activity (units x 10^-4)*</th>
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<tbody>
<tr>
<td>pgC-CAT</td>
<td>9.7</td>
</tr>
<tr>
<td>pgC-CAT + pHXT</td>
<td>9.8</td>
</tr>
<tr>
<td>pgC-CAT + pSG424-ICP0</td>
<td>76.3</td>
</tr>
<tr>
<td>pgC-CAT + pD19T</td>
<td>7.8</td>
</tr>
<tr>
<td>pgC-CAT + pKST</td>
<td>6.0</td>
</tr>
<tr>
<td>pgC-CAT + pSG424-ICP0 + pHXT</td>
<td>73.9</td>
</tr>
<tr>
<td>pgC-CAT + pSG424-ICP0 + pKST</td>
<td>12.6</td>
</tr>
<tr>
<td>pgC-CAT + pSG424-ICP0 + pD19T</td>
<td>8.7</td>
</tr>
<tr>
<td>pgC-CAT + pSG424-ICP0 + pD19T (1:5 dilution)</td>
<td>19.2</td>
</tr>
<tr>
<td>pgC-CAT + pSG424-ICP0 + pD19T (1:10 dilution)</td>
<td>33.8</td>
</tr>
<tr>
<td>pgC-CAT + pSG424-ICP0 + pD19T (1:20 dilution)</td>
<td>57.1</td>
</tr>
</tbody>
</table>

* Equimolar amounts of plasmid DNAs were transfected into Vero cells, except in those experiments which employed the indicated molar dilutions of pD19T plasmid. CAT assays were performed on cellular extracts, and n-butyryl-l-^C-chloramphenicol products were isolated and quantified using liquid scintillation as described in Methods. Counts were converted to units of CAT activity by the preparation of a standard curve using purified CAT (Promega). Values represent the mean total CAT activity in a 60 mm dish of transfected cells as determined by a minimum of four experiments.

Table 2. Inhibition of HSV-1 replication by dominant negative mutants of ICP0 and VP16

<table>
<thead>
<tr>
<th>Cotransfected plasmid</th>
<th>Plaque count*</th>
</tr>
</thead>
<tbody>
<tr>
<td>pHXT</td>
<td>179</td>
</tr>
<tr>
<td>pSG424-ICP0</td>
<td>296</td>
</tr>
<tr>
<td>pD19T</td>
<td>6</td>
</tr>
<tr>
<td>pVP16T</td>
<td>61</td>
</tr>
</tbody>
</table>

* Plaques were generated by cotransfection of 2 \( \mu \)g of gradient-purified HSV-1 genomic DNA with 2 \( \mu \)g of the indicated plasmid into Vero cells. Transfections were performed in duplicate as described in Methods.
cotransfections of 0.2 μg of pNL4-3, which contains a cloned HIV proviral genome (Adachi et al., 1986), with 0.1 μg of the indicated ICP0 plasmid (or with no additional plasmid) were carried out in HeLa cells. The amount of HIV capsid protein p24 in culture supernatants was determined. The mean production of p24 protein in those transfections where pHXT and, surprisingly, pSG424-ICP0 were cotransfected into HeLa cells (unpublished results). The apparent inability of the wild-type ICP0 gene to mediate promoter trans-activation capability have been reported previously for ICP0 in studies which employed the HIV long terminal repeat promoter (Ostrove et al., 1987) and other promoters (Everett, 1986, 1988b; Sekulovich et al., 1988), and appear to be the result of a number of variables which are inherent in transient assay systems (Everett, 1988b). Nevertheless, these results demonstrate that the ability of the pD19T mutant to repress viral gene expression promiscuously also allows it to inhibit the replication of two completely unrelated viruses, indicating that this polypeptide may possess powerful broad spectrum antiviral properties.

The structure of the pD19T mutant suggests that only exons 1 and 2 are required for the dominant negative phenotype, since all but four amino acids of the third exon have been deleted in this derivative. Inspection of the ICP0 gene sequence (Perry et al., 1987) revealed that a failure to splice intron 2 from the primary ICP0 transcript would result in a truncated protein which would be structurally very similar to the pD19T mutant. This derivative would contain all 241 amino acids encoded by exons 1 and 2, plus an additional 21 amino acids derived from translation of the unspliced second intron. To investigate the properties of this hypothetical polypeptide, a truncated ICP0 gene was constructed which expressed this protein. The ICP0 mutant contained in plasmid pKST (Fig. 1) has lost all of the exon 3 coding sequences as well as the splice acceptor site for intron 2, so that the coding sequences in intron 2 cannot be spliced out. As a result, pKST encodes the same 262 amino acid protein which would be generated if intron 2 was not removed from the primary ICP0 transcript. When tested in cotransfection assays with the wild-type ICP0 gene, the pKST mutant was found to possess a dominant negative phenotype which was nearly identical to that of the pD19T mutant (Table 1). Thus, HSV-1 can potentially encode its own trans-dominant mutant of the ICP0 protein through an alternative splicing mechanism. Since this polypeptide can repress the trans-activation functions of ICP0, and is partially derived from the coding sequences for ICP0, it has been given the designation ICP0R. Further studies are under way to demonstrate the existence of this protein in HSV-1-infected cells.

**Discussion**

The carboxy-terminal truncation mutant pD19T was the first reported dominant negative mutant of the ICP0 protein (Weber & Wigdahl, 1992). ICP0 therefore joins the growing list of HSV-1 proteins for which dominant
negative mutants have been described (Friedman et al., 1988; Kwong & Frenkel, 1989; Shepard et al., 1990; Gao & Knipe, 1991; Smith et al., 1991). Given the number of other HSV-1 proteins which are known to require protein-protein interactions for function (Claesson-Welch & Spear, 1986; Johnson et al., 1988; Koff & Tegtmeyer, 1988; Crute et al., 1989; Gottlieb et al., 1990), it is likely that many more such mutants will be isolated in the future. Most of these derivatives have been shown to inhibit HSV-1 replication by interfering with essential activities mediated by their wild-type counterparts during infection (Friedman et al., 1988; Shepard et al., 1990; Gao & Knipe, 1991; Smith et al., 1991), making dominant negative mutants an exciting new prospect in anti-HSV-1 therapy. The dominant negative mutant of ICP0 encoded by pD19T was also found to behave as a powerful suppressor of HSV-1 replication (Table 2). However, the ability of the pD19T mutant to repress viral gene expression promiscuously also allowed it to inhibit the replication of a second completely unrelated virus (Fig. 2), indicating that this polypeptide may possess powerful broad spectrum antiviral properties as well.

It is important to note that since the pD19T mutant is such a strong suppressor of gene expression, it may also mediate a deleterious effect on the host cell itself; the potentially cytotoxic properties of this protein are under investigation at the present time. However, no cytopathology or decreased viability were observed in Vero cells which were transfected with concentrations of pD19T that were sufficient to prevent HSV-1 replication (unpublished results). Toxicity problems which arise could be overcome by devising antiviral strategies which ensure that the pD19T mutant gene is targeted only to virus-susceptible tissues, that it can be expressed only in cells which contain an actively replicating virus, and that it fails to be expressed once it has eliminated an infection. Numerous methods of virus- and non-virus-mediated tissue-specific gene delivery systems have been developed (Mann et al., 1983; Geller & Breakefield, 1988; Felgner & Rhodes, 1991) which would make it possible to direct pD19T into a limited set of cells. The pD19T gene could also be expressed from a promoter that is functional only in the presence of specific viral regulatory proteins; this would ensure that only those cells which contain replicating virus would be susceptible to the action of this protein. As the infection subsides and viral trans-activator proteins disappear, the expression of the repressor gene will diminish and any cytotoxic effects on the host cell should be minimized. By this combination of approaches, the powerful repression activity of the pD19T mutant could be specifically and exclusively directed against the replication of a single virus species.

The potential ability of HSV-1 to generate its own version of the pD19T mutant product, ICP0R, by alternative splicing of the primary ICP0 transcript suggests that this protein may play an important role in the down-regulation of viral gene expression during infection. The use of alternative splicing in creating truncated polypeptides which possess activities antagonistic to that of their wild-type parents appears to be a common regulatory strategy in many systems (Lambert et al., 1987; Misra & Rio, 1990; Roman et al., 1991). However, ICP0R has not been detected in previous analyses of virus-encoded polypeptides, and studies of ICP0 transcript structure have indicated that the removal of the intron 2 sequences which encode the carboxy-terminal region of ICP0R is a highly efficient process during productive infection in cell culture (Perry et al., 1986). Nevertheless, it is still possible that intron 2 splicing may be suppressed in certain tissues in vivo, and that the resulting production of ICP0R may lead to abortive infection, restrictions in host range or latency. For example, the establishment of latent infection in neuronal cells by HSV-1 may be a direct result of inefficient removal of intron 2; this may be mediated by a lack of availability of the required splicing factors, or by an alteration in the secondary structure of the primary ICP0 transcript induced by binding of the virus-encoded latency-associated transcript (LAT) RNAs (Deatly et al., 1987; Puga & Notkins, 1987; Rock et al., 1987; Stevens et al., 1987). These findings also raise the interesting possibility that other herpesviruses utilize a similar repressor protein as a means of down-regulating gene expression during infection, since the ICP0 homologues present in three other herpesviruses are considerably smaller than ICP0 and show sequence similarity only to the region of the gene which encodes ICP0R (Perry et al., 1986; Cheung, 1991; van Santen, 1991). Moreover, one of these ICP0 homologues, the product of gene 61 of varicella-zoster virus, manifests repression characteristics in transient expression assays which are remarkably similar to those of ICP0R (Nagpal & Ostrove, 1991).

Everett (1991) has constructed a derivative of HSV-1 in which the two introns of ICP0 were removed by site-directed mutagenesis. This virus, which should theoretically be unable to synthesize ICP0R since it lacks the intron 2 coding sequences, was recently found to establish, maintain and reactivate from latent infections in mice with the same ability as a wild-type virus (Natarajan et al., 1991). These results suggest that ICP0R, should it exist, plays an unimportant role in HSV-1 latency and infection in vivo. However, the explant coculture procedure used to reactivate the latent virus in these experiments has been shown previously to give results which differ from those of other systems: for example, LAT deletion viruses show a
modest inhibition in reactivation frequency using explant cocultivation in the mouse eye model (Javier et al., 1988; Leib et al., 1989b; Steiner et al., 1989), but exhibit a much more dramatic inability to reactivate in vivo when iontophoresis of epinephrine is employed in the rabbit eye model (Hill et al., 1990). Thus, it is still possible that a virus unable to make ICPOr might exhibit significant replication defects if studied in other systems. Another interpretation of these results is that reactivation from the latent state may depend upon an interplay between a number of different virus-encoded factors (e.g. the LAT RNAs, ICP0, ICP0R), each of which is dispensable for reactivation when removed from the HSV-1 genome individually.

Although some of these ideas are speculative, it is clear from the strength of the repression induced by pD19T (Table 1) that any tissue which acts to drive even low levels of ICP0R synthesis should be able to mediate a dramatic inhibition in HSV-1 gene expression. Moreover, the proposed ability to induce expression of the ICP0R protein by prevention of normal splicing suggests a novel strategy for future antiviral therapies: antisense oligonucleotides directed against the intron 2 splicing signals should increase the production of the ICP0R repressor while they reduce synthesis of the ICP0 trans-activator, both of which would lead to suppression of viral gene expression. The possible roles and uses of this novel inhibitor protein in HSV-1 infections are under investigation at the present time.

Although the potential antiviral properties of pD19T have been emphasized in this work, it should be noted that further characterization of this mutant will almost certainly help to unravel the unknown mechanism by which ICP0 activates gene expression. In several mutational analyses, ICP0 function has been shown to be highly sensitive to disruptions in the coding sequence of the second exon (Everett, 1987, 1988a; Cai & Schaffer, 1989; Chen et al., 1991). The dominant negative mutant of ICP0 in pD19T is encoded almost entirely by this same region. Thus, the second exon probably encodes a critical domain that interacts with the unknown cellular protein through which ICP0 mediates its activation and the pD19T mutant mediates its repression. Further studies with this domain encoded by pD19T may facilitate the identification and purification of this cellular target.

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