The cellular RING finger protein PML is not a functional counterpart of the herpes simplex virus type 1 RING finger protein Vmw110

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Herpes simplex virus type 1 (HSV-1) immediate early protein Vmw110 (also known as ICP0) is required for the fully efficient expression of viral genes during onset of lytic growth and for normal reactivation from latency. Both Vmw110 and the cellular protein PML are members of the RING finger family of zinc binding domain proteins, a family which includes an increasing number of examples from a wide evolutionary range. The function of the RING finger domain is unknown, and the question arises whether the RING finger (like several other examples of conserved domains) fulfils similar functions in these diverse proteins. Another link between Vmw110 and PML is that at early times of HSV-1 infection Vmw110 migrates to distinct nuclear structures which contain the PML protein. In order to test the possibility that PML and Vmw110, or their RING finger domains, fulfil similar functions, we have constructed recombinant viruses that express either intact PML, or a chimeric Vmw110 protein which contains the PML RING finger in place of its own. The results indicate that the PML and Vmw110 RING fingers are not functionally interchangeable, and that PML is not a cellular functional counterpart of Vmw110.

Introduction

Herpes simplex virus type 1 (HSV-1) immediate early protein Vmw110 is required for fully efficient onset of lytic virus growth and reactivation from latency (Stow & Stow, 1986; Everett, 1989; Cai & Schaffer, 1989; Harris, 1989; Lieb et al., 1989; Clements & Stow, 1989; Cai et al., 1993). In transfection assays, Vmw110 is a potent and promiscuous activator of gene expression (for a review, see Everett et al., 1991). It has recently been demonstrated that Vmw110 is a member of a family of proteins which includes representatives from all the alphaherpes viruses so far investigated and an increasing number of cellular proteins of diverse evolutionary origin and function (Freemont et al., 1991; Freemont, 1993). Characteristic of the members of this family is a sequence motif called the C3HC4 zinc binding domain, or RING finger. The structure of this domain is quite unlike other previously characterized zinc finger motifs, and its function and target remain obscure (Everett et al., 1993a; Barlow et al., 1994). However, the RING finger domain of Vmw110 is crucial for the functions of Vmw110 (Everett, 1988, 1989; Harris et al., 1989).

We have recently shown that early in infection, Vmw110 localizes to discrete nuclear structures, referred to herein as ND10, and that these structures include the cellular RING finger protein PML (Maul et al., 1993; Maul & Everett, 1994; Everett & Maul, 1994). The normal functions of ND10 and the PML protein are unknown, but in a disorder known as promyelocytic leukaemia a chromosomal translocation results in the N-terminal part of PML (including its RING finger domain) being linked to the retinoic acid receptor-α (RAR-α) (Goddard et al., 1991; de The et al., 1991; Kakizuka et al., 1991; Kastner et al., 1992). The PML–RAR-α fusion has been implicated in the development of the proliferation disorder, since in leukaemic cells the normal localization of PML in ND10 is disrupted and both this disruption and the leukaemia can be reversed by treatment with retinoic acid (Dyck et al., 1994; Koken et al., 1994; Weis et al., 1994).

Since PML and Vmw110 both include a RING finger domain, and since they both localize to ND10, it seemed possible that they were involved in similar functions. We set out to test this possibility by constructing plasmids and recombinant HSV-1 viruses that expressed either intact PML or a hybrid Vmw110 that contained the PML RING finger in place of its own.

Methods

Plasmids. Plasmid p111 expresses intact Vmw110 from the IE-1 promoter in a pUC9 background, whereas p10FXE (derived from p111) has a deletion in the Vmw110-coding sequences which removes
codons 106-149. Plasmid p175 contains the Vmw175-coding region under the control of the simian virus 40 early promoter (Everett, 1988). Plasmid pIEPML was constructed by inserting the 1.75 kb NcoI-PvuII fragment containing the cDNA coding sequence of the PML-1 isoform (Kakizuka et al., 1991) between the NcoI and SalI sites of p111. The NcoI site includes the translational initiation codon of PML whereas the PvuII site is located 82 bp 3' of the translational stop codon of this isoform. The IE-1 promoter and 3' processing signals remain in this plasmid. Plasmid p110PMLZ was constructed by utilizing a 5' PCR primer which had an XhoI site 5' of codons 47-52 of the PML-coding region, and a 3' primer which commenced at codon 90 and had a Smal site 3' of codon 94. PCR amplification of the 48 codon region between the two primers was conducted using a PML cDNA plasmid clone as template. The PCR product was cut with XhoI and Smal and inserted between the XhoI and Adal sites of p111. The junction regions were designed so as to maintain the Vmw110 reading frame. This construct has 10 residues of PML prior to the first cysteine of the RING finger (replacing the 10 corresponding residues of Vmw110) and three PML residues after the last coordinating cysteine (replacing the corresponding three residues of Vmw110), flanking the PML RING finger domain. The entire PML region and flanking sequences in p110PMLZ were re-sequenced to ensure that no point mutations had been introduced during PCR amplification.

**Viruses and cells.** HSV-1 Glasgow strain 17 syn + was the parental virus strain for all mutant virus constructions. The Vmw110 null mutant d11403 (Stow & Stow, 1986) and the Vmw110 RING finger deletion mutant virus FXE (Everett, 1989) have been described previously. The PML expression virus IEPML and RING finger domain swap virus PMLZ were constructed as described below. All viruses were propagated and titered on BHK cells grown in the Glasgow modification of Eagle's medium (GMEM) supplemented with 10% tryptose phosphate broth, 10% new born calf serum (NBCS), 100 U/ml penicillin and 100 μg/ml streptomycin. HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2.5% NBCS, 2.5% fetal calf serum and antibiotics as above.

**Transfection of cells and CAT assays.** BHK cells in 50 mm dishes were transfected by the calcium phosphate co-precipitation technique exactly as previously described (Everett, 1986, 1988) with plasmid pgDCAT (4 μg) as indicator and increasing amounts of activator plasmids p111, p110FXE, pIEPML and p110PMLZ. For transfections in HeLa cells, 4 μg of plasmid p175 was included in each mixture. The total amount of plasmid DNA in each transfection (12 μg) was made constant with pUC9. Two days after transfection, cell extracts were prepared and assayed for CAT activity as described (Everett, 1988). The product and substrate, separated on TLC plates, were quantified prepared and assayed for CAT activity as described (Everett, 1986, 1988) with plasmid pgDCAT (4 μg) as indicator and increasing amounts of activator plasmid. The results represent averages from at least four independent experiments.

**Construction and isolation of recombinant viruses.** Plasmids pIEPML and p110PMLZ were linearized by digestion with ScaI (which cuts once within the vector sequences) and co-transfected into BHK cells with infectious d11403 DNA (a virus lacking most of the IE-1-coding region). Progeny plaques were propagated in Linbro wells, then total infected cell DNA was prepared and screened by Southern blot hybridization. Positive clones were purified by three rounds of plaque purification. Viral DNA from the final stocks was analysed by Southern blotting.

**Antibodies.** Monoclonal antibodies (MAbs) 11060 and 10810, which recognize different epitopes within Vmw110, have been described previously (Everett et al., 1993b). MAb 5E10 (Stuurman et al., 1992) recognizes PML (Dyck et al., 1994).

**Immunofluorescence.** BHK cells were seeded at a density of 0.5 × 10⁶ cells per ml into 24-well Linbro dishes containing glass coverslips. Cells were washed with PBS at the indicated times after infection, then fixed with paraformaldehyde (3.75% in 10 mm-PIPES pH 6.8, 100 mm-NaCl, 100 mm-sucrose, 3 mm-MgCl₂, 1 mm-EDTA) and permeabilized with 0.5% NP40 in PBS. The primary antibodies were diluted in PBS containing 1% NBCS. Anti-Vmw110 MAb 11060 was used at a dilution of 1:2000 and anti-PML MAb 5E10 (Stuurman et al., 1992) was used at 1:20. Goat anti-mouse FITC-labelled secondary antibody (Sigma) was used at the concentration recommended by the supplier. After staining, coverslips were mounted and examined in a Nikon Microphot-SA microscope with the 40 x objective lens and appropriate filters.

**Western blots.** Protein samples from infected cells were boiled in SDS gel-loading buffer and separated down 7.5% SDS-polyacrylamide gels using a glucose-based running buffer in a Bio-Rad Mini-Protein II gel kit, using reagents and methods as recommended by the supplier. Separated protein bands were transferred to nitrocellulose using the compatible Bio-Rad Western blotting kit. The filters were blocked by incubation with 3% gelatin in TBS containing 0.05% Tween-20, then incubated with the appropriate antibody dilution (MAB 11060 at 1/10000; MAB 5E10 at 1/10). Bound antibody was detected by the Amersham ECL method after incubation with goat anti-mouse Ig horseradish peroxidase-conjugated immunoglobulin, as recommended by the supplier.

**Results**

*Neither PML nor a hybrid Vmw110 protein containing the PML RING finger domain activate gene expression in transfection assays*

The similarities between the RING finger domains of PML and Vmw110, and the fact that both PML and...
Vmw110 co-localize at least transiently in the ND10 nuclear structure, led us to investigate whether the two proteins or their RING finger domains had interchangeable functions. Accordingly, recombinant plasmids were constructed which express either PML or a hybrid domain swap protein under the control of IE-1 regulatory signals. Plasmid p110PMLZ expressed a Vmw110 protein with amino acid residues 106-159 replaced by PML residues 47-94, and plasmid pIEPML contains the PML-coding region (Fig. 1). Plasmids pIEPML and p110PMLZ were used in transfection assays to investigate whether they could activate gene expression either by themselves or in synergy with another HSV-1 IE gene product, Vmw175, in a fashion analogous to that of normal Vmw110 (Everett, 1986, 1988). Either BHK cells (Fig. 2; for activation in the absence of Vmw175) or HeLa cells (Fig. 3; for activation in its presence) were transfected with plasmid pgDCAT as indicator and increasing amounts of either p110PMLZ or pIEPML. The positive control was p111, which expresses wild-type Vmw110, and the negative control was p110FXE, a RING finger deletion mutant of Vmw110 which retains some residual activity owing to the presence of a functional C-terminal domain (Everett, 1988). The results showed that neither PML nor the PMLZ domain swap activated gene expression above the levels seen with the negative control.

Neither PML nor a hybrid Vmw110 protein containing the PML RING finger substitute for Vmw110 function during virus infection

We constructed recombinant viruses expressing PML and the domain swap protein since transfection assays do not always give an exact reflection of the events during normal virus infection. Plasmids pIEPML and p110PMLZ were linearized and co-transfected with d11403 viral DNA, which has a deletion removing almost the entire Vmw110-coding region (Stow & Stow, 1986). Progeny virus clones were screened for the presence of the required sequences, plaque purified three times, and stocks prepared. Southern blot analysis of the resultant isolates indicated that the structure of the IE-1 region was as expected in both cases (Fig. 4).

The characteristics of Vmw110-deficient viruses include a yield reduction of 10- to 100-fold in single-step growth experiments, and a relatively poor plaquing efficiency in HFL cells (Stow & Stow, 1986; Everett, 1989). Both the PMLZ and IEPML viruses were as defective as virus FXE (the Vmw110 RING finger deletion mutant virus) in single-step growth curves (Fig. 5). In addition, both were defective in plating efficiency on HFL compared to BHK cells, showing a 12- to 15-fold reduction compared to the 20-fold reduction of virus FXE. This presents further evidence that PML is not a functional homologue of Vmw110.
It is possible that the junctions of the domain swap were inappropriate for correct folding of the hybrid protein, since the junction of Vmw110 and PML residues...
on the C-terminal side lies in a β-strand which contributes to the stability of the RING finger core (Barlow et al., 1994). Although it was not possible to determine the conformation of the RING finger in the domain swap protein, we found that virus PMLZ produced normal amounts of full-length hybrid protein as detected by
Fig. 8. Localization of PML during infection with virus IEPML. (a) shows uninfected BHK cells stained with MAb 5E10 to detect PML in ND10; Hep2 cells give similar patterns (Maul et al., 1993). In (b) Hep2 cells were infected with virus IEPML and PML protein was detected with MAb 5E10 16 h post-infection. Confocal microscopy was used to visualize PML protein in a cross section of the nuclear membrane. Bar marker in (b) represents 10 μm.

western blot, and the pattern of minor degradation products was not significantly different from that of normal Vmw110 (Fig. 6a, b). Virus IEPML expressed readily detectable amounts of PML (Fig. 6c).

Intracellular localization of the virus-expressed PML and PMLZ proteins

One further property of the recombinant viruses and the hybrid protein was investigated. Wild-type Vmw110 transiently co-localizes with PML in the punctate ND10 structures early in infection, later becoming more diffuse and spread throughout the cell (Maul & Everett, 1994; Everett & Maul, 1994). It was of interest to determine what effect the RING finger domain swap had on the distribution of Vmw110 during infection. BHK cells infected with viruses 17+, PMLZ and FXE were stained to detect the localization of Vmw110 8 h after infection. Virus PMLZ produced abnormal punctate cytoplasmic and nuclear structures very similar to those of RING finger-deletion FXE (Maul & Everett, 1994), whereas the wild-type virus showed mostly diffuse cytoplasmic and nuclear staining (Fig. 7). The similarities between the PMLZ and FXE staining patterns again suggests that the PML domain swap fails to complement the loss of the Vmw110 RING finger.

High-level expression of PML during infection with virus IEPML allowed further investigation of the properties of the PML protein itself. At early times of infection, some of the newly synthesized PML protein appeared to be localized in ND10 structures, since they became larger and more intensely stained (data not shown). At later times of infection, some infected cells with large amounts of nuclear PML (detected by MAb 5E10) also exhibited prominent staining of the nuclear membrane (Fig. 8b). This confirms our earlier observation that PML can be present in the nuclear membrane (Maul & Everett, 1994).

Discussion

The similarities between the RING finger zinc binding domains of Vmw110 and PML, and the fact that both proteins can be located in the ND10 nuclear structures, suggested it was possible that PML might be a cellular functional counterpart of Vmw110. The results presented here show that this is not the case.

In contrast to the extensive analysis of the effects of the PML–RAR-α fusion proteins on gene expression from retinoic acid-responsive promoters, there is very little data on the functions of PML itself. The results in this paper strongly suggest that PML is unable to activate gene expression in transient assays. This conclusion is supported by unpublished data cited by Perez et al. (1993). Another recent report has suggested that PML might be a promoter-dependent repressor of gene expression (Mu et al., 1994). However, it remains possible that the various PML isoforms that arise from differential splicing (Kakizuka et al., 1991; Kastner et al., 1992; Pandolfi et al., 1992) might have different functions.
Considering the PMLZ domain swap protein, it is probable that the differences in the implied exposed surface regions of the Vmw110 and PML RING finger domains (Everett et al., 1993; Barlow et al., 1994) are largely responsible for the failure of the PML domain to complement the loss of the Vmw110 RING finger, and it is likely that these two domains contact different targets. Despite the high positional conservation of the zinc-coordinating residues in the RING finger domain family, it now seems unlikely that RING finger domains fulfill the same role in each of the family members (Barlow et al., 1994). However, it remains likely that the functions of PML and Vmw110 are in some way connected through interactions with the ND10 structure. Given the potential importance (direct or indirect) of ND10 to the control of cellular proliferation in promyelocytic leukemia, and to the functions of Vmw110 during HSV-1 infection, it will be of great interest to pursue further studies on the roles of ND10 structures and their constituent proteins.

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