The equine herpesvirus 1 gene 63 RING finger protein partially complements Vmw110, its herpes simplex virus type 1 counterpart

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All alpha herpesviruses of known DNA sequence have been found to encode a protein with similarities to immediate early protein Vmw110 (ICP0) of herpes simplex virus type 1 (HSV-1). The conserved portion of this family of proteins is a characteristic zinc binding module, known as a RING finger or C3HC4 domain. Examples of RING finger domains occur in many other proteins of diverse evolutionary origin and function. Recently, the solution structure of the equine herpesvirus 1 (EHV-1) RING finger protein, encoded by gene 63, has been solved. To investigate whether this structure could be considered to be a paradigm of herpesvirus RING domains, we have constructed a recombinant HSV-1 which expresses the EHV-1 gene 63 protein (EHVg63) in place of Vmw110. Comparison of the growth properties of the recombinant with those of wild-type and Vmw110-defective viruses indicates that EHVg63 is able to fulfill partially, but not completely, the roles of Vmw110 during virus growth in tissue culture.

After primary infection, HSV-1 attains a life-long latent state and affected individuals suffer from recurrent episodes of virus activity of varying duration and periodicity (for a review see Roizman & Sears, 1990). The control of viral gene expression during the contrasting lytic and latent states is therefore of interest to both the molecular biologist and the clinician. Lytic virus infection is characterized by the expression of at least 75 distinct genes (McGeoch et al., 1993 and references therein) which can be divided into the broad temporal classes of immediate early (IE) early and late. At least three of the IE gene products are involved in the control of viral gene expression. One of the most intriguing of these, named Vmw110 or ICP0, is encoded by IE gene 1. Vmw110 is of great interest since it is a general activator of gene expression in transfection assays, it is required for fully efficient lytic growth in tissue culture and it has been implicated in the process of reactivation from latency (reviewed by Everett et al., 1991). The mechanisms by which Vmw110 achieves these functions are unknown but an attractive hypothesis is that Vmw110 can influence the balance between the latent and lytic states.

Mutational analysis of Vmw110 has revealed that it includes a cysteine-rich region which is very important for full protein activity (Everett, 1986, 1988, 1989; Harris et al., 1989). This region has since been found to be a zinc binding domain of unusual structure, and examples of related domains have been found in a large number of viral and cellular proteins. The characteristic pattern of cysteine and histidine residues in this domain family has led to the name of the C3HC4 domain, but it is more frequently referred to as the ‘RING finger’ after the first protein in which it was recognized (Freemont et al., 1991; Freemont, 1993). All the alpha herpesviruses for which appropriate DNA sequence is available have been found to encode a protein related to Vmw110 by virtue of their possession of a RING finger sequence. However, sequence similarity between Vmw110 and other regions of the herpesvirus RING finger proteins is very limited and whether they constitute a family of proteins related by function is uncertain. The only other member of the herpesvirus RING finger protein family to have been studied in any depth is the product of varicella-zoster virus (VZV) gene 61. There are conflicting reports about the ability of this protein to activate or repress gene expression (Nagpal & Ostrove, 1991; Moriuchi et al., 1993) but despite the limited identity between Vmw110 and the VZV gene 61 protein, it has been reported that cell lines expressing the latter complement the growth of HSV-1 mutants which fail to express functional Vmw110 (Moriuchi et al., 1992). A recent study also suggests that a functional chimeric protein may be made by exchanging the N-terminal portion of the VZV gene 61...
Fig. 1. The structures of the plasmids and viruses used in this work. (a) The uppermost line depicts the HSV-1 IE-1 transcript and DNA segment with key restriction sites marked. The locations of the 775 residue Vmw110 coding region and the RING finger domain (marked C3HC4) are indicated. Plasmid p111 contains the SacI-HpaI region cloned into pUC9. Below is a representation of plasmid p110FXE, which has an in-frame deletion which removes residues 106-149; the first and last cysteines of the Vmw110 RING finger are at residues 116 and 156. Plasmid p1EEHVg63, shown below p110FXE, has the complete EHV-1 gene 63 coding region inserted between the NcoI and SalI sites of p111. The NcoI-DraI coding region, which includes the EHVg63 RING finger, is indicated. The two domain swap vector plasmids are shown below: pENDA110 has the coding sequence of the first 63 residues of EHVg63 (which includes the complete EHVg63 RING finger, whose first and last cysteine residues are at residues 8 and 46) linked to residue 159 of Vmw110; in p110ENX, the region encoding the first 105 residues of Vmw110 has been added to the 5' end of the coding region in pENDA110. This last construct includes all the promoter, splicing and 3' processing signals of the normal IE-1 gene and transcript. (b) Construction and Southern blot characterization of recombinant virus 17Eg63. The upper panels show a comparison of the bands revealed by IE-1-specific and EHV-1 gene 63-specific probes after digestion of strain 17 (17+), parent virus d11403, recombinant virus 17Eg63 and parent plasmid p1EEHVg63 DNAs with restriction enzymes as indicated. The diagnostic bands are indicated by arrows. Detailed restriction maps of the normal and recombinant IE-1 regions are shown below, from which the sizes of the expected SacI-HpaI and SacI-NcoI fragments can be calculated. The 1.39 kb SacI and the 1.6 kb SacI-HpaI fragments in 17Eg63 hybridize to both HSV and EHV probes as they span the junctions at the 5' and 3' ends of the coding sequences, respectively. Shorter exposures showed the presence of the two bands in the right-hand panel, whereas the 1.6 kb band is poorly detected by the HSV probe because it includes only 0.45 kb of HSV DNA. The 1.6 kb SacI-HpaI fragment is not present in the parent p1EEHVg63 plasmid, since the HpaI site is not present. The 1.75 kb NcoI fragment contains only HSV DNA. The 4.6 kb SacI-HpaI IE-1 region fragment in strain 17 is reduced to 2.4 kb in d11403 because of the deletion of the XhoI-SalI region; the absence of the 2.4 kb band in 17Eg63 indicates that both copies of the IE-1 region contain the EHV coding sequences. The SalI site marked in brackets on the hybrid gene map has been destroyed during the cloning manipulations; it corresponds to the HSV IE-1 SalI site. Unattributed bands of high apparent molecular mass in the plasmid digests are vector plasmid fragments.
Table 1. Activation of gene expression by plasmids expressing Eg63 and the hybrid domain swap proteins*

<table>
<thead>
<tr>
<th>Activator plasmid...</th>
<th>HeLa cells pgDCAT plus p175</th>
<th>BHK cells pSS80, no p175</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.05 0.2 10 40</td>
<td>0.05 0.2 10 40</td>
</tr>
<tr>
<td>p11</td>
<td>4 1 8.3 14.0 14.0</td>
<td>1.3 3.7 5.3 10.5</td>
</tr>
<tr>
<td>p110FXE</td>
<td>1.0 1.8 5.1 &lt;1</td>
<td>1.9 1.2 5.0 2.5</td>
</tr>
<tr>
<td>pIEEHVg63</td>
<td>ND &lt;1 &lt;1 1.4 1.4</td>
<td>1.7 36 59 7.5</td>
</tr>
<tr>
<td>pENDA110</td>
<td>ND 2.4 6.0 4.3 0.8</td>
<td>1.2 1.2 2.5 2.5</td>
</tr>
<tr>
<td>p110ENX</td>
<td>ND 1.9 5.6 1.5 0.8</td>
<td>0.7 2.5 2.5 2.5</td>
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*HeLa or BHK cells were transfected by the calcium phosphate method with the relevant reporter plasmid (4 μg per 50 mm plate) either in the presence or absence of plasmid p175 (4 μg per plate). The indicated amounts of the activator plasmids were included, and the total amount of plasmid DNA was normalized in each transfection using pUC9. The cells were washed 16 h after transfection and extracts prepared 24 h later. Aliquots of the extracts were incubated with [14C]chloramphenicol and the substrate and products were separated by thin-layer chromatography. The protein content of each extract was determined using the Bio-Rad protein assay kit. The details of the above methods were as described previously (Everett, 1988). The amount of radioactivity in the substrate and product bands was quantified using a Molecular Dynamics Phosphorimager and ImageQuant software and the percentage conversion of substrate to product was normalized according to the protein concentrations of each sample. Positive control p11 and negative control p100FXE titrations were included in each experiment and as far as possible the plasmids were analysed in parallel. The results represent the averages from two to four independent titration experiments. ND, Not determined.

The solution structure of the RING finger domain of equine herpesvirus 1 (EHV-1) gene 63 protein (EHVg63) has been solved and comparison of the primary sequences of the RING domains of EHVg63 and Vmw110 suggests that the two probably adopt very similar topologies (Barlow et al., 1994). Based on this homology modelling, single amino acid substitution mutations within the helix region of the Vmw110 RING finger were constructed and found to have profound effects on the ability of Vmw110 to activate gene expression in transfection assays and to enhance viral growth (Barlow et al., 1994; R. Everett and others, unpublished results). While these results suggest that there may be significant similarities in the structure and function of the herpesvirus RING finger proteins, they do not address whether the proteins are interchangeable in the context of normal virus growth. We have explored this question by making chimeric proteins in which the minimal RING finger domain of EHVg63 has been exchanged with that of Vmw110, and by making an HSV-1 recombinant virus containing the EHV-1 gD promoter region linked to the chloramphenical protein (which contains the RING finger domain) with that of Vmw110 (Moriuchi et al., 1994).

Plasmid p100FXE is a derivative of p11 which contains the complete 332 residue EHV-1 gene 63 coding region on an NcoI–EcoRV fragment inserted between the NcoI and SalI sites of p11. Since the NcoI sites in both the EHV-1 and HSV-1 genes contain their respective ATG initiation codons, this plasmid constitutes an almost precise reading frame swap of the two related genes; the transcriptional and processing control signals remain those of HSV-1. Plasmid pENDA110 has the NcoI–DraI fragment of EHV-1 gene 63, encoding the N-terminal 63 residues of the RING finger region of EHVg63, inserted in place of the NcoI–AluI fragment of p111, and therefore expresses a hybrid protein with the first 3 residues of EHVg63 linked to residues 159–775 of Vmw110. Plasmid p110ENX is a derivative of pENDA110 in which sequences encoding the first 105 residues of Vmw110 have been replaced, thereby expressing a hybrid protein in which the 55 residue Vmw110 RING finger region has been replaced by the 63 residue EHVg63 RING finger region. These plasmids are shown schematically in Fig. 1 (a).

The ability of EHVg63 and the domain swap proteins to activate gene expression was assessed using transfection assays. Vmw110 activates gene expression in synergy with the major HSV-1 transactivator Vmw175, especially in HeLa cells (Everett, 1986). Using the reporter plasmid pgDCAT, which contains the HSV-1 gD promoter region linked to the chloramphenical...
acetyltransferase (CAT) coding region, this assay indicated that EHVg63 was ineffective (Table 1). The two domain swap plasmids showed no significant activity above that of the RING finger deletion control, p110FXE. It has been observed that Vmw110 strongly activates the ICP6 (or R1) promoter (Desai et al., 1993). Using reporter plasmid pSS80, which contains the ICP6 promoter linked to CAT, plasmid pEEHVg63 achieved moderate levels of activation of gene expression in BHK cells (Table 1) but the domain swap plasmids were again not significantly active.

One interpretation of these data is that the RING domain of EHVg63 is not able to substitute for that of Vmw110. However, despite the fact that the inserted region of EHVg63 is able to fold properly (Barlow et al., 1994) it is not possible to be certain that the RING domain, or its junction regions, are folded correctly in the context of the entire hybrid protein. In contrast, the intact EHVg63 protein activated gene expression in at least one situation; indeed it behaved in a manner similar to that of C-terminal deletion mutants of Vmw110 which retain some ability to activate gene expression, but not in synergy with Vmw175 (Everett, 1988). It may be pertinent that there is little identity between the C-terminal sequences of Vmw110 and EHVg63.

Because of this positive result we set out to determine whether EHVg63 could substitute for Vmw110 in the context of virus infection in tissue culture. Linearized pEEHVg63 plasmid DNA was co-transfected into BHK cells with infectious HSV-1 Vmw110-null mutant d11403 DNA; the progeny viruses were plaque purified and screened for the presence of EHVg63 coding sequences by Southern blotting. The methods employed were exactly as described previously (Everett, 1989). After three rounds of plaque purification, stocks of recombinant virus 17Eg63 were prepared, and its IE-1 region analysed by Southern blotting. The results indicated that both copies of R~ contained an IE-1 region with the EHVg63 coding sequences (Fig. 1b). As a control, a resucuant virus (17Eg63R) was constructed by cotransfecting 17Eg63 DNA with linearized plasmid p111. Virus 17Eg63R was isolated after three rounds of plaque purification by screening progeny plaques by Southern blotting; it contains the normal IE-1 gene in place of the EHVg63 sequences in 17Eg63.

The two simplest phenotypes of Vmw110-deficient viruses in culture are reduced growth in single-step growth experiments, as measured by plaque assay and reduced plaquing efficiency in human fetal lung (HFL) cells compared to BHK cells (Stow & Stow, 1986). The growth of recombinant 17Eg63 was intermediate between wild-type strain 17 virus and the Vmw110 RING finger deletion virus FXE, whereas the resucuant virus 17Eg63R grew normally (Fig. 2). The three- to five-fold reduction in virus yield of 17Eg63 was reproducible over several repeated experiments. The conclusion that EHVg63 at least partially substitutes for Vmw110 was reinforced by comparison of the plaquing efficiencies of 17Eg63 in BHK and HFL cells. The average ratio of the plaquing efficiencies of 17Eg63 in the two cells types (1:2) was not significantly different from that of strain 17 (1:4), whereas the ratio obtained with Vmw110 null mutant d11403 in the same experiments was 23:2. The d11403 null mutant and the FXE RING finger deletion virus behave identically in these assays (Everett, 1989).

The results presented in this report clearly show that the HSV-1 and EHV-1 RING finger proteins are at least partially functionally interchangeable in the context of virus infection. The remaining growth defect of 17Eg63 may be a consequence of the absence from EHVg63 of sequences with obvious similarity to the C-terminal 180 residues of Vmw110. This region of Vmw110 has been shown to be required for normal interactions between cellular proteins and nuclear structures (named ND10) which contain a number of cellular proteins, including PML; these interactions correlate with fully efficient virus growth (Everett, 1989; Maul et al., 1993; Everett & Maul, 1994; Maul & Everett, 1994; Meredith et al., 1994, 1995). This interpretation is supported by the observation that, unlike strain 17, virus 17Eg63 failed to disrupt the PML-containing ND10 nuclear domain in infected cells (data not shown). Despite the improved plaquing efficiency of HSV-1 Vmw110-null mutants in cell lines which express the VZV homologue, the gene 61 protein (Moriuchi et al., 1992) it seems likely that a recombinant virus which contains VZV gene 61 in place of gene IE-1 would also exhibit partial growth defects due to the absence of sequences homologous to the Vmw110 C-terminal region. Given that this part of Vmw110 is involved in cellular interactions, it is tempting to speculate that the differences between the equivalent alpha herpesvirus proteins represent their differing host interactions. It is interesting that the C-terminal region of Vmw110 is conserved only with its HSV-2 counterpart in the alpha herpesvirus protein family.

The fact that virus 17Eg63 grows more efficiently than virus FXE suggests that the two RING domains have similar functions, since this region is the only clearly conserved part of the two proteins. However, despite confidence that the inserted EHVg63 RING finger region in the Vmw110 domain swap proteins is capable of folding independently, it is not possible to conclude that the two RING fingers either do or do not contact the same targets. The lack of function of the hybrid proteins indicates either that they do not contact the same target, or that the context of the RING finger in relation to the remainder of the parent protein needs to be more precise than was achieved with the junctions formed in these
were harvested at 4, 8, 16 and 24 h after infection and the progeny parallel plates were infected at a multiplicity of 2 p.f.u. per cell. Plates viruses were titrated on BHK cells. The vertical axis gives the total amount of virus per plate on a log scale. Symbols: O, 17Eg63; @, 17Eg63R; ■, FXE; □, 17°.

Fig. 2. Single step growth curves of strain 17 (17+), 17Eg63, 17Eg63R and RING finger deletion virus FXE (Everett, 1989). BHK cells on parallel plates were infected at a multiplicity of 2 p.f.u. per cell. Plates were harvested at 4, 8, 16 and 24 h after infection and the progeny viruses were titrated on BHK cells. The vertical axis gives the total amount of virus per plate on a log scale. Symbols: O, 17Eg63; •, 17Eg63R; ■, FXE; □, 17°.

experiments. The implication is that the RING finger, unlike many DNA binding or transcriptional activation domains, is not a simple moveable element. This conclusion is supported by similar Vmw110 domain swap experiments using minimal RING finger domains from a number of cellular proteins, none of which yielded an active hybrid Vmw110 protein (Everett et al., 1995; D. O’Rourke & P. O’Hare, personal communication). However, in apparent contrast to the minimal domain swap constructs described above, Moriuchi et al. (1994) have shown that a hybrid protein comprising the initial 105 residues of the VZV gene 61 protein (which includes its RING finger) linked to the C-terminal two-thirds of Vmw110 has a partial restoration of function. The converse hybrid protein was also able to activate gene expression. These data suggest that the RING domain might have to be in a context of a larger portion of the parent protein in order to be active.

To understand in more detail the complex nature of the functions of Vmw110 and its various domains, it will be essential to determine the target of its RING finger and how it relates to the remainder of the protein and to the interactions mediated by the C-terminal domain.

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References


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