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Point Mutations in the Herpes Simplex Virus Type 1 Vmw110 RING Finger Helix Affect Activation of Gene Expression, Viral Growth, and Interaction with PML-Containing Nuclear Structures

ROGER EVERETT,1*, PETER O’HARE,2 DELIA O’ROURKE,2 PAUL BARLOW,3 AND ANNE ORR1

Medical Research Council Virology Unit, Glasgow G11 5JR, Scotland,1 and Department of Biochemistry, University of Oxford, Oxford OX1 3QU,3 and Marie Curie Research Institute, Oxford, Surrey RH8 0TL,2 England

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Herpes simplex virus type 1 immediate-early protein Vmw110 (also known as ICP0) has been implicated in the control of the balance between the lytic and latent states, but the precise mechanisms by which it exerts its effects are unknown. Vmw110 includes a characteristic zinc binding domain, termed the C4HC4 domain or RING finger, which is essential for its function. The solution structure of a related herpesvirus RING finger domain suggested that an amphipathic alpha helix might be an important functional component of the RING finger. In this paper, we show that the equivalent region of Vmw110 is important for virus growth in tissue culture and for the normal interaction of Vmw110 with nuclear structures which include the PML protein.

Herpes simplex virus type 1 (HSV-1) is an important human pathogen with the intriguing ability to replicate lytically in the periphery and to establish a latent infection in sensory neurons. The virus encodes at least 75 distinct genes (27, 28) which are regulated differentially during lytic growth and latency. During the lytic cycle, all viral genes are expressed in a temporal cascade in which the five immediate-early (IE) genes are transcribed first, followed by the early and late classes (see reference 30 for a review). During latency, only the latency-associated transcripts, whose function remains obscure, have been detected (17, 33). An understanding of the regulatory mechanisms which distinguish the lytic and latent states of the virus is of both clinical and biological importance.

Several of the five IE gene products have significant or essential roles in the regulation of lytic viral gene expression. Of these, the product of IE gene 1 (IE-1) (Vmw110 or ICP0) appears to be involved in both lytic growth and latency and is able to activate gene expression in transfection assays (see references 8 and 15 for reviews). Viruses with lesions in Vmw110 have multiplicity-dependent and cell-type-dependent defects in the onset of the lytic cycle (10, 32, 34) and are unable to reactivate latent virus in an in vitro latency system (20, 31). Such viruses also reactivate inefficiently in mouse latency models (3, 4, 23). Mutational analysis of Vmw110 has established that a RING finger zinc binding domain is very important for Vmw110 activity (9, 10, 18–20). The solution structure of the RING finger domain of a related herpesvirus protein illustrates that it comprises a triple antiparallel beta sheet, on one side of which is embedded an amphipathic alpha helix (1, 12). Two zinc atoms are coordinated at either end of the helix, which extends basic and polar side chains into solvent. We surmised that these hydrophilic side chains would be required for the function of the herpesvirus RING finger. This prediction was supported by the observation that amino acid replacements in the potential helix region reduced Vmw110 activity in transfection assays, when acting in synergy with HSV-1 IE protein Vmw175 (1).

To extend these studies, we have investigated whether mutations in the potential Vmw110 helix region disrupt its ability to activate gene expression in the absence of Vmw175, and we have constructed recombinant viruses which express the mutant Vmw110 proteins. Since Vmw110 becomes associated with nuclear structures which contain the PML protein (referred to herein as ND10) (6, 14, 21, 22, 25, 26, 36), we have also investigated the interactions between the mutant Vmw110 proteins and ND10.

Mutations in the potential RING finger helix reduce activation of gene expression by Vmw110. The RING finger domain of the equine herpesvirus type 1 (EHV-1) gene 63 protein includes an amphipathic alpha helix with exposed polar and basic side chains (1). Comparison of primary sequences shows that charged and polar residues are present in the Vmw110 RING finger at positions similar to those in the EHV gene 63 RING finger helix (Fig. 1). In all the herpesvirus members of the protein family there is a tryptophan residue 4 residues from the zinc-coordinating cysteine at the start of the potential helix region. We have shown that mutation of Vmw110 residues lysine 144, glutamine 148, and asparagine 151 affects the ability of the protein to activate gene expression in transfection assays in the presence of the major HSV-1 transactivator, Vmw175 (1). We wished to determine what effect these mutations had on the ability of Vmw110 to activate gene expression when acting alone; in addition, the effect of mutating the conserved tryptophan was investigated.

Vmw110 strongly activates expression of genes which include the HSV-1 UL39 promoter in both transfection assays and during virus infection (5). Therefore, we used a plasmid with the UL39 promoter linked to chloramphenicol acetyltransferase to assay the effects of substitution mutations in the Vmw110 RING finger. Surprisingly, mutation of the tryptophan at position 146 to an alanine residue had little effect on the activity of Vmw110 (Fig. 2). Presumably, structural stability can be maintained without the interactions which W-146 makes with the core of the domain. The K-to-E mutation at position 144 (K144E) had the most substantial effect, similar to
that of the RING finger deletion mutant p110FXE. The N151D mutation also had a significant effect, while the activity of Q148E was reduced only about twofold (Fig. 2). We conclude that mutations in the potential RING finger helix region reduce the intrinsic ability of Vmw110 to activate gene expression, but the extent of the effect depends on the nature of the mutation.

These results differ slightly from those obtained with the same Vmw110 mutant plasmids acting on a gD promoter reporter in the presence of Vmw175 (1). The Q148E mutant was less active in the latter system, while the K144E mutant was more active. Variations in the details of results obtained with different transfection protocols have been well established (15); it is interesting that the results obtained with the UL39 reporter construct reported in this paper reflect more closely the effects of the mutations on virus growth than those obtained with the gD reporter in the presence of Vmw175.

Mutations in the potential RING finger helix of Vmw110 affect growth of HSV-1 virus in tissue culture. Infectious viral d11403 DNA, which carries a deletion of the majority of the Vmw110 coding region (34), was cotransfected into cells with plasmids containing the mutant IE-1 genes. Progeny plaques were screened for the presence of a reconstituted IE-1 region, and clones were purified as previously described (10). Silent coding changes were introduced into the K144E, Q148E, and N151D viruses to form an NsiI site for screening purposes. However, the actual mutagenic base changes could not be detected by restriction enzyme analysis. To prove that the viruses encoded the mutant proteins, their complete RING finger coding regions were amplified by PCR and the products were sequenced directly. In all cases, the expected mutation was detected and there was no evidence of other alterations.

HSV-1 viruses which fail to express Vmw110, or which express Vmw110 proteins with an inactivated RING finger, have a 1- to 2-log reduction in growth efficiency in single-step growth curves (10). The extent of the reduction correlates inversely with the growth rate of the indicator cells (11). The single-step growth kinetics of viruses K144E, W146A, Q148E, and N151D in BHK cells were determined in comparison with wild-type virus and the mutant virus FXE, from which the Vmw110 RING finger has been deleted (10). The results clearly show that the K144E and N151D mutations were as deleterious for virus growth in BHK cells as removal of the entire RING finger domain (Fig. 3). The W146A and Q148E mutations had little, if any, significant effect. Therefore, the potential helix region of the Vmw110 RING finger is important for HSV-1 growth in tissue culture. Interestingly, the effects of the individual mutations on virus growth mirror their effects on activation of the UL39 promoter in transfected HeLa cells (Fig. 2).

All of the mutant viruses expressed normal amounts of full-length Vmw110, which suggests that the single-residue substitutions had not caused gross conformational alteration or instability of the mutant proteins (Fig. 4).

Mutations in the potential RING finger helix affect the interaction of Vmw110 with discrete nuclear domains. At early times of HSV-1 infection, wild-type Vmw110 is located in part in discrete nuclear structures (ND10) which contain PML, sp100, and other uncharacterized cellular proteins. At later times of infection, the cellular ND10 structures are disrupted, an effect which requires a functional Vmw110 RING finger domain (25, 26). Similarly, in transfected cells which express wild-type Vmw110, detection of PML in ND10 becomes much more difficult, indicating either that PML is being dispersed or that conformational changes occur so as to obscure the epitope recognized by the 5E10 anti-PML monoclonal antibody (14). When the Vmw110 RING finger domain is deleted, PML and mutant Vmw110 stably colocalize in transfected cells (14).

To assess the effects of the RING finger helix mutations on the interactions between Vmw110 and ND10, BHK cells were transfected with pl111, pl10FXE, and plasmids expressing Vmw110 proteins with substitution mutations in the potential RING finger helix region. The cells were stained for Vmw110 and PML 16 h later; representative results from several hundred Vmw110-positive cells are presented in Fig. 5. Wild-type Vmw110 drastically reduced the detection of PML (Fig. 5A and B). In fact, PML was undetectable in many cells expressing wild-type Vmw110, but a field where faint PML fluorescence remains visible has been selected so as to enable comparison between the images at the two different wavelengths. In contrast, the RING finger mutant p110FXE stably colocalized with the ND10 structures (Fig. 5K and L). The RING finger helix mutations exhibited a range of phenotypes. Mutant K144E was similar to FXE, with extensive colocalization of mutant Vmw110 and PML in ND10 structures (Fig. 5C and D). Mutant N151D also failed to disperse PML and colocalized at ND10 (Fig. 5E and F). However, mutant Q148E exhibited intermediate phenotypes, with some cells which expressed the mutant Vmw110 protein retaining PML in ND10, while in

FIG. 1. An alignment of the RING finger helix regions of the HSV-1 Vmw110 (upper line) and EHV-1 gene 63 (lower line). The residues that are relevant to this study are shown in boldface type, and their coordinates within the parent proteins are indicated. The coordinating cysteine and histidine residues are underlined. Residues within the helix and adjacent regions and other conserved residues are indicated by the single-letter code. Nonconserved residues are indicated by X.

FIG. 2. Single amino acid substitutions in the HSV-1 Vmw110 RING finger helix affect its ability to activate gene expression. Gene expression from the UL39 promoter in HeLa cells was activated by increasing amounts of pl11, expressing wild-type Vmw110 (29), and the single-point mutant plasmids are compared with p110FXE, which lacks the RING finger region (9). Transfections and chloramphenicol acetyltransferase assays were performed exactly as previously described (7, 9). In all experiments, four amounts of each test plasmid were used, and positive (p111) and negative (p10FXE) control titrations were included. The results presented are the averages from four independent repeat experiments of each complete titration.
other cells PML staining was much reduced (compare the amounts of PML detected in the two Vmw110-positive cells; Fig. 5G and H). The W146A mutant protein exhibited a localization phenotype closer to that of the wild type, but even with this mutation, retention of PML seemed to occur in some cells (Fig. 5I and J; compare the relative Vmw110 and PML signals in the two cells at the bottom right of panel J). Similar experiments were conducted with virus-infected cells, with equivalent results (data not shown).

It is interesting that the degree of abnormality of the mutant proteins in this assay correlates directly with their effects on the activation of gene expression and the growth of the mutant viruses (Fig. 2 and 3). This suggests that ND10 structures constitute an important part of the mechanism of action of Vmw110. However, it is not possible at this stage to determine if Vmw110 actually functions at these sites or whether the disruption of ND10 releases factors which operate elsewhere. It is also possible that ND10 structures in some way inhibit viral gene expression and that their disruption by Vmw110 overcomes this effect.

What is the function of the RING finger domain? The RING finger family of proteins includes a wide range of members from evolutionarily diverse sources (18, 19). The domain includes a core sequence of CXHXXCXXC, which comprises two metal coordinating doublets separated by a short beta strand (1, 2). Given that the spacing of this sequence is invariant in the family, it is likely that the positions of the two zinc atoms are also highly conserved. Immediately following this core region in EHV-1 gene 63 lies an alpha helix. Examination of the corresponding sequences of other members of the family indicates that many are also likely to form an alpha helix at this point (1). However, even among the herpesvirus proteins, the charged residues do not occupy exactly the same positions within the primary sequence of the predicted helix (1), which implies significantly different spatial positions of the side chains. When the predicted helix regions of other members of the RING finger family are considered, the diversity of the positions and the nature of the polar and charged residues (1) strongly suggest that, if RING finger domains bind to other macromolecules, their individual targets may differ considerably. Molecular modelling based on the topological similarity between part of the RING finger structure and a TFIH-1-type zinc finger (1) has shown that the EHV-1 gene 63 RING domain could not bind in the major groove of normal DNA because of extreme steric clashing between other parts of the RING domain and the DNA helix (24).

The results presented here and elsewhere (14, 25, 26) indicate that the Vmw110 RING finger is required for modification of ND10 nuclear structures, suggesting that the role of the Vmw110 RING finger concerns complex interactions between the components of ND10. Since ND10 structures have been found to be closely associated with the nuclear matrix (35), these interactions may include not only the contacts between the various protein constituents of ND10 but also the interactions between these proteins and the nuclear scaffold. The mutants described in this paper may provide useful tools with which to try to dissect these connections.

FIG. 3. Single-step growth curves of wild-type HSV-1 (strain 17+) and the Vmw110 mutant viruses in BHK cells. The cells were infected at a multiplicity of infection of 1 PFU per cell, and parallel plates were harvested at 4, 8, 16, and 24 h after infection. Titers of progeny virus were determined by using BHK cells. The results represent the averages from two independent experiments.

FIG. 4. Expression of Vmw110 by wild-type and mutant viruses. BHK cells were infected with the viruses as indicated at a multiplicity of infection 5 PFU per cell and harvested 16 h later. Samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting with monoclonal antibody 11060 (13). The position of full-length Vmw110 protein (as previously determined with respect to other characterized marker proteins) is indicated.
FIG. 5. Intracellular localization of wild-type and mutant forms of Vmw110 16 h after transfection (16) of BHK cells. The left-hand panels show staining of representative fields of cells with monoclonal antibody 5E10 (35) to detect PML (6), while the right-hand panels show the same fields of cells stained with rabbit r95 serum (13) to detect Vmw110. (A and B) p111 (wild-type Vmw110); (C and D) p110K144E; (E and F) p110N151D; (G and H) p110Q148E; (I and J) p110W146A; (K and L) p110FXE (RING domain deletion). The methods used were exactly as described previously (14).
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