Examination of determinants for intranuclear localization and transactivation within the RING finger of herpes simplex virus type 1 IE110k protein

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The herpesvirus regulatory protein IE110k possesses a cysteine-rich, RING finger motif required for its role in transactivation and virus replication. IE110k also localizes to subnuclear compartments termed PODs (PML oncogenic domains). Localization to PODs induces redistribution of the proteins associated with this nuclear compartment, including the cellular RING finger protein, PML. Here we construct a series of deletions, RING domain swaps and point mutations to analyse specific requirements within the IE110k RING finger for subnuclear localization, redistribution of PML and transactivation and we examine the relationship between these activities. We find that IE110k localizes to distinct nuclear subdomains that are more numerous than the cellular PODs and that mutation of two residues within a predicted loop of the RING finger, or replacing the IE110k RING finger with a RING finger from a cellular gene abrogates the ability of IE110k to localize to these extra compartments and traps IE110k in the original PODs. We further demonstrate that RING fingers from the cellular genes mdm-2 and Bmi I, when placed within IE110k, alter the nuclear distribution of IE110k, do not transactivate, and do not redistribute PML. We also demonstrate that the majority of wild-type IE110k, like PML, is associated with the nuclear matrix. Although substitutions and deletions within the RING finger abolish transactivation, these mutant proteins remain tightly associated with the matrix. These results further dissect the determinants involved in different aspects of nuclear compartmentalization of IE110k and are discussed in relation to PML, PODs and the IE110k RING finger.

Introduction

The herpes simplex virus (HSV) regulatory protein IE110k is a member of the RING finger family of proteins defined by the presence of a novel subclass of cysteine/histidine-rich, zinc-chelating domain, the RING domain (Freemont et al., 1991). Proteins containing the conserved RING finger domain have been observed in yeast, Drosophila, mammals and in a number of DNA viruses, and have been associated with diverse processes including the regulation of gene expression, inhibition of apoptosis and DNA repair (Freemont, 1993). IE110k functions independently as a broad spectrum transactivator of gene expression (Everett, 1986; Gelman & Silverstein, 1985; O’Hare & Hayward, 1985; O’Hare et al., 1986), is required for normal levels of expression of all classes of HSV genes (Everett et al., 1991; Sacks & Schaffer, 1987; Stow & Stow, 1986) and has a role in virus reactivation (Cai et al., 1993; Clements & Stow, 1989; Leib et al., 1989). The exact mechanism by which IE110k functions remains poorly understood despite extensive mutational analysis of the protein. However, analysis of IE110k mutants in transient transfections and recombinant viruses has demonstrated that mutations within the RING finger motif are among the most debilitating for functional activity (Chen et al., 1991; Everett, 1987, 1988).

During a virus infection IE110k transiently localizes to novel subnuclear compartments termed PODs (PML oncogenic domains), also known as ND10 (Ascoli & Maul, 1991; Dyck et al., 1994; Everett, 1987; Gelman & Silverstein, 1986; Knipe & Smith, 1986; Weis et al., 1994). The term POD derives from the presence within these domains of the protein PML, which like IE110k, contains a RING finger motif. Fusion

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of the PML gene to the retinoic acid receptor gene plays a causal role in the development of acute promyelocytic leukaemia (Goddard et al., 1991; Kakizuka et al., 1991; Pandolfi et al., 1991) and results in disruption of the PODs (Dyck et al., 1994; Koken et al., 1994; Weis et al., 1994). Retinoic acid treatment of the leukaemogenic cells results in the reappearance of PODs, differentiation of cells and remission of disease. This has suggested a possible direct link between normal cell growth and the organization of PODs.

The PODs are associated with the nuclear matrix fraction of the cell (Chang et al., 1995; Stuurman et al., 1992). The normal function of PML protein may be connected with the proper organization of the nuclear matrix and/or these subnuclear organelles may represent matrix-associated repositories of regulatory components. Interestingly, previous results have shown that not only does IE110k localize to PODs but that this also results in the redistribution of constituent components including PML (Everett & Maul, 1994; Maul et al., 1993).

The conservation of a RING finger in a cellular and viral protein showing related subnuclear localization prompted us to examine further determinants within the RING finger and explore the relationship between localization to PODs, redistribution of PML, matrix association and transactivation activity of IE110k. We asked whether the related motifs from cellular genes would function for transactivation in the background of the IE110k protein and examined the subcellular localization of these proteins and their effects on PML and PODs. We also analysed a series of IE110k RING finger variants for transactivation, nuclear localization, PML redistribution and nuclear matrix association. We found that IE110k forms more numerous punctate foci than there were original POD foci in a cell and furthermore, that like PML, IE110k is tightly associated with the nuclear matrix. However, neither the formation of the extra foci nor the matrix association requires the RING motif. Surprisingly, a mutation within the RING finger, 10 amino acids N-terminal to the first cysteine of the RING finger (residue 156), without altering the amino acid sequence. A unique Xhol site was present at the 5’ end of the RING finger, 10 amino acids N-terminal to the first cysteine residue.

### Construction of chimeric domain swap proteins

PCR primers were designed to amplify each heterologous RING finger. The primers contained the appropriate sequences linked to IE110k-specific sequences with 5’ and 3’ Xhol and HindIII sites respectively. The PCR product was digested to insert the heterologous RING fingers into IE110k in-frame. This basic method was modified by varying the extent of IE110k sequence in the PCR primers used to amplify the heterologous RING fingers. Plasmid identities were confirmed by sequencing.

- pDR29, pDR30 and pDR31 were all constructed using a strategy to replace exactly the same number of amino acids flanking the core RING finger that were removed during the digestion of p27 with Xhol/R. The chimeras are summarized in Fig. 1(a):
  - 7018, ATTACCTCGAGAGAGCTTGGACATCACAAATAGGACAATACTTGCGGCAGGGCTTATT 6885,
  - 7019, CGGCCTGGCTTTGTCTACATGACTGGCAGGGCTTATT 6886,
  - ATTACCTCGAGAGAGGCCGGTGATTAATTTTAAATTTGAG 6887,
  - GACCGGAGCTTTGTCTACATGACTGGCAGGGCTTATT 6888,
  - ATTACCTCGAGAGAGTTTGCCCCTTAATGCCATTGAACCTG 6889,
  - ATTACCTCGAGAGAGTTTGCCCCTTAATGCCATTGAACCTG 6890,
  - GACCGGAGCTTTGTCTACATGACTGGCAGGGCTTATT 6891,
  - ATTACCTCGAGAGAGTTTGCCCCTTAATGCCATTGAACCTG 6892,
  - GACCGGAGCTTTGTCTACATGACTGGCAGGGCTTATT 6893,
  - ATTACCTCGAGAGAGTTTGCCCCTTAATGCCATTGAACCTG 6894,
  - GACCGGAGCTTTGTCTACATGACTGGCAGGGCTTATT 6895.

### Construction of deletions and alterations in the IE110k RING finger

pDR27 (C153A, C156A) was constructed by digestion of pDR27 with Xhol and HindIII, mung bean nuclease treatment, and religation to create an in-frame fusion of IE110k lacking amino acids 105–160 inclusive. pDR40 (A129–130) was created using a PCR cloning strategy. Primers 7404/7405 were designed to amplify the IE110k RING finger and contained IE110k sequences and unique Xhol and SpeI restriction sites that allowed insertion of the mutant RING finger back in-frame into IE110k.

- pDR42 (C153A, C156A) contains mutations of the amino acids cysteine 153 and cysteine 156. The 5’ primer, 7479, contained the IE110k HindIII site and a mutation of bases in the cysteine 153 and 156 codons to create alanine codons; the 3’ primer, 7404, contained wild-type (wt) IE110k sequence including the unique Xhol site.
- pDR47 (A129–130, P125A) contains a mutation of the proline residue at 125 in a pDR40 background (i.e. also containing a deletion in C129 and D130). The pDR27 was digested with Xhol and SpeI and the primers 7404, containing wt IE110k sequence and the unique Xhol site, and 7708, containing both the C129 and D130 deletion and the mutation changing the proline codon to an alanine codon, were used to amplify the RING finger from the pDR40 construct:
  - 7404, CCCCCCCTCAGAGAGCCAGCCCGGAGCCACG,
  - 7405, AGCGGTTGCATGCACTGGCAAGGTTGCGAGGGTGGCCG,
  - 7479, GTACACAAAGCTTTGCCGACCAAGGCTCGCAGCGAG

### Methods

**Construction of IE110k expression vectors.** The construct pDR27 contains the IE110k cDNA linked to the cytomegalovirus (CMV) immediate early promoter. To facilitate the RING finger motif swapping experiments we removed the unique HindIII site from the pCMV19a vector and introduced a G–T mutation at bp 480, which created a unique HindIII site in the IE110k cDNA coding sequence, two amino acids C-...
Fig. 1. (a) Summary of the conservation of a RING motif in each of the proteins of the alphaherpesvirus family, and in the heterologous proteins tested in this work. The position of the RING finger is indicated by the hatched box and the numbers represent the position of the first and last cysteine within each RING motif. The number of amino acids in each protein is indicated to the right of each schematic. (b) The first and last cysteine of the IE110k RING motif are numbered 116 and 156 respectively and aligned with the corresponding regions of the other herpesvirus proteins, the cellular proteins mdm-2 and Bmi I and the baculovirus protein PE-38. The extent of the RING finger motif is indicated by the stippled bar above the protein sequences. The two extra residues in the IE110k and IE118k (HSV-2) from optimum alignment of the region, together with the conserved tryptophan at 146 and the core cysteines at 153 and 156 are indicated by arrows. Substitutions in these residues were made in the variants indicated in (c) and described in the text. Asterisks indicate residues which are conserved in at least four of the six RING motifs. (c) Site-directed mutations and deletions within the IE110k RING motif as described in the text.

7708, AGCGGTGCATGCAGGAAGGTGCAGTGAGGCCCAGATCTCATCCGT.

The mutations in the IE110k RING finger are summarized in Fig. 1(c).

■ Transfections and CAT assays. Transfections were performed in Vero cells by the calcium phosphate precipitation method modified by the use of BES [N,N-bis(2-hydroxyethyl)-2-aminoethanesulphonic acid]-buffered saline (pH 7.06) as previously described (Batchelor & O’Hare, 1992). The reporter constructs p38kCAT, pTKCAT, p110kCAT and pLATCAT have all been described (Batchelor & O’Hare, 1992; O’Hare & Goding, 1988). The total amount of DNA was equalized to 2 µg in all cases by the addition of pUC19 carrier DNA.

■ Immunolocalization studies. Vero cells were plated at 2 × 10⁵ cells per 35 mm well, each containing a glass coverslip. Approximately 40 h after transfection (500 ng of each construct), the cells were washed in PBS and fixed with methanol. Primary antibodies, diluted in PBS, were the monoclonal antibody 5E10 for endogenous PML (Stuurman et al., 1992), PK monoclonal antibody for transfected PML, and the polyclonal antibody against the N-terminus of IE110k (Mullen et al., 1994). The fluorochrome-conjugated secondary antibody, diluted in PBS and 10% calf serum, was added after washing. Cells were visualized with a Nikon microscope using the Nikon plan APO 60/1.4 oil lens and the image was scanned and processed using a Bio-Rad MRC 600 laser scanning confocal microscope. Illustrations were compositied using Adobe Photoshop software.

■ Biochemical fractionation. Nuclear matrix fractionation was performed according to the method described by Capco et al. (1982). Transfected cells were resuspended in 250 µl buffer containing 0.5% Triton X-100, 100 mM PIPES pH 6.8, 1 mM EDTA, 1 mM MgCl₂, incubated on ice for 5 min and centrifuged at 12000 r.p.m. for 2 min. Supernatants were mixed with 250 µl 2× SDS lysis buffer, while pellets were resuspended in 250 µl buffer containing 10 mM PIPES pH 6.8, 300 mM sucrose, 100 mM KCl, 3 mM MgCl₂, 1 mM EDTA. DNase I and RNase (25 µg) were added and mixtures were incubated at 33 °C for 60 min. After centrifugation at 12000 r.p.m. for 2 min the supernatants were treated as before and corresponding pellets were resuspended in 250 µl 250 mM ammonium sulphate, incubated on ice for 5 min and centrifuged at 12000 r.p.m. for 2 min. The resulting supernatants were treated as before and pellets were resuspended in 250 µl 2× SDS lysis buffer, while pellets were resuspended in 250 µl buffer containing 10 mM PIPES pH 6.8, 300 mM sucrose, 100 mM KCl, 3 mM MgCl₂. The supernatants were mixed with 250 µl 2× SDS lysis buffer, while final pellets were lysed in 500 µl 1× SDS lysis buffer. Equal fractions were boiled and analysed by SDS-PAGE. For Western blotting, IE110k and variants were detected with rabbit polyclonal antibody (1:350) to an N-terminal peptide. Epitope-tagged PML was detected with a monoclonal antibody (PK
antibody, 1:2000) to the SV5 tag. VP16 was detected with the monoclonal antibody LP1. Lamin A/C was detected with rabbit antibody (1:250) supplied by Novacast Laboratories. Horseradish peroxidase-coupled anti-rabbit and anti-mouse antibodies were used at dilutions of 1:3000 and detected using the ECL detection system according to the manufacturer’s instructions (Amersham).

Results

IE110k transactivation and nuclear localization

The conservation of the RING finger motif within IE110k of HSV-1, other herpesvirus homologues and several cellular genes relevant to this work is summarized schematically in Fig. 1. Primary sequence similarities in the core RING domain between the viral genes are illustrated in Fig. 1(b) together with some of the variants examined in this work in Fig. 1(c).

Typical results demonstrating the broad spectrum transactivation by IE110k, for which the RING finger is critical (see below), are shown in Fig. 2. Representative promoters from each of the classes of HSV genes (including late, data not shown) were activated to similar levels upon cotransfection with the IE110k expression vector pDR27 (which contains the cDNA for IE110k under the control of the CMV IE enhancer/promoter region). Parallel examination of the localization of IE110k in such transactivation assays is shown in Fig. 2(b). The IE110k protein was present in a punctate pattern within the nuclei (Fig. 2b), consistent with previous results (Everett, 1987; Gelman & Silverstein, 1986; Knipe & Smith, 1986), and in IE110k-positive cells the endogenous PML protein present in the punctate pattern representing PODs had disappeared (Fig. 2b, PML panel and merged). However, a distinct difference between the subnuclear distribution of IE110k and PML was observed (Fig. 2b, compare distribution of PML in untransfected cell with distribution of IE110k in transfected cell). We noted that IE110k, in addition to displacing PML, formed abundant punctate foci, which were much more numerous than the original endogenous PODs within the nucleus. This ability of IE110k to form numerous foci was highly reproducible and represented a distinct qualitative feature of IE110k (see below).

The IE110k RING finger is required for transactivation and redistribution of PML

We wished to examine the transactivation activity and intranuclear distribution of the specific deletion of the RING finger, and the effect of substitution of highly conserved zinc co-ordinating cysteines. In the IE110k expression vector pDR33 the entire RING domain has been deleted from amino acids 105–160, while in pDR42, alanines have been substituted for two of the zinc co-ordinating cysteines (Fig. 1c). The efficiencies of transactivation of these mutants were compared to that of wt IE110k (pDR27). The results (Fig. 3) demonstrate that deletion of the RING finger, or the substitution of cysteines 153 and 156, resulted in loss of transactivation. Control Western blots of transfected cell extracts demonstrated that the mutant IE110k encoded in pDR42, and the deleted variant in pDR33 were synthesized in equivalent amounts to the wt protein (see Fig. 7). The complete lack of activity of pDR42 due to the two cysteine substitutions reinforces the critical requirement for a RING finger domain within IE110k for its broad spectrum transactivation.

We next examined the effect of the RING finger deletion and core cysteine substitutions on IE110k localization, and distribution of PML, in cells transfected with these IE110k variants. Precise deletion of the complete RING finger region had virtually no effect on the distribution of IE110k (Fig. 3b, pDR33). This variant was distributed in a punctate pattern within the nucleus indistinguishable from that seen with wt IE110k. The difference was observed when examining PML (Fig. 3b, PML). Clearly PML was not displaced from its punctate foci and a subset of the mutant IE110k was colocalized in these foci. The extra IE110k-positive, PML-negative foci can clearly be seen in the merged image together with the subset of foci positive for both proteins (Fig. 3b, pDR33 merged). Mutation of the conserved cysteines of the RING finger had a subtle but distinct effect compared to the RING finger deletion. The DR42 mutant IE110k was present in a diffuse distribution which formed a background to less numerous punctate foci (Fig. 3c). These latter punctate foci colocalized precisely with the endogenous PODs from which, again in contrast to the situation with wt IE110k, PML was not displaced. The localization with the PODs is visualized in the merged image showing the yellow foci with the surrounding diffuse nuclear pattern of the mutant IE110k (Fig. 3c, merged). Thus, while the lack of PML disruption and the presence of IE110k in the POD foci was similar for both variants, the pattern of IE110k outside the PODs was distinct; for the RING finger deletion the pattern was punctate and reflected the wt, while the cysteine substitution mutant was unable to form these distinct foci being present in a diffuse distribution. Thus, neither the RING finger of IE110k per se, nor PML disruption is required for the formation of the extra IE110k-positive foci. (The reason for the diffuse background pattern of the pDR42 substitution mutant compared to the apparently normal foci of the RING deletion mutant is presently unclear but may be due to some pleiotropic effect of the cysteine substitutions, see Discussion.) However, the RING finger is required for PML disruption. Furthermore, normal formation of the IE110k extra foci in the absence of PML disruption is clearly not sufficient for transactivation.

Cellular RING fingers do not complement IE110k function

To test whether heterologous RING motifs could functionally substitute for the IE110k RING finger in the background of IE110k we constructed chimeric polypeptides which contained the RING finger motifs from two cellular proteins, Bmi I (pDR31), which co-operates with c-myc to form B-lymphomas (Haupt et al., 1991; van Lohuizen et al., 1991),
Fig. 2. (a) Generalized activation of expression by IE110k. Vero cells were transfected with target CAT constructs containing various herpesvirus promoters as indicated. The constructs 38kCAT and TKCAT contain delayed early promoters and were transfected in 0.5 μg amounts, IE110kCAT contains the constitutively stronger IE promoter for the IE110k gene itself and was transfected in 50 ng amounts as was the construct with the latency-associated promoter, LATCAT. The various reporter constructs were transfected without (-) or together with the IE110k expression vector pDR27 (1 or 10 ng), where the total amount of transfected DNA was equalized with carrier DNA. (b) Sub-nuclear compartmentalization of IE110k and PML. Vero cells were transfected with pDR27 (0.1 μg) and IE110k localization was assessed with a rabbit polyclonal antibody against the N terminus of IE110k, detected with Texas red-conjugated anti-rabbit antibody, while endogenous PML (green) was detected with the monoclonal antibody 5E10 and fluorescein-conjugated anti-mouse antibody. No overlap in detection was observed for the two channels. The cell within the PML field which was expressing IE110k is indicated with an arrow. The cells shown are typical of those for the nuclear localization of IE110k.

Fig. 3. (a) Mutation of core cysteines of the RING motif. Vero cells were transfected with a target CAT construct containing the HSV TK promoter (0.5 μg) and 1 ng, 10 ng or 100 ng of wt (pDR27), the RING finger deletion variant (pDR33) and the cysteine-substituted variant (pDR42). In each case the total amount of transfected DNA was equalized with pUC19 DNA. Extracts were made approximately 40 h after transfection and equal amounts were assayed for CAT activity. (b) Sub-nuclear compartmentalization of IE110k variants, and the effects on endogenous PML in Vero cells. IE110k localization (red) for the RING finger deletion variant (pDR33; b) and the cysteine-substituted variant (pDR42; c), was assessed with a rabbit polyclonal antibody against the N terminus of IE110k, detected with Texas red-conjugated anti-rabbit antibody, while endogenous PML (green) was detected with the monoclonal antibody 5E10 and fluorescein-conjugated anti-mouse antibody. The cells within the PML field which were expressing IE110k are indicated with an arrow. The right hand panel illustrates the merged fields. Colocalization at any point will be represented by yellow.

and mdm-2 (pDR29), which interacts with the tumour suppressor protein p53 and inhibits the transactivation property of this protein (Boddy et al., 1994; Momand et al., 1992). The RING finger from the baculovirus regulatory protein PE-38 (Krappa & Knebel, 1991) was also tested (pDR30).

The activity of these constructs (summarized in Fig. 4a), was compared to the wt IE110k on a number of different target promoters in transfection assays in Vero cells. Typical results from an experiment using a delayed early promoter linked to the CAT reporter are illustrated in Fig. 4(b). None of the
chimeric proteins exhibited significant transactivation of any of the target reporter constructs, while in parallel transfections the parental IE110k construct resulted in 20–30-fold activation. Control experiments again demonstrated similar levels of synthesis of the chimeric proteins compared to the parental IE110k protein (data not shown).

We next examined the effect of the heterologous RING fingers on subnuclear localization and interaction with the PODs. The RING-like motif from mdm-2 exhibited a surprising phenotype. This chimeric protein was localized to the nucleus and was targeted to the PODs. However, it did not form additional foci, nor was it able to displace PML from PODs (Fig. 5a, pDR29, PML and merged). This protein was therefore targeted precisely and exclusively to the original cellular PODs with no additional IE110k-specific distribution. The chimeras containing the RING finger from Bmi I (Fig. 5b) and PE-38 (data not shown) exhibited an intermediate pattern where the protein was clearly associated with the PODs and PML was not displaced, but some additional foci containing only the IE110k chimera were also detected together with diffuse staining (Fig. 5b, pDR31, PML and merged). Thus, otherwise normal RING domains from heterologous proteins do not substitute for the IE110k motif in the function of disruption of PML and again, although lack of PML disruption was not the only effect of the swaps on nuclear localization, it correlated with lack of transactivation.

**Residues unique to the HSV RING finger are specifically required for transactivation**

Fig. 1(b) illustrates a comparison of the primary sequences of the RING finger motifs from HSV-1 IE110k, its homologue from HSV-2, other alphaherpesvirus homologues, and Bmi I, mdm-2 and PE-38. A tryptophan residue (Fig. 1b, arrowed) is conserved among all the viral RING motifs but not in the cellular proteins. Although this conservation looks significant we have previously shown that substitution of this residue with alanine has no effect on transactivation by IE110k nor on subnuclear localization (Everett et al., 1995c). Interestingly, compared to the other virus members, the RING motifs of both the HSV-1 IE110k protein and the HSV-2 homologue IE118k contain two extra residues (including a cysteine) between the second and third core cysteines of the motif (Fig. 1b, arrowed). In the equine herpes virus (EHV-1) homologue whose structure has been characterized (Barlow et al., 1994), this region would be within the junction between the first major loop and a β-strand. To examine the relevance of the extra residues present in IE110k, we constructed pDR40, which contains a deletion of...
the cysteine 129 and aspartic acid 130 (Fig. 1c). Deletion of residues 129/130 had a dramatic effect, resulting in complete loss of transactivation function (Fig. 6a). Furthermore, although the protein was detected in nuclear foci (Fig. 6b), an interesting phenotype was observed for this mutant IE110k in the localization studies. The pDR40 variant was still nuclear and still retained a punctate distribution, but these foci were fewer in number and precisely colocalized with PML in the PODs, from which PML was not displaced (Fig. 6b, pDR40, PML and merged). Analysis of numerous fields of transfected cells showed that the POD-restricted phenotype of the pDR40 mutant was a completely reproducible qualitative feature which contrasted with the numerous foci observed for wt IE110k protein. Identical results were obtained with pDR47 (Fig. 1), a derivative of pDR40, in which a proline (residue 125) had been substituted to alanine, in addition to the CD deletion. The phenotype of restricted POD localization was very similar to that obtained with the pDR29, the mdm-2 chimeric protein.

Nuclear matrix association of PML, IE110k and RING finger variants

PML was originally characterized as a protein that bound tightly to the nuclear matrix of cells in biochemical fractionations (Chang et al., 1995). Nuclear matrix association of IE110k has not been previously addressed. We wished to determine if IE110k was also matrix associated and what effect the mutations may have. Fig. 7 shows the results of fractionation of cells transfected with each of the IE110k wt and RING finger variants, for total protein (Fig. 7, top panel) and IE110k protein (Fig. 7, bottom panel, Western blot). Detergent treatment of IE110k-transfected cells extracted most of the total cellular protein (Fig. 7, top, lane 2) and solubilized a significant fraction of IE110k (Fig. 7, bottom, lanes 2), which was presumably due to the cytoplasmic overexpressed protein seen in certain cells by immunofluorescence. Subsequent single-step high salt extraction of the nuclear pellet indicated that the majority of IE110k remained within the pelleted fraction (data not shown). To analyse IE110k partitioning more rigorously we performed a sequential step extraction of the nuclear material after detergent treatment, with DNase and RNase (Fig. 7, lanes 3), ammonium sulphate (lanes 4), and 2 M NaCl (lanes 5). This procedure solubilizes most of the nuclear protein and the remaining salt-insoluble fraction is indicative of nuclear matrix association (lanes 6). Most nuclear proteins are extracted after the high salt plus DNase/RNase treatment (lane 3, top panel) or ammonium sulphate treatment (lane 4, note the quantitative extraction of histones in this fraction). In contrast, IE110k (lower panels, IE110k) was not extracted in these fractions but was highly enriched in the 2 M NaCl pellet material (IE110k, lane 6) which contained less than 2% of the total protein (top panel, lane 6). After stripping the blot we analysed the behaviour of the nuclear matrix-associated lamin A/C and found it quantitatively associated with the same fraction as IE110k, in the 2 M NaCl pellet (lamin panel, lanes 1–6). As a control we tested the distribution of transfected VP16 which was completely extracted in the detergent fraction, in agreement with its cytoplasmic staining observed in immunofluorescence studies (Elliott et al., 1995). These results indicate that for nuclear IE110k, the punctate localization (which represents the majority of nuclear protein), reflects an association with the nuclear matrix.

We next compared the behaviour of IE110k in fractionation with that of PML. Endogenous PML has proved difficult to detect by Western blot analysis and we therefore analysed the partitioning of epitope-tagged PML expressed from a transfected plasmid. The behaviour of PML in the salt extraction of the remaining nuclear fraction was similar to that of IE110k (Fig. 7, PML, lanes 3–6) with the exception that a fraction of PML was extracted after DNase/RNase treatment (PML, lane 3). This represented a reproducible difference in that IE110k was never extracted with DNase or RNase treatment. However, the majority of the nuclear PML partitioned to the high salt pellet fraction (PML, lane 6). Thus, both IE110k and PML were highly enriched in the nuclear matrix.

To investigate the role of the RING finger in the association of IE110k with the nuclear matrix we examined the biochemical fractionation of a number of the RING finger variants described above. The pDR33 construct, which exhibited the normal punctate distribution (Fig. 3), partitioned exactly like the wt IE110k protein (Fig. 7, bottom panel DR33). Surprisingly for pDR42 which, although exhibiting a partial punctate pattern, was present in the nucleus in a largely diffuse pattern, the nuclear protein also partitioned with the matrix fraction (Fig. 7, DR42). While levels of IE110k from pDR40 were comparable to those of wt and the other mutants, slightly less was extracted in the cytoplasm (Fig. 7, pDR40). However, like wt, most of pDR40 IE110k was present in the nucleus and partitioned to the matrix fraction (Fig. 7, pDR40).

All of the RING finger variants examined lacked transactivation activity but remained tightly associated with the nuclear matrix despite the range of distributions observed in immunofluorescence studies. The RING finger of IE110k is clearly not the signal involved in IE110k recruitment to PODs nor its tight association with the nuclear matrix.

Discussion

The herpesvirus regulatory protein IE110k possesses a cysteine-rich, zinc-binding RING finger motif required for its transactivation activity and examine the relationship between these activities.
We provide evidence that IE110k, like PML, is associated with the nuclear matrix. However, despite the conservation of the RING motif, other RING fingers were not functionally interchangeable with the IE110k RING finger and the RING finger was required neither for nuclear matrix association, nor for POD localization. While nuclear matrix association has not previously been addressed, our results are consistent with those obtained from experiments in which the RING finger from the PML protein itself failed to function in the context of the IE110k protein (Everett et al., 1995b). Although little is known about the specific biochemical function of RING finger domains, the conservation of specific residues indicates that these domains may have similar types of roles within the various proteins that contain them. Clearly, however, they are not interchangeable, indicating that certain features are selective. The specific charge and distribution of polar residues within the presumptive $\alpha$ helical region of the IE110k RING finger have been suggested to be important for transactivation (Barlow et al., 1994; Everett et al., 1993, 1995b). This distribution is not well-conserved in the cellular RING fingers, and could account for their inability functionally to replace the IE110k motif. It is also noteworthy that the deletion of residues D. O’Rourke and others

Fig. 5. Effect of heterologous RING motifs from (a) mdm-2 (pDR29) or (b) Bmi I (pDR31) on subnuclear compartmentalization and PML in Vero cells. IE110k localization (red) for the RING motif swaps (pDR29, a; pDR31, b), was assessed with a rabbit polyclonal antibody against the N terminus of IE110k, detected with Texas red-conjugated anti-rabbit antibody, while endogenous PML (green) was detected with the monoclonal antibody 5E10 and fluorescein-conjugated anti-mouse antibody. The cells within the PML field which were expressing IE110k are indicated with an arrow. The right hand panel illustrates the merged fields. Colocalization at any point will be represented by yellow.

Fig. 6. Two residues within the RING motif required for transactivation, displacement of PML and formation of extra subnuclear motifs. (a) Transfections and (b) immunolocalization with the pDR40 variant in Vero cells were performed as for Fig. 2. Precise colocalization of the pDR40 variant and PML can be clearly seen in the merged left panel.
129/130, which abolishes transactivation in HSV IE110k, paradoxically makes the alignment with other alphaherpesvirus RING domains better. Therefore, it could be that the actual functional domain comprises the RING domain together with some additional determinant. In other herpesviruses with a subtly different RING domain, there could be a compensating change in the additional determinant. This would be consistent with previous results showing that the EHV-1 RING domain did not function in the context of IE110k (Everett et al., 1995a). It would also not be inconsistent with the results of Moriuchi et al. (1994) who reported that the RING domain from varicella zoster virus gene 61 did function in the context of IE110k, since these workers used larger regions encompassing the RING domain in the swaps. Precise splicing of the RING domain from one herpesvirus protein into the other will be necessary to answer these questions. If such chimeras consistently do not function, but activity can be restored by including flanking regions, then it may be that the RING domain functions in concert with other determinant(s).

The IE110k deletions and point mutations demonstrate that the RING motif is not the determinant of POD localization of IE110k, nor is it required to form the extra IE110k domains, but it is required for both the dispersal of PML from PODs and for transactivation. Surprisingly, the mutation of the conserved cysteines of the RING finger in the DR42 mutant IE110k resulted in a diffuse distribution which formed a background to PODs from which PML was not displaced. Similarly, replacing the IE110k RING finger with cellular RING fingers or making point mutations or deletions within the IE110k RING finger all altered the subnuclear distribution of IE110k with respect to its ability to localize efficiently to the extra IE110k-specific domains. However, these variants still localized to the nucleus and furthermore retained nuclear matrix association, indicating that the determinants for POD localization and nuclear matrix association differ.

In contrast to the effects of mutation within the RING finger, the deletion of the entire RING finger did not interfere with the ability of IE110k to localize to these domains in the

Fig. 7. IE110k and PML partition to the nuclear matrix fraction. Transfected COS cells were detergent extracted and the nuclei were further fractionated in a stepwise fashion. Relative protein abundance in the fractions (equal cell equivalents) is shown in the top panel as follows: lane 1, total sample; lane 2, Triton X-100 soluble fraction; lane 3, DNase/RNase soluble fraction; lane 4, ammonium sulphate soluble fraction; lane 5, 2 M NaCl soluble fraction; lane 6, 2 M NaCl salt pellet. The arrows in lane 3 indicate the added DNase and RNase. In addition the quantitative extraction of histones (filled dots) was observed after the ammonium sulphate step. The behaviour of the endogenous lamin A/C component of the nuclear matrix is shown in the lower panel of Western blots, as well as the behaviour of exogenous VP16 protein (cytoplasmic).
nucleus. One interpretation of these data is that, while the RING finger is the determinant involved in PML disruption, the determinant for localization to the extra IE110k-specific domains resides elsewhere in the protein and this determinant is affected by the disruption of the RING motif structure in the IE110k mutations and swaps, but not by precise deletion of the motif.

In this regard the results of Mullen et al. (1994) are informative. Although the numbers of IE110k foci and the fate of PML were not addressed in their work, these workers demonstrated that deletions within and adjacent to the RING finger, e.g. Δ106–134, Δ136–177 and Δ106–177, retained nuclear punctate distribution. Furthermore they showed that transfer of the N terminus of IE110k (minimally residues 1–105) could transfer nuclear punctate distribution to the otherwise diffuse localization of the IE175k protein. However, inclusion of the RING finger, i.e. residues 1–244, significantly improved punctate localization of the chimeras. One way to reconcile these data which would be consistent with all the results would be that the structure or presentation of the extreme N-terminal determinant was influenced by the RING finger. In this model, within the context of the IE110k protein, precise removal of the RING finger would not be as deleterious for presentation of the N-terminal region while mutations within the RING domain would impinge on this region. Likewise, in chimeric proteins such as those constructed by Mullen et al. (1994), the RING finger would augment or influence proper presentation of the N terminus. This notwithstanding it is clear that within IE110k the N-terminal region, including the RING finger, is not sufficient for nuclear POD localization and previous results have shown that mutations elsewhere in the protein, notably within the C-terminal regions, also affected subnuclear localization (Chen et al., 1991; Everett, 1988; Everett & Maul, 1994). It is possible that these variants also alter conformation of the N-terminal motif, perhaps because the C-terminal region is involved in dimerization of IE110k and this too may be important for presentation of the determinant.

Localization of viral proteins to PODs and displacement of POD proteins have now been observed for a number of viruses (Ahn & Hayward, 1997; Koriath et al., 1996; Carvalho et al., 1995; Doucas et al., 1996; Kelly et al., 1995; Maul et al., 1993; Puvion-Dutilleul et al., 1995; Szekely et al., 1996). This suggests that it may be a critical requirement for the successful infection of host cells. Alternatively, two proteins with potential roles in proteolysis have been shown to be components of at least some of the PODs (Boddy et al., 1996; Everett et al., 1997) and it is therefore possible that part of the cellular response to infection may be in targeting viral proteins to PODs, e.g. for ubiquitin-mediated destruction. However, it is difficult to define a common feature of those viral proteins (e.g. HSV IE110k, human CMV IE1, Epstein–Barr virus EBNA 5 and adenovirus E4-ORF3) which have been reported to localize to or affect PML and PODs. Of these, IE110k is the only one which contains a RING finger domain and for IE110k this domain is essential for PML disruption and transactivation. It remains possible therefore that, unlike IE110k, for other viral proteins POD localization is a passive event or at least not necessarily involved in their regulatory function. To date no direct correlation has been established.

The results of this work demonstrate nuclear matrix association of IE110k and show that determinants other than the RING finger are required for this association and for POD localization. However, the mechanism of action of IE110k, which exhibits broad spectrum transactivation, correlates with its effect on a pre-existing nuclear compartment, the POD, for which determinants within the RING finger are critically required. RING finger motifs from related proteins do not functionally substitute for the IE110k RING finger. The phenotype of mutants localizing exclusively to PODs, versus the numerous extra foci seen for the parent protein, indicate that IE110k may traffic through the PODs to extra, matrix-associated sites. Whether these sites are formed upon or in association with specific cellular proteins (but excluding PML) remains to be determined.

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References


O’Hare, P., Mosca, J. & Hayward, G. S. (1986). Multiple trans-acting proteins of herpes simplex virus that have different target promoter specificities and exhibit both positive and negative regulatory functions. *Cancer Cells* 4, 175–189.


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