Replacement of the herpes simplex virus type 1 Vmw175 DNA binding domain with its varicella-zoster virus counterpart results in a protein with novel regulatory properties that can support virus growth

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The alphaherpesviruses encode major immediate early transactivator proteins that are essential for the expression of later classes of viral genes. We have previously shown that the extensive sequence similarity between the herpes simplex virus type 1 (HSV-1) and varicella-zoster virus (VZV) members of the family (proteins Vmw175 and VZV140k) extends to function, since a virus which expresses VZV140k in place of Vmw175 is able to grow, albeit at reduced efficiency. We have also shown that the DNA binding characteristics of the isolated DNA binding domains of Vmw175 and VZV140k are related but distinct. In order to assess whether the different DNA binding properties of the two proteins are responsible for the differences in their individual transcriptional regulatory functions, we constructed a plasmid and an HSV-1 virus in which the VZV140k DNA binding domain coding sequences replace those of Vmw175. The characteristics of the resultant hybrid protein in transfection assays and during virus infection suggest that the nature of the DNA binding domain plays a significant role in the transactivation and repression properties of the Vmw175 family of proteins.

Introduction
The alphaherpesviruses comprise an extensive family of neurotropic viruses with representatives which infect a wide range of hosts. A characteristic feature of these viruses is the expression of a major transcriptional transactivator protein, of which the prototype is Vmw175 from herpes simplex virus type 1 (HSV-1) (McGeoch et al., 1986). Vmw175 (also known as ICP4) is one of five immediate early (IE) genes of HSV-1 and is essential for the expression of later classes of viral genes (for reviews, see Fields et al., 1996). Intensive study of Vmw175 and its varicella-zoster virus (VZV) counterpart, VZV140k, has established that both are DNA binding proteins with related, but distinct DNA binding properties (Faber & Wilcox, 1986; Michael et al., 1988; Michael & Roizman, 1989; Imbalzano et al., 1990; DiDonato et al., 1991; Flanagan et al., 1991; Smiley et al., 1992; Gu & DeLuca, 1994). More recent evidence highlights the requirement for DNA binding for Vmw175 action since a tripartite complex of Vmw175, TFIID and TFIIB forms cooperatively on promoter sequences containing both a Vmw175 binding site and a TATA box (Smith et al., 1993). Furthermore, we have shown that a single amino acid substitution within the DNA binding domain of VZV140k that results in loss of DNA binding function also destroys the ability of the mutant VZV140k protein to transactivate (Tyler et al., 1994).

It has been established that the sequence similarity between Vmw175 and VZV140k is reflected in partial functional equivalence, since cell lines expressing VZV140k complement Vmw175 mutant HSV-1 viruses (Felser et al., 1988), and a virus (HSV140) which expresses VZV140k in place of Vmw175 grows (with reduced efficiency) in tissue culture (Disney & Everett, 1990). Among the many potential explanations as to
why HSV140 has reduced growth potential is the interesting possibility that the differing DNA binding properties of Vmw175 and VZV140k might contribute to their individual transcriptional regulatory properties. For example, it has been established that, in transfection assays, VZV140k is a more powerful and promiscuous transactivator than Vmw175 (Everett, 1984; Inchauspe & Ostrove, 1989; Cabirac et al., 1990). It is possible that the efficient transactivation by VZV140k may be a consequence of its relaxed DNA binding specificity compared to that of Vmw175 (Tyler & Everett, 1993).

In addition to their roles in transactivation, the major regulatory proteins of the alphaherpesviruses also repress specific target promoters. For instance, Vmw175 autoregulates its own IE3 transcription unit by binding to sequences which overlap the transcriptional initiation site of that gene (DeLuca & Schaffer, 1985; O’Hare & Hayward, 1985; Muller, 1987; DeLuca & Schaffer, 1988; Roberts et al., 1988; Michael & Roizman, 1993). This repression function of Vmw175 appears to be a consequence of Vmw175 positioned over the IE3 cap site, therefore blocking communications between the upstream activator proteins and the pre-initiation complex (Gu et al., 1995; Kuddus et al., 1995). Intriguingly, VZV140k can also bind to the Vmw175 binding site spanning the HSV-1 IE3 cap site (Wu & Wilcox, 1991; Tyler & Everett, 1993), yet does not repress IE3 gene expression, although VZV140k does autoregulate expression from its own promoter (Disney et al., 1990). Since the specificity and details of the Vmw175 interaction with sequences at the IE3 cap site differ from those of VZV140k (Wu & Wilcox, 1991; Tyler & Everett, 1993), it is possible that the distinction between the two proteins is a consequence of their differing DNA binding properties.

In order to investigate the contribution of the differing DNA binding characteristics of Vmw175 and VZV140k to their individual regulatory functions, we have constructed a hybrid gene which expresses a Vmw175 protein with its DNA binding domain replaced with that of VZV140k. The ability of the hybrid protein to repress the IE3 promoter and activate gene expression was compared to the activities of the parental proteins in transfaction assays. In addition, a recombinant HSV-1 virus (HSV12DS) was constructed which expresses the hybrid protein instead of Vmw175. The results showed that replacement of the Vmw175 DNA binding domain with the VZV140k equivalent destroyed the ability of Vmw175 to autoregulate its own expression in both transfection and infection experiments. In addition, the VZV140k DNA binding domain conferred stronger transactivation properties on Vmw175 in transfection assays. Interestingly, the increased transactivation activity of the hybrid protein was not reflected in more efficient virus growth. Indeed, HSV2DS gave lower yields than wild-type HSV-1. The simplest interpretation of our data is that the DNA binding domain of VZV140k confers altered regulatory properties to Vmw175 that now more closely resemble those of full-length VZV140k. These data support and extend the view that the DNA binding activities of the alphaherpesvirus major regulatory proteins make a significant contribution to their specific transcriptional regulatory activities that in turn help define the characteristics of the virus life cycle.

Methods

■ Plasmids and bacteria. The following plasmids have been described previously: plasmid p175 contains the complete HSV-1 IE3 transcription unit linked to the SV40 early promoter/enhancer region (Everett, 1986); plasmid pI9 contains a 12 bp EcoRI oligonucleotide linker inserted into codon 252 of the Vmw175 open reading frame (Patersen & Everett, 1988a); plasmid p140SV contains the complete VZV gene 62 coding region (which encodes VZV140k) and 3’ transcriptional control sequences, linked to the SV40 early promoter/enhancer region (Disney et al., 1990); plasmid pL10/11 is a derivative of p175 which contains a small deletion just 5’ of the DNA binding domain coding sequences and, more importantly for the experiments described in this paper, a point mutation which removes the second of two BamHI sites just 3’ of the DNA binding domain coding sequence, but maintains the coding potential (Allen, 1993: p58T7β2 is a T7 expression vector essentially identical to p58T7α (Tyler & Everett, 1993) except for a frameshift in the coding region; plasmids pgDCAT, pIE3CAT and p140CAT are reporter plasmids used to assess the activation of gene expression (Everett, 1986; Paterson & Everett, 1988a; Disney et al., 1990). All plasmids were propagated in E. coli strain DH5α and purified by density gradient centrifugation.

■ Construction of hybrid plasmid p175R2DS. A PCR primer was synthesized with 17 bases homologous to VZV140k sequences (codons 417–421), linked to an EcoRI site and 7 additional random bases at its 5’ end. A second primer included 17 homologous bases (VZV140k codons 668–673) linked to a BamHI site followed by 6 random bases at its 5’ end. The primers were designed to amplify VZV140k codons 417–673 with the 5’ EcoRI and 3’ BamHI sites able to link in frame to Vmw175 coding sequences. The PCR product was excised from a gel, cut with EcoRI and BamHI, and ligated to a PvuII–EcoRI fragment of plasmid pI9 (see above), which contains part of the vector sequences and the promoter and the 5’ part of gene IE3 in plasmid p175, and the BamHI–PvuII fragment of pL10/11 (see above), which contains the 3’ part of the IE3 gene and the remainder of the vector sequences. The resulting plasmid, p175R2DS, is similar to the parent Vmw175 expression plasmid, p175, except that codons 253–521 of Vmw175 have been replaced by codons 417–673 of VZV140k. In addition, there are three additional residues (proline-arginine-isoleucine) between codons 251 and 417 of Vmw175 and VZV140k, respectively, which arise from the original EcoRI linker insertion into Vmw175 codon 252.

■ Transfections and CAT assays. HeLa cells were transfected by the calcium phosphate co-precipitation technique, and cell extracts were prepared for assay of chloramphenicol acetyltransferase (CAT) activity as previously described (Tyler et al., 1994). Each experiment used several different amounts of activator plasmid, and the total amount of DNA in the transfection mixture was kept constant by addition of pUC9. The titration transfection experiments were independently repeated on a number of occasions to ensure that the relative effectiveness of each construct was reproducible.

■ Cells and viruses. Baby hamster kidney (BHK) cells were grown in Glasgow Modified Eagle’s Medium (GMEM) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 10% newborn calf serum (NBCS) and 10% tryptose phosphate broth. HeLa cells for transfection
Results

Construction of plasmid expressing a hybrid Vmw175–VZV140k protein

The construction of a properly folded hybrid protein, even by using closely related sequences, is usually complicated by a lack of structural information to use as a guide. In this case, several criteria were relevant. (i) Vmw175 and VZV140k are highly related, especially in their DNA binding domains (Everett et al., 1993). (ii) The Vmw175 DNA binding domain can be readily excised from the parent protein as a protease resistant and active polypeptide, implying that the sequences surrounding the domain are of open structure (Everett et al., 1990). (iii) Both the Vmw175 and VZV140k DNA binding domains can be expressed in bacteria as separate and active entities, implying that their correct folding does not require sequences from other parts of the protein (Wu & Wilcox, 1990, 1991; Everett et al., 1991; Tyler & Everett, 1993). (iv) Mutations in Vmw175 flanking the DNA binding domain have little effect on Vmw175 activity. Specifically, insertion of a 12 bp EcoRI linker into codon 252 of Vmw175 has little effect on its activity either in transfection assays (Paterson & Everett, 1988a), or in the context of intact virus (Allen, 1993). (v) A Vmw175 polypeptide extending between residues 253 and 523 has a DNA binding specificity very similar to that of the intact protein in both gel retention and footprinting assays (Everett et al., 1991; Tyler & Everett, 1993). A comparable VZV140k polypeptide encompassing residues 417–647 has similar properties (Tyler & Everett, 1993). On the basis of these considerations, PCR primers were designed to amplify the VZV140k coding sequence between residues 417 and 673 in such a way as to allow the replacement of Vmw175 residues 253–521. The details of this procedure are given in the methods section, and are depicted in Fig. 1. The VZV140k insert includes the whole of the highly conserved DNA binding domain. Like p175, the SV40 early promoter/enhancer...
region is present in p175R2DS instead of the HSV-1 IE3 promoter.

To ensure that the PCR did not introduce frameshift or deleterious point mutations within the amplified segment, the product was also cloned into the p585T7b2 expression vector, which enables high level expression of the inserted sequence in bacteria. The 175R2DS DNA binding domain polypeptide was expressed and partially purified as described (Tyler & Everett, 1993); it was found to be of the expected size and to have DNA binding properties indistinguishable from those of the previously characterized bacterially expressed VZV140k DNA binding domain (data not shown).

The 175R2DS hybrid protein has regulatory properties distinct from those of Vmw175 and VZV140k

The two main differences between Vmw175 and VZV140k in transfection assays are, firstly, that VZV140k fails to repress the HSV-1 IE3 promoter and, secondly, that VZV140k activates a wide variety of target promoters much more strongly than Vmw175 by itself. To determine the effects of the domain swap in these two assays, plasmids expressing Vmw175 (p175), VZV140k (p140SV) and the 175R2DS hybrid protein (p175R2DS) were transfected in increasing amounts into HeLa cells with either of two CAT indicator plasmids. Plasmid pgDCAT, which contains the HSV-1 gD promoter, was used as a representative target to monitor activation. Plasmid pIE3CAT was used to monitor the effects of the three proteins on the IE3 promoter. Plasmids p175, p140SV and p175R2DS all use the SV40 early promoter region to drive expression of the activator, and Western blot analysis verified that the three activators were expressed as intact proteins in the transfected cells (data not shown).

The results showed that p140SV gave high levels of activation of the gD promoter, while p175 is a much poorer activator by itself in this particular transfection system (Fig. 2).
A hybrid HSV-1 Vmw175–VZV140k protein

Fig. 4. Southern blot analysis of hybrid virus HSVR2DS. DNA from two independent final purified stocks of HSVR2DS was cut with restriction enzymes as indicated and compared to the rescuant virus HSVR2DSR and the parent plasmid p175R2DS. The tracks in the left-hand panel were probed for VZV sequences using p140 (which contains the whole of VZV gene 62), while the right-hand panel was probed for HSV sequences with p175. Below, a schematic of the hybrid gene is shown, with coding regions as boxes and non-coding DNA as a bar. The p140 probe detects only those fragments which include VZV sequences, namely the 1·8 kb BamHI fragment and its 0·77 kb BamHI–EcoRI sub-fragment which is composed entirely of VZV140k sequences (shown as hatched boxes in the schematic) (left-hand panel). The identities of these fragments were confirmed by comparison with fragments from their parental plasmid DNAs (centre panel). The p175 probe detects the 1·8 kb BamHI band of HSVR2DS (which increases to 1·84 kb in the rescuant virus HSVR2DSR as the latter has 15 more codons) and its 1·03 kb BamHI–EcoRI sub-fragment. The p175 probe also hybridizes to flanking IE3 fragments in the short repeat region of the genome which give rise to the multiple higher molecular mass bands in the right-hand panel.

Fig. 5. Growth curve comparison of strain 17 (●), HSVR2DS (■), HSV140 (□) and the rescuant viruses HSVR2DSR (○) and HSV140R (□). BHK cells on parallel plates were infected with the viruses as shown at an m.o.i. of 2 p.f.u. per cell. The cells and medium were harvested at the indicated times after infection and virus titres were determined using BHK cells. The results of a typical experiment are shown; the differences in growth potential were reproducible in repeated experiments.

Interestingly, p175R2DS gave an intermediate phenotype, with activation levels at about 25 % of those seen with p140SV and significantly higher than those with p175 (Fig. 2). Importantly, this result confirms that the hybrid protein is folded into an active conformation.

It has been shown previously that Vmw175 represses the IE3 promoter in plasmid pIE3CAT, while VZV140k slightly activates it (Disney et al., 1990). In this study, similar results were observed, with p140SV activating the IE3 promoter by a small, but reproducible degree at low input doses (Fig. 3a). In contrast to p175, plasmid p175R2DS failed to significantly repress the IE3 promoter even at high doses, while slight, but reproducible activation of the IE3 promoter by p175R2DS was observed at low input doses (Fig. 3a). The slight repression of the IE3 promoter observed at high doses of both p140SV and p175R2DS is probably due to promoter competition, since a plasmid carrying the SV40 promoter alone also shows similar repression at equivalent high doses (data not shown). These results suggest that exchanging the DNA binding domain modified the properties of the hybrid protein to resemble more closely those of VZV140k.

In contrast to the results with the IE3 promoter, the domain swap protein expressed by plasmid p175R2DS repressed the VZV gene 62 promoter in plasmid p140CAT to an extent similar to that achieved by both Vmw175 and VZV140k (Fig. 3b).
Fig. 6. Time-course of expression of selected viral proteins by HSVR2DS. Vero cells were infected at an m.o.i. of 5 p.f.u. per cell with HSVR2DS, HSVR2DSR and strain 17+ and the cells were harvested 4, 8, 16 and 24 h later. Proteins were separated on 7.5% SDS gels and transferred to nitrocellulose by Western blotting. The nitrocellulose filter was incubated with antibodies as shown, and bound antibodies were detected by the ECL system (Amersham). The filter was stripped and re-probed with multiple antibodies. In (f), the same samples were analysed on a parallel gel and the proteins were stained with Coomassie blue. The arrows in panels (a–e) indicate the full-length protein, while smaller bands are breakdown products which are efficiently detected by the very sensitive ECL method. The major capsid protein (ICP5; UL19) and glycoprotein B (gB; UL27) bands are indicated in (f); other abundant viral proteins can also be seen, especially in the 24 h HSVR2DS track. mi, Mock infected.
infected with HSV-1 strain 17 and monitored the production of progeny virus in a single-step growth curve in tissue culture. Parallel plates of BHK cells were co-transfected with linearized plasmid p175R2DS to allow the isolation of a virus which expressed the hybrid protein. The selected final isolate (virus HSVR2DS) was stable and had a restriction pattern in the IE3 region entirely consistent with the presence of the expected domain swap in both copies of the IE3 gene (Fig. 4). In turn, infectious DNA from virus HSVR2DS was co-transfected with linearized plasmid p175R2DS to construct a virus, named HSVR2DSR, in which the IE3 sequences had been repaired to re-establish a normal IE3 gene.

**Construction of an HSV-1 virus which expresses the 175R2DS hybrid protein in place of Vmw175**

The prediction from the above experiments is that a recombinant HSV-1 virus which expresses the hybrid 175R2DS protein in place of Vmw175 might activate viral gene expression more efficiently than wild-type HSV-1. In addition, due to the failure of the 175R2DS protein to repress the IE3 promoter, such a virus would be expected to express higher levels of the hybrid Vmw175 protein. In a manner analogous to the method of construction of HSV140 (Disney & Everett, 1990), co-transfection of the IE3 deletion mutant D30EBA viral DNA with linearized plasmid p175R2DS allowed the isolation of a virus which expressed the hybrid protein. The selected final isolate (virus HSVR2DS) was stable and had a restriction pattern in the IE3 region entirely consistent with the presence of the expected domain swap in both copies of the IE3 gene (Fig. 4). In turn, infectious DNA from virus HSVR2DS was co-transfected with linearized plasmid p175 DNA to construct a virus, named HSVR2DSR, in which the IE3 sequences had been repaired to re-establish a normal IE3 gene. Southern blots showed that, compared to the plasmid DNA controls, both HSVR2DS and HSVR2DSR had the expected restriction fragments which hybridized to the relevant HSV-1 and VZV sequence probes (Fig. 4).

**Virus HSVR2DS expresses several viral proteins more abundantly than wild-type virus**

To examine the properties of HSVR2DS in more detail, we monitored the efficiency of expression of a selection of IE and early gene products during the productive cycle. The results presented were obtained using Vero cells, and essentially similar results were obtained in BHK cells (not shown). Cells in Linbro wells were infected with viruses HSVR2DS, HSVR2DSR and HSV-1 strain 17 at an m.o.i. of 5.p.f.u. per cell and parallel wells were harvested after 4, 8, 16 and 24 h of infection. The viral proteins were separated by SDS–PAGE and detected by Western blotting. Initially, the efficiency of Vmw175 expression was monitored by probing with monoclonal antibody 58S, which recognizes an epitope near the C terminus of Vmw175 (Showalter et al., 1981). The three viruses expressed a Vmw175 polypeptide of very similar gel mobility, but HSVR2DS reproducibly expressed significantly higher amounts, especially at late times of infection (Fig. 6b). The Vmw175-related polypeptide expressed by HSVR2DS was shown to include the VZV140k DNA binding domain by probing the same blot with r109 serum (Tyler et al., 1994), which was raised against a bacterially expressed polypeptide equivalent to the VZV140k insert in virus HSVR2DS (Fig. 6a). The higher levels of expression of the hybrid Vmw175 protein by HSVR2DS are entirely consistent with its failure to repress the IE3 promoter in transfection assays (Fig. 3). This conclusion is also consistent with the observed higher levels of expression of VZV140k protein by virus HSV140 (Disney & Everett, 1990).

We were interested to see the effects of the hybrid protein on expression of other viral proteins. For this reason, similar blots were probed to detect the amounts of selected viral proteins: IE polypeptide Vmw110; ICP6 or R1, an early polypeptide with some IE characteristics; and UL42, an early polypeptide. The results showed that HSVR2DS expressed...
higher amounts of Vmw110 than either HSVR2DSR or HSV-1 strain 17+ (Fig. 6c), while there was little significant difference in the amounts of R1 expressed by the three viruses (Fig. 6d). The fact that the R1 promoter has been shown to be unresponsive to Vmw175, but instead appears to be activated by Vmw110 (Desai et al., 1993) may be relevant. The levels of UL42 expressed by the three viruses were not significantly different (Fig. 6e), but Coomassie staining of the protein gels indicated that several abundant viral polypeptides were expressed by HSVR2DS at significantly higher levels, compared to the other two viruses (Fig. 6f). This was particularly clear in the 24 h samples, but the same phenomenon was also visible in the 16 h samples, and to a limited extent in the 8 h samples. The advantage of using Coomassie staining for this experiment is that, despite lower sensitivity, several viral proteins could be examined on the same gel and their amounts compared directly. Care was taken to ensure that the input multiplicities were equal in these experiments, and it was determined that the particle to p.f.u. ratios of the three virus stocks were also similar. Therefore, the hybrid protein appears to activate expression of several viral polypeptides more efficiently than normal Vmw175 during virus infection, which is consistent with the higher levels of activation that it achieved in transfection assays.

Discussion

The results presented in this paper re-emphasize the functional equivalence of Vmw175 and VZV140k, and illustrate that their properties are partially determined by the nature of their DNA binding domains. The VZV140k DNA binding domain has a more relaxed sequence specificity than that of Vmw175 (Tyler & Everett, 1993). This property might allow VZV140k to interact more efficiently with its target promoters and thus explain its apparently greater activation potential in transfection assays. The properties of the DNA binding domain swap hybrid protein are compatible with this suggestion. At the HSV-1 IE3 cap site, in contrast to its Vmw175 equivalent, the VZV140k domain does not bend the DNA and the two proteins give DNAse I footprints of different characteristics (Everett et al., 1992; Tyler & Everett, 1993). Since the positioning of Vmw175 at a binding site in the vicinity of the IE3 transcriptional initiation site is crucial for the ability of Vmw175 to repress IE3 transcription (Roberts et al., 1988; Michael & Roizman, 1993; Koop et al., 1993), it is possible that the differences in detail of VZV140k binding at this site might also explain why VZV140k activates, rather than represses, the IE3 promoter. Indeed, a recent analysis of the Vmw175-induced DNA distortions that occur at the IE3 cap site suggested that Vmw175-induced DNA bending may play a role in repression of the IE3 promoter (Kuddus et al., 1995). It is possible that the inability of VZV140k to introduce a DNA bend at this site is responsible for the failure of both VZV140k and the hybrid protein to down-regulate IE3 expression (Tyler & Everett, 1993).

However, the 175R2DS domain swap protein is not as efficient an activator as the VZV140k protein itself. This could be explained either by inappropriate folding of the hybrid protein, or by the absence of highly efficient activator sequences that are present in VZV140k but not in Vmw175. The former explanation seems unlikely, since the sequences immediately flanking the Vmw175 DNA binding domain are not crucial for function (Paterson & Everett, 1988a, b; Shepard et al., 1989), and the available information indicates that this is probably also true for VZV140k (Baudoux et al., 1995). The latter explanation is supported by the identification of a very strong activation sequence within residues 9–86 of the N terminus of VZV140k (Cohen et al., 1993; Perera et al., 1993) which is apparently not conserved in Vmw175. Therefore, it seems that the high activation potential of VZV140k might be explained by its possession of this activation sequence and also by the characteristics of its DNA binding domain.

The properties of the 175R2DS domain swap protein in transfection assays were reflected by the increased efficiency of expression of several viral polypeptides by the corresponding HSVR2DS virus. For example, the Vmw175 protein itself was detected at increased levels at later times during infection by HSVR2DS as compared to wild-type HSV-1. Obviously, there are many factors which influence the levels of viral polypeptide synthesis during infection, and the situation during HSVR2DS infection is further complicated by the presumably increased levels of polypeptides that would normally be down-regulated by Vmw175, but given the complexity of the controls that are operating, it is striking that substitution of the highly conserved DNA binding domain of Vmw175 can have such a significant effect upon gene expression during infection. It is interesting that the higher levels of viral gene expression achieved by HSVR2DS do not result in increased numbers of infectious virus particles. Instead, a reduced virus yield as compared to wild-type HSV-1 was observed, indicating that virus yield is not merely a function of the level of viral protein production, but is a carefully regulated process. This is exemplified here by the reduced virus production that results when gene expression is mis-regulated as a consequence of replacing the Vmw175 DNA binding domain with that of VZV140k.

In conclusion, the inference from these data is that the specific details of the DNA binding activities of Vmw175 and VZV140k play a significant part in regulating the specificity of their transactivation and repression properties that, in turn, determine the highly efficient production of infectious herpes-virus virions.

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